Effects of Ghrelin Administration on Left Ventricular Function, Exercise Capacity, and Muscle Wasting in Patients With Chronic Heart Failure

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Background—Ghrelin is a novel growth hormone–releasing peptide that also induces vasodilation, inhibits sympathetic nerve activity, and stimulates feeding through growth hormone–independent mechanisms. We investigated the effects of ghrelin on left ventricular (LV) function, exercise capacity, and muscle wasting in patients with chronic heart failure (CHF).

Methods and Results—Human synthetic ghrelin (2 μg/kg twice a day) was intravenously administered to 10 patients with CHF for 3 weeks. Echocardiography, cardiopulmonary exercise testing, dual x-ray absorptiometry, and blood sampling were performed before and after ghrelin therapy. A single administration of ghrelin elicited a marked increase in serum GH (25-fold). Three-week administration of ghrelin resulted in a significant decrease in plasma norepinephrine (1132±188 to 655±134 pg/mL; $P<0.001$). Ghrelin increased LV ejection fraction (27±2% to 31±2%; $P<0.05$) in association with an increase in LV mass and a decrease in LV end-systolic volume. Treatment with ghrelin increased peak workload and peak oxygen consumption during exercise. Ghrelin improved muscle wasting, as indicated by increases in muscle strength and lean body mass. These parameters remained unchanged in 8 patients with CHF who did not receive ghrelin therapy.

Conclusions—These preliminary results suggest that repeated administration of ghrelin improves LV function, exercise capacity, and muscle wasting in patients with CHF. (***Circulation.* 2004;110:3674-3679.**)

Key Words: growth substances ■ heart failure ■ hormones ■ nutrition

Left ventricular (LV) remodeling (dilatation and wall thinning) and cardiac cachexia (body weight loss and muscle wasting) often are observed in patients with end-stage chronic heart failure (CHF).[1,2] Growth hormone (GH) and its mediator, insulinlike growth factor-1 (IGF-1), are anabolic hormones that are essential for skeletal and myocardial growth and metabolic homeostasis.[3,4] Earlier studies have shown that GH supplementation may have beneficial effects on LV myocardial structure and function in some patients with CHF,[5] although the importance of GH resistance[6] and neutral results of randomized trials also have been reported.[7,8]

Ghrelin is a novel GH-releasing peptide that was isolated from the stomach and has been identified as an endogenous ligand for the growth hormone secretagogue receptor.[9] Therefore, we believed that administration of ghrelin may induce beneficial changes in LV function and energy metabolism in patients with CHF via a GH-dependent mechanism. On the other hand, growth hormone secretagogue receptor mRNA is detected not only in the hypothalamus and pituitary but also in the heart and blood vessels,[10] implying direct cardiovascular effects of ghrelin. Wiley and Davenport[11] have demonstrated that ghrelin is an endothelium-independent vasodilator in isolated human arteries. We have shown that intravenous administration of ghrelin decreases systemic vascular resistance and increases cardiac output in patients with CHF.[12] Furthermore, ghrelin induces a positive energy balance by stimulating food intake[13,14] and adiposity[15] through GH-independent mechanisms. These findings raise the possibility that ghrelin administration may have beneficial effects in cachectic patients with CHF. In fact, we recently have demonstrated that treatment with ghrelin improves not only LV function but also cardiac cachexia in rats with CHF.[16] In humans, however, the potential effects of ghrelin as a therapeutic agent for CHF remain unknown.

Thus, the purposes of this study were as follows: (1) to investigate whether repeated administration of ghrelin im-

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TABLE 1. Patient Characteristics

	Control Group (n=8)	Ghrelin Group (n=10)
Age, y	74±2	75±2
Sex, M/F	6/2	7/3
Body mass index, kg/m^2	19.0±1.1	19.0±0.9
Cause of CHF, n		
Dilated cardiomyopathy	4	4
Ischemic cardiomyopathy	1	3
Hypertensive heart disease	2	1
Valvular heart disease	1	2
NYHA functional class, n		
III	8	9
IV	0	1
LVEF, %	28±2	27±2
Presence of cardiac cachexia, n	6	8
Medication use, n		
Digoxin	6	9
ACE inhibitors	7	9
A II blockers	2	2
β-Blockers	6	7
Diuretics	7	10

LVEF indicates LV ejection fraction; A II, angiotensin II. Data are mean±SEM.

proves LV myocardial structure and function in patients with CHF, (2) to examine whether ghrelin improves exercise capacity in such patients, and (3) to examine whether ghrelin induces anabolic effects in patients with CHF.

Methods

Study Subjects

Eighteen patients with CHF (13 men, 5 women; mean age, 75 years; range, 63 to 80 years) were included in this study. Inclusion criteria were as follows: (1) LV ejection fraction <35% as assessed by cardiac catheterization, (2) a stable clinical condition, and (3) clinical evidence of heart failure despite conventional therapy. Exclusion criteria were the presence of any of the following: (1) chronic renal impairment (serum creatinine level ≥2.0 mg/dL), (2) significant liver dysfunction, (3) evidence of malignant diseases, (4) active infection, (5) hematologic abnormalities, or (6) systolic blood pressure <90 mm Hg. Ten patients with CHF (ghrelin group) received repeated administrations of ghrelin. Although this study was neither randomized nor placebo controlled, 8 patients with CHF who did not receive ghrelin (control group) were studied to exclude time-course effects during hospitalization. Patients in the ghrelin group were admitted only for the study. Those in the control group had been in hospital for diagnostic examination and stayed for 3 weeks for the study. There was no significant difference in demographic, clinical, or hemodynamic data at baseline between the ghrelin and control groups (Table 1). Eight patients in the ghrelin group and 6 patients in the control group were defined as exhibiting cardiac cachexia, as reported previously.[17] The weight loss in cachectic patients amounted to 6.4±0.4 kg or 11.8±0.7% loss of previous body weight during 14±2 months. The ethics committee of the National Cardiovascular Center approved the study, and all patients gave written informed consent.

Preparation of Human Ghrelin

Human synthetic ghrelin was obtained from the Peptide Institute Inc. This peptide is not commercially available. Ghrelin was dissolved in distilled water with 4% D-mannitol and sterilized by passage through a 0.22-μm filter (Millipore Co). Ghrelin was stored in 2-mL volumes, each containing 200 μg ghrelin. The chemical nature and content of the human ghrelin in vials were verified by high-performance liquid chromatography and radioimmunoassay. All vials were stored frozen at −80°C from the time of dispensing until the time of preparation for administration.

Study Protocol

This study was performed while patients were in a stable clinical condition during hospitalization. Ghrelin (2 μg/kg, 10 mL solution) was administered intravenously over 30 minutes at a constant rate. The infusion was repeated twice a day (before breakfast and before dinner) for 3 weeks. Study patients in both groups remained hospitalized for 3 weeks. Echocardiography, cardiopulmonary exercise testing, dual x-ray absorptiometry, hand-grip test, and blood sampling were performed at baseline and after 3 weeks of treatment with ghrelin (ghrelin group) or without ghrelin (control group). Long-term medication, including digitalis, diuretics, ACE inhibitors, and β-blockers, was kept constant during this study protocol.

Echocardiographic Studies

Echocardiography was performed by an investigator blinded to treatment allocation. Two-dimensional targeted M-mode tracings were obtained at the level of the papillary muscles with an echocardiographic system equipped with a 3.5-MHz sector scan probe (SONOS 2000, Hewlett Packard). LV wall thickness, dimensions, and fractional shortening were measured according to the recommendations of the American Society of Echocardiology from at least 3 consecutive cardiac cycles. LV end-diastolic volume, end-systolic volume, and ejection fraction were calculated with a modified version of Simpson's method.[18]

Cardiopulmonary Exercise Testing

Cardiopulmonary exercise testing was performed in all patients except 1, who underwent a 6-minute walk test as recommended by attending physicians. The patients exercised seated on a cycle ergometer. The work rate was then increased by 15 W/min up to their symptom-limited maximum. Breath-by-breath gas analysis was performed with an AE280 (Minato Medical Science).[19] Exercise capacity was evaluated by peak oxygen consumption (peak $\dot{V}o$). Ventilatory efficiency during exercise was represented by the $\dot{V}E$–$\dot{V}CO_2$ slope.[19]

Food Intake and Body Mass Analyses

Food intake for 3 consecutive days was assessed before ghrelin administration and during the last week of ghrelin therapy. Food intake was semiquantitatively assessed by a calorie count based on a 10-point scale method (0=null intake, 10=full intake or 1800 kcal), which was averaged for 3 days. Dual x-ray absorptiometry (DPX-L, Lunar Radiation) was repeated in all patients to examine changes in lean body mass, fat mass, and bone mineral content. Hand-grip strength was determined with a dynamometer.

Blood Sampling and Assay

Blood samples were taken from the antecubital vein the morning after an overnight fast. Serum GH and IGF-1 were measured by immunoradiometric assay (Ab Bead HGH Eiken, Eiken Chemical Co, Ltd, sensitivity=0.1 ng/mL; Somatomedin CII Bayer, Bayer Medical Ltd, sensitivity=0.3 ng/mL). Plasma norepinephrine and epinephrine were measured by high-performance liquid chromatography (HLC8030, Tosoh Co, sensitivity=6 pg/mL). Serum cortisol and insulin were measured by enzyme immunoassay (AIA-PACK CORT, sensitivity=0.2 μg/dL; AIA-PACK IRI, sensitivity=2.0 μU/mL, Tosoh Co). Serum tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) were measured by enzyme immunoassay (Quantikine HS, R&D Systems Inc, sensitivity=0.18 pg/mL; TFB kit, TFB Co, Ltd, sensitivity=0.3 pg/mL). Plasma renin and aldosterone were measured with radioimmunoassay kits (RENIN RIABEAD, sensitivity=0.1 ng/mL; ALDOSTERONE RIAKIT II, sensitivity=2.0

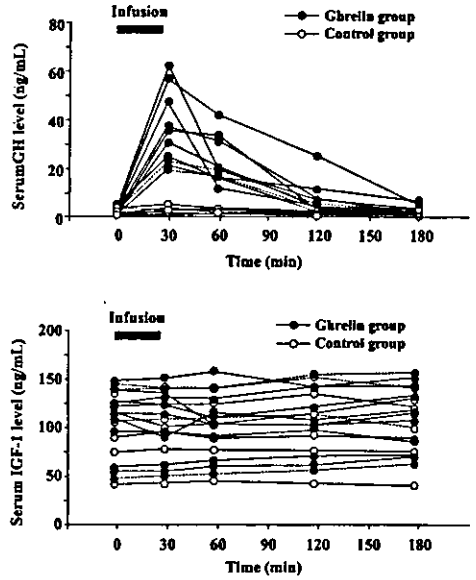

Figure 1. Changes in serum GH and IGF-1 after single administration of ghrelin. Solid line indicates cachectic patients; dotted line, noncachectic patients.

ng/dL, DAINABOT Co). Plasma brain natriuretic peptide (BNP) was measured by immunoradiometric assay (SHIONORIA BNP, sensitivity=4.0 pg/mL).

Statistical Analysis

Numerical values are expressed as mean±SEM. Comparisons of parameters between the 2 groups were made by unpaired Student's *t* test. Comparisons of the time course of serum GH and IGF-1 between the 2 groups were made by 2-way ANOVA for repeated measures, followed by the Newman-Keuls test. Comparisons of changes in parameters during the 3-week follow-up between the 2 groups were also made by 2-way ANOVA for repeated measures, followed by the Newman-Keuls test. A value of $P<0.05$ was considered significant.

Results

Administration of ghrelin transiently caused stomach rumbles in 6 patients and a slight feeling of being warm and sleepy in 4 subjects. Two patients felt slightly thirsty during ghrelin infusion. Other than these minor complaints, all subjects tolerated 3-week administration of ghrelin without incident. After 3-week administration of ghrelin, NYHA functional class improved in 4 patients and was unchanged in 6 patients. No change in NYHA functional class was observed in patients who did not receive ghrelin.

Effects of Ghrelin on Somatotropic Function

A single administration of ghrelin markedly increased serum GH level (baseline, 1.4±0.4; peak, 35.0±5.0 ng/mL; $P<0.001$; Figure 1). This elevation lasted >60 minutes after the start of ghrelin infusion. Serum IGF-1 level tended to increase 3 hours after the start of ghrelin infusion (101±12 to 110±12 ng/mL; $P=0.08$). Three-week administration of ghrelin tended to increase basal serum IGF-1 level (99±13 to 116±13 ng/mL; $P=0.07$). There was no significant difference in basal serum GH level between before and after 3 weeks of ghrelin therapy (2.0±0.8 to 1.2±0.3 ng/mL; P=NS).

Effects of Ghrelin on Food Intake, Body Weight, and Lean Body Mass

Administration of ghrelin significantly increased food intake (Figure 2). Three-week administration of ghrelin tended to increase body weight (49.6±2.7 to 50.4±2.7 kg; $P=0.09$). No development of edema was observed during ghrelin therapy. Dual x-ray absorptiometry demonstrated that treatment with ghrelin significantly increased lean body mass in patients with CHF (38.3±2.1 to 39.1±2.1 kg; $P<0.05$). Ghrelin did not significantly alter bone mineral content (2243±191 to 2265±189 g; P=NS) or fat mass (8877±1353 to 8748±1311 g; P=NS). Hand-grip strength was increased significantly by ghrelin therapy (20.5±1.7 to 22.7±2.0 kg; $P<0.01$). All of these parameters remained unchanged in patients who did not receive ghrelin.

Effects of Ghrelin on Cardiac Structure and Function

Neither heart rate nor blood pressure was significantly changed by 3-week administration of ghrelin (Table 2). Ghrelin increased LV ejection fraction (27±2% to 31±2%; $P<0.05$) in association with a decrease in LV end-systolic volume and an increase in LV mass (Figure 3), although ghrelin did not significantly alter LV end-diastolic volume. All of these parameters remained unchanged in patients who did not receive ghrelin.

Effects of Ghrelin on Exercise Capacity and Ventilatory Efficiency

Three-week administration of ghrelin significantly increased peak workload and peak $\dot{V}o$ during exercise (739±127 to 801±126 mL/min; $P<0.05$; Figure 4). Treatment with ghrelin did not significantly alter the $\dot{V}_E$–$\dot{V}_{CO_2}$ slope. In 1 patient

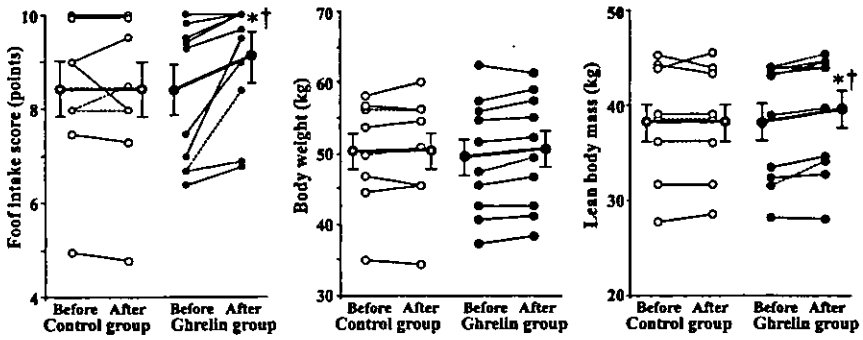

Figure 2. Food intake, body weight, and lean body mass before and after 3-week administration of ghrelin. Food intake was described semiquantitatively with 10-point scale method (0=null intake, 10=full intake). Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

TABLE 2. Physiological and Echocardiographic Measurements

	Control Group	Ghrelin Group
Heart rate, bpm		
Before	77±3	78±3
After	76±3	74±3
Mean arterial pressure, mm Hg		
Before	79±4	81±2
After	80±3	78±3
LVDd, mm		
Before	65.6±3.2	66.6±2.5
After	64.4±3.7	63.7±3.3
LVDs, mm		
Before	55.1±3.0	56.9 ± 2.9
After	53.9±3.6	52.8 ± 3.4*
FS, %		
Before	16.1±1.2	14.8±1.7
After	16.0±1.3	17.3±2.3
AWT diastole, mm		
Before	10.0±0.8	9.5±1.0
After	10.1±0.9	10.0±1.0*
PWT diastole, mm		
Before	9.2±0.4	9.3±0.6
After	9.4±0.4	9.9±0.5*†

LVDd indicates LV end-diastolic dimension; LVDs, LV end-systolic dimension; FS, fractional shortening; AWT, anterior wall thickness; and PWT, posterior wall thickness. Data are mean±SEM.

*$P<0.05$ vs before; †$P<0.05$ vs respective control group.

who did not undergo cardiopulmonary exercise testing, the distance walked in 6 minutes increased from 300 m to 410 m with ghrelin treatment. Exercise parameters remained unchanged without ghrelin.

Effects of Ghrelin on Sympathetic Nerve Activity

Three-week administration of ghrelin significantly decreased plasma norepinephrine and epinephrine (Figure 5). Treatment with ghrelin significantly decreased plasma BNP level (Table 3). Ghrelin did not significantly alter circulating glucose, insulin, cortisol, TNF-α, or IL-6. Neither plasma renin activity nor plasma aldosterone level was changed significantly. All of these parameters remained unchanged in patients who did not receive ghrelin.

Discussion

Ghrelin is a novel GH-releasing peptide that acts through a mechanism independent of that of hypothalamic GH-releasing hormone.[9] The GH-releasing effect of ghrelin has been shown to be more potent than that of GH-releasing hormone.[20] In fact, in the present study, ghrelin infusion elicited potent GH release in patients with CHF. Three-week administration of ghrelin increased LV ejection fraction in association with an increase in LV mass, which is consistent with findings from a previous experimental study in rats.[16] Plasma BNP level, a marker for LV function and wall stress, was decreased by ghrelin therapy. GH and its mediator, IGF-1, have been shown to enhance physiological compensatory hypertrophy in rats with CHF, resulting in a decrease in LV wall stress, leading to improvement in cardiac function.[21] Thus, ghrelin may also improve cardiac function partly through GH-dependent mechanisms. On the other hand, Baldanzi et al[22] have shown that ghrelin inhibits apoptosis of cardiomyocytes and endothelial cells through activation of extracellular signal-regulated kinase-1/2 and Akt serine kinases. Furthermore, stimulation of GHS-R by hexarelin has been shown to prevent cardiac damage after ischemia-reperfusion in hypophysectomized rats.[23] When these results are considered together, improvement in cardiac function by ghrelin therapy may be related to direct effects of ghrelin on myocardium. Importantly, ghrelin significantly decreased plasma norepinephrine levels in the present study. It is possible that improvement in cardiac function may lead to attenuation of sympathetic nerve activity. Interestingly, a recent study has demonstrated that ghrelin acts directly on the central nerve system to decrease sympathetic nerve activity.[24] Thus, inhibitory effects of ghrelin on sympathetic nerve activity may contribute to a decrease in plasma norepinephrine, which may have beneficial effects on cardiac performance in patients with CHF.

In the present study, 3-week administration of ghrelin improved exercise capacity in patients with CHF, as indicated by an increase in peak workload and peak $\dot{V}o$. A decrease in peak $\dot{V}o$ in patients with CHF is attributable not only to an inadequate increase in cardiac output during exercise, which is a central effect, but also to muscle wasting, a peripheral effect. Recently, we have shown that infusion of ghrelin increases cardiac output in patients with CHF.[12] In the present study, ghrelin increased lean body mass and muscle strength. These results suggest that ghrelin may improve exercise capacity through both central and peripheral effects.

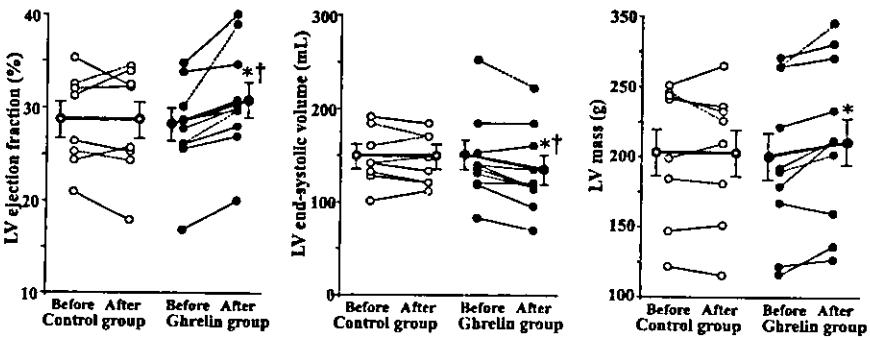

Figure 3. LV geometry and function before and after ghrelin therapy. Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

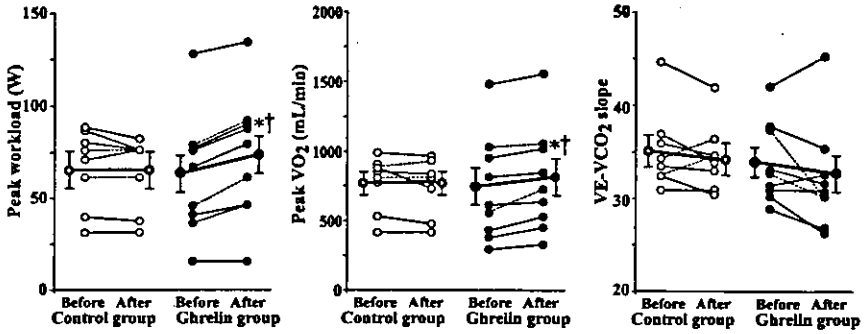

Figure 4. Exercise capacity and ventilatory efficiency before and after ghrelin therapy. Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

Cardiac cachexia, a catabolic state characterized by weight loss and muscle wasting, occurs frequently in patients with end-stage CHF[25] and is a strong independent risk factor for mortality in such patients.[26] Recently, we have shown that plasma ghrelin level is increased in cachectic patients with CHF as a compensatory mechanism in response to anabolic-catabolic imbalance.[17] In the present study, 3-week administration of ghrelin tended to increase body weight and significantly increased lean body mass and muscle strength. These results suggest that treatment with ghrelin improves muscle wasting in patients with CHF. These effects may be mediated, at least in part, by GH/IGF-1, which is considered essential for skeletal muscle. Earlier studies have shown that ghrelin induces orexigenic effects via activation of neuropeptide Y neurons in the hypothalamic arcuate nucleus.[13,14] In the present study, intravenous administration of ghrelin increased food intake in patients with CHF, which may contribute to anabolic effects of ghrelin. Tschop et al[15] have shown that administration of ghrelin induces adiposity through a GH-independent mechanism. In the present study, however, ghrelin did not significantly increase fat mass. This difference may be explained by the high dose of ghrelin (>2000-fold) used by Tschop et al. Ghrelin itself decreases fat utilization and increases fat, whereas GH decreases fat tissue and increases lean tissue. Thus, in the present study, ghrelin-induced GH may have attenuated an increase in fat and enhanced an increase in lean tissue.

The major limitation of this pilot trial relates to the lack of a randomized, placebo-controlled group. Patients in the control group were not treated identically because a placebo infusion was not performed. Nonetheless, this study was performed while patients were in a stable clinical condition during hospitalization. In addition, 8 patients in the control group were studied to exclude time-course effects during hospitalization. On the basis of the results of this study, a double-blind, randomized, and placebo-controlled study should be conducted. Second, this clinical study did not clarify mechanisms of increased LV ejection fraction by ghrelin therapy. Further studies are necessary to examine which mechanism predominantly contributes to improvement in LV ejection fraction.

Except for a few minor complications, long-term treatment with ghrelin was tolerated well in patients with CHF. Although a preliminary study documented the beneficial effects

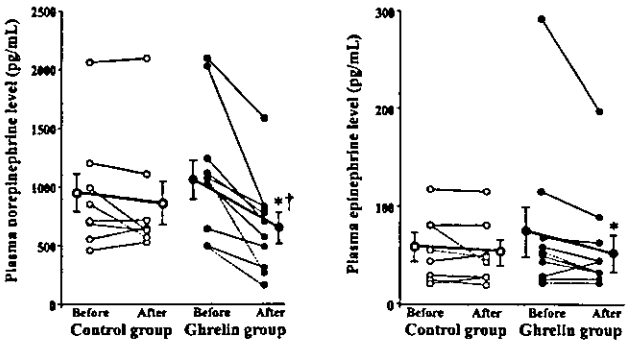

Figure 5. Plasma levels of norepinephrine and epinephrine before and after ghrelin therapy. Data are mean±SEM. Solid line indicates cachectic patients; dotted line, noncachectic patients. *$P<0.05$ vs before; †$P<0.05$ vs respective control group.

TABLE 3. Hormone Analysis in Patients With CHF

	Control Group	Ghrelin Group
BNP, pg/mL		
Before	180±53	238±59
After	181±62	190±60*
Fasting glucose, mg/dL		
Before	105±5	101±4
After	102±6	102±7
Insulin, μU/mL		
Before	6.0±1.4	3.9±0.7
After	6.8±2.0	5.5±1.2
Cortisol, μg/dL		
Before	15.5±1.9	17.9±1.6
After	14.5±2.6	17.2±1.5
TNF-α, pg/mL		
Before	5.3±0.9	5.7±0.8
After	5.4±0.9	5.6±0.8
IL-6, pg/mL		
Before	3.2±0.5	3.8±0.7
After	3.4±0.5	3.6±0.7
Renin, ng·mL^{-1}·h^{-1}		
Before	9.3±4.6	7.3±3.0
After	10.1±4.1	6.9±3.7
Aldosterone, ng/dL		
Before	11.6±4.1	15.0±4.7
After	12.7±4.1	11.9±4.2

Data are mean±SEM.
*$P<0.05$ vs before.

of GH,[5] controlled studies in humans have been predominantly negative.[7,8] Nevertheless, ghrelin has been shown to have GH-independent effects, stimulating vasodilation,[10–12] reversing cachexia,[13–15] and inhibiting sympathetic nerve activity[24] and myocyte apoptosis.[22] Thus, ghrelin may have additional therapeutic potential compared with GH supplementation. Ghrelin improved cardiac function and exercise capacity in not only cachectic CHF patients but also noncachectic ones. Nevertheless, the best candidates may be cachectic CHF patients because ghrelin stimulates feeding and improves muscle wasting.

Conclusions

These preliminary results suggest that repeated administration of ghrelin improves LV structure and function, exercise capacity, and muscle wasting in patients with CHF. Thus, administration of ghrelin may be a new therapeutic approach for the treatment of CHF.

Acknowledgments

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

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

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

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

Key Words: peptides ■ angiogenesis ■ peripheral vascular disease

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

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

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Methods

Animal Model of Hindlimb Ischemia

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

MNC Transplantation and AM Infusion

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

Assessment of Blood Perfusion

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

Histological Assessment

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

Monitoring of Transplanted MNCs in Ischemic Hindlimb Muscle

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

In Situ Detection of MNC Apoptosis

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

In Vitro Apoptosis Assay

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

Adhesion Assay

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

Cell ELISA

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

EPC Culture Assay

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

Fluorescence-Activated Cell Sorting Analysis

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

Real-Time Polymerase Chain Reaction

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

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

In Vitro Matrigel Assay

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

Measurements of Cytokines

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

Migration Assay

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

Statistical Analysis

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

Results

Blood Perfusion and Capillary Density

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

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

Differentiation of Transplanted MNCs

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

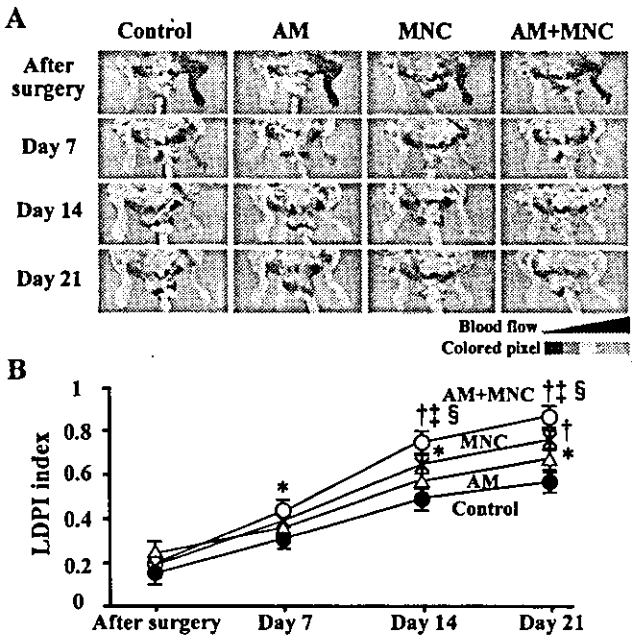

Figure 1. A, Representative examples of serial laser Doppler perfusion images. Blood perfusion of ischemic hindlimb increased notably in AM+MNC group (red to yellow). B, Quantitative analysis of hindlimb blood perfusion with LDPI index, ratio of ischemic to nonischemic hindlimb blood perfusion. Data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

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

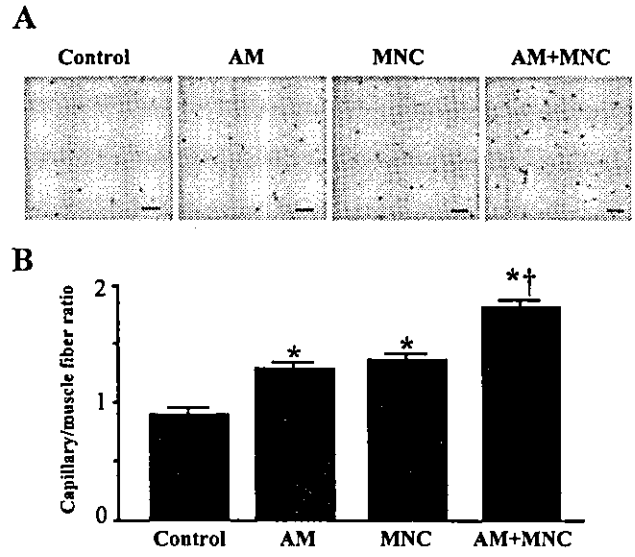

Figure 2. A, Representative photographs of alkaline phosphatase staining in ischemic hindlimb muscles. Capillary density in AM+MNC group was markedly higher than that in other groups. B, Quantitative analysis of capillary density in ischemic hindlimb muscles. Data are mean±SEM. *$P<0.01$ vs control;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

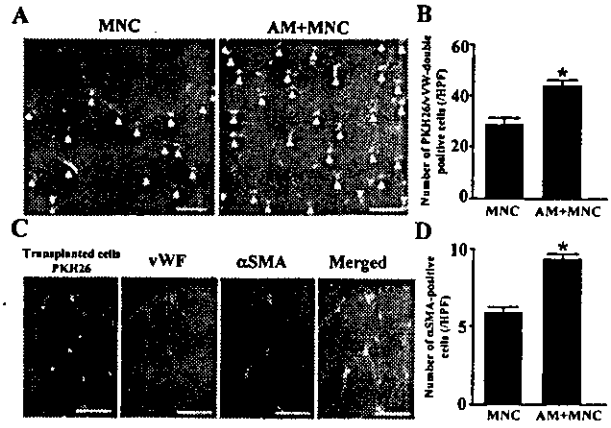

Figure 3. In vivo differentiation of transplanted MNCs. A, Representative photographs of MNC-derived vascular structures in MNC and AM+MNC groups. Red fluorescence (PKH26)–labeled MNCs were transplanted into ischemic thigh muscle. PKH26 (red)/vWF (blue) double-positive cells (pink, arrows) were frequently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

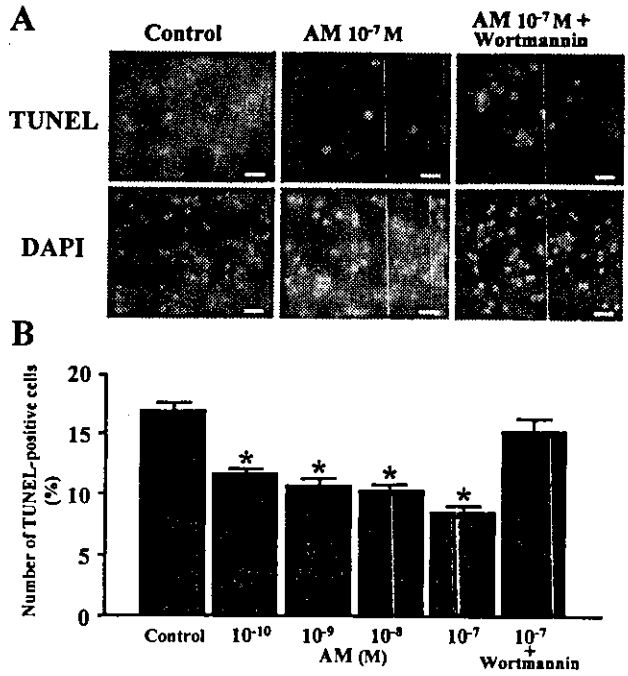

Figure 4. Apoptosis assay. A, Apoptosis of MNC was detected by TUNEL assay (green). Nuclei of MNC were stained with DAPI (blue). AM inhibited MNC apoptosis in serum-free medium. B, Quantitative analysis. AM decreased percentage of TUNEL-positive cells in dose-dependent manner. Pretreatment with wortmannin,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

Antiapoptotic Effect of AM on MNCs

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

Effect of AM on MNC Adhesiveness

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

Effect of AM on EPC Expansion

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

Effects of AM on Tube Formation and SMC Migration

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

Discussion

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

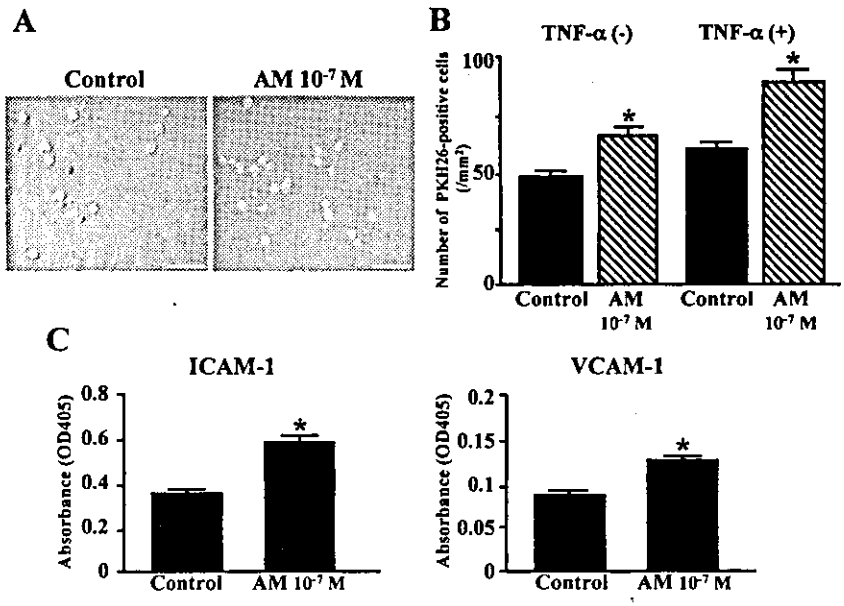

Figure 5. A and B, Adhesion assay. Representative photographs of red fluorescence–labeled MNC adhesion to HUVEC monolayer with and without AM (A). Quantitative analysis of MNC adhesion (B). Bars: 50 μm. C, Surface expression of ICAM-1 and VCAM-1 in HUVECs with or without AM. Data are mean±SEM. TNF indicates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

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

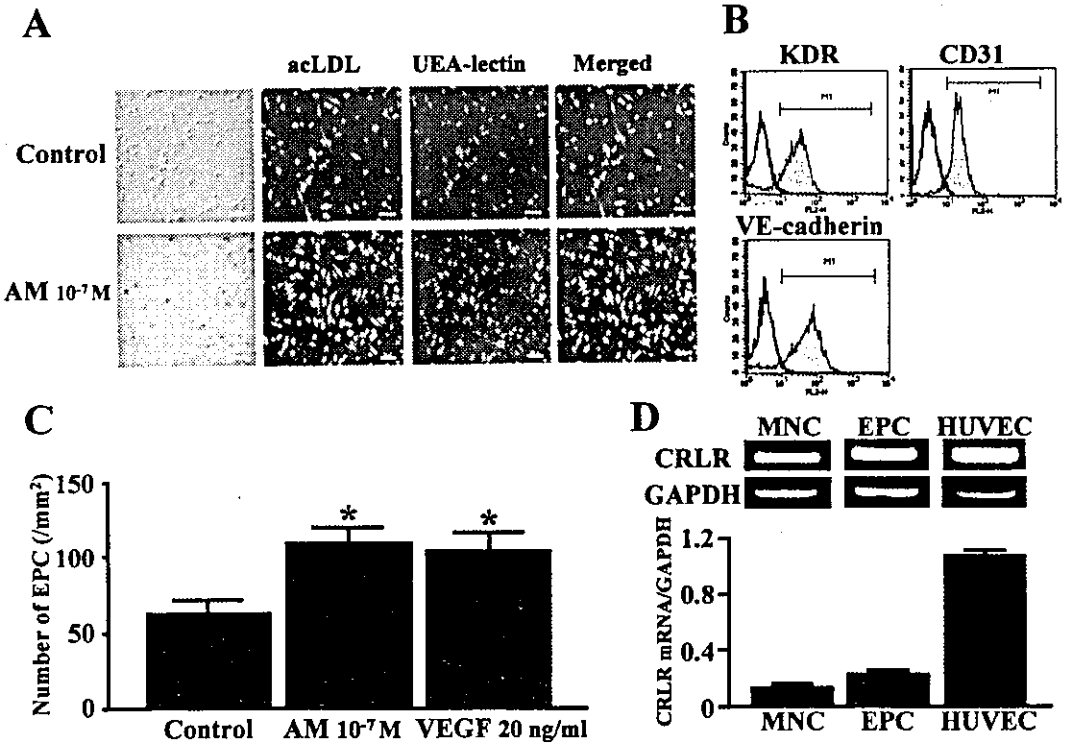

Figure 6. A through C, EPC culture assay. Cultured adherent cells took up DiI-acLDL (red) and FITC-labeled lectin (green) in same fields (A). Fluorescence-activated cell sorting analyses revealed that most adherent cells expressed KDR, VE cadherin, and CD31 (B). Culture of MNCs with AM significantly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

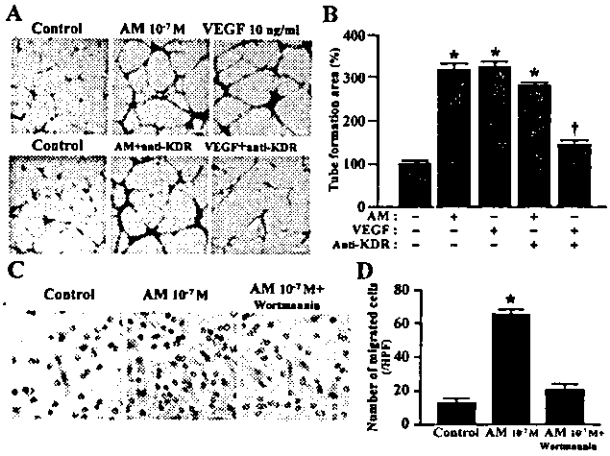

Figure 7. A and B, Matrigel assay. Representative photographs of tube formation (A). Quantitative analysis of tube formation area (B). Data are mean±SEM. *P<0.01 vs control; †P<0.01 vs VEGF. Bars: 20 μm. C and D, Migration assay. Representative photographs of migrated SMCs (C). Quantitative analyses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

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

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

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

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

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

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

Conclusions

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

Acknowledgments

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

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

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

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

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

Key Words: angiogenesis ■ apoptosis ■ stroke

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

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

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Materials and Methods

Stroke Model

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

MSC Preparation

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

MSC Transplantation and AM Infusion

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

Detection of MSC Differentiation in Ischemic Hemisphere

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

Neurological Assessment

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

Measurement of Infarct Size

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

Assessment of Angiogenesis

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

Detection of Apoptosis in Ischemic Penumbra

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

Statistical Analysis

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

Results

Engraftment and Differentiation of Transplanted MSCs

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

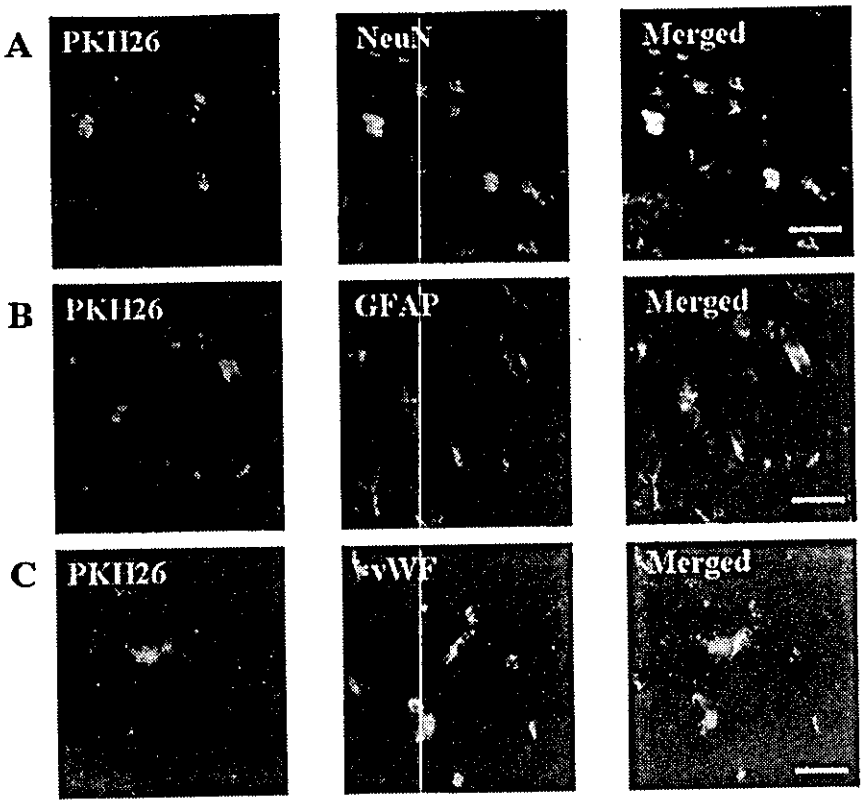

Figure 1. Engraftment and differentiation of transplanted MSCs. PKH26-labeled MSCs were frequently observed in ischemic penumbra. Some PKH26-positive MSCs (red) expressed neuronal marker (NeuN; green; A), astrocyte marker (GFAP; green; B), or endothelial cell marker (vWF; green; C). Bars=20 μm.

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

Neurological Assessment

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

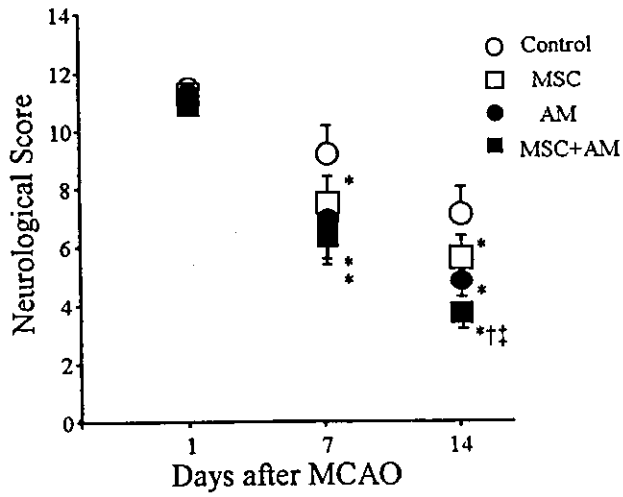

Figure 2. Neurological score on days 1, 7, and 14 in the control group, MSC group, AM group, and MSC+AM group. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs MSC group; ‡$P<0.05$ vs AM group.

Infarct Size and Physiological Data

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

TABLE 1. Percent Infarct Size to the Contralateral Hemisphere

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

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

Data are mean±SEM.

*$P<0.05$ vs control group.

†$P<0.05$ vs MSC group.

‡$P<0.05$ vs AM group.

TABLE 2. Percent Increase of Body Weight

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

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

Data are mean±SEM.

*$P<0.05$ vs control group.

Angiogenic Potency of AM and MSCs

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

Antiapoptotic Effects of AM on Neuronal Cells and Transplanted MSCs

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

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

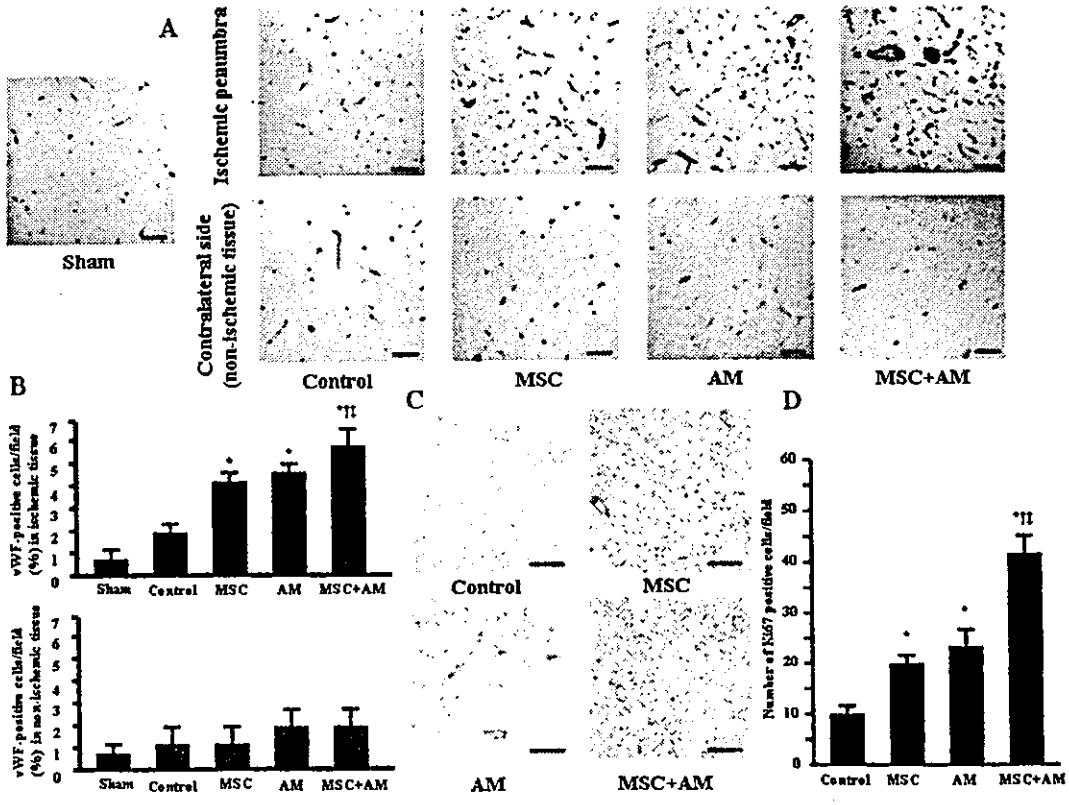

Figure 3. A, Representative photomicrographs of vWF staining in ischemic penumbra (top) and in contralateral nonischemic tissue (bottom). Bars=25 μm. B, Quantitative analysis of angiogenesis using the area of vWF staining in ischemic penumbra (top) and in nonischemic tissue (bottom). C, Representative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

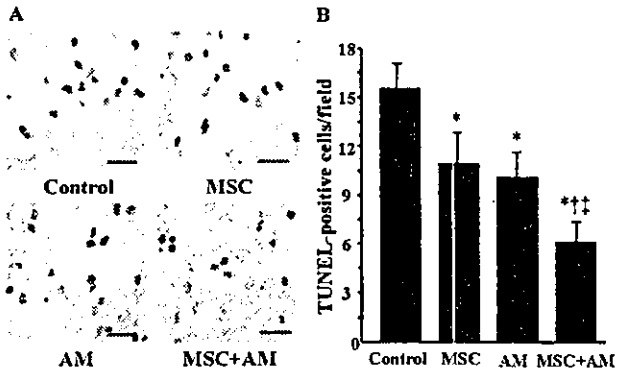

Figure 4. A, Representative photomicrographs of TUNEL staining in ischemic penumbra. The number of TUNEL-positive cells (DAB; brown) in the MSC+AM group was markedly lower than that in the other 3 groups. B, Quantitative analysis of the number of TUNEL-positive cells. Data are mean±SEM. *$P<0.05$ vs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

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

Discussion

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

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

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

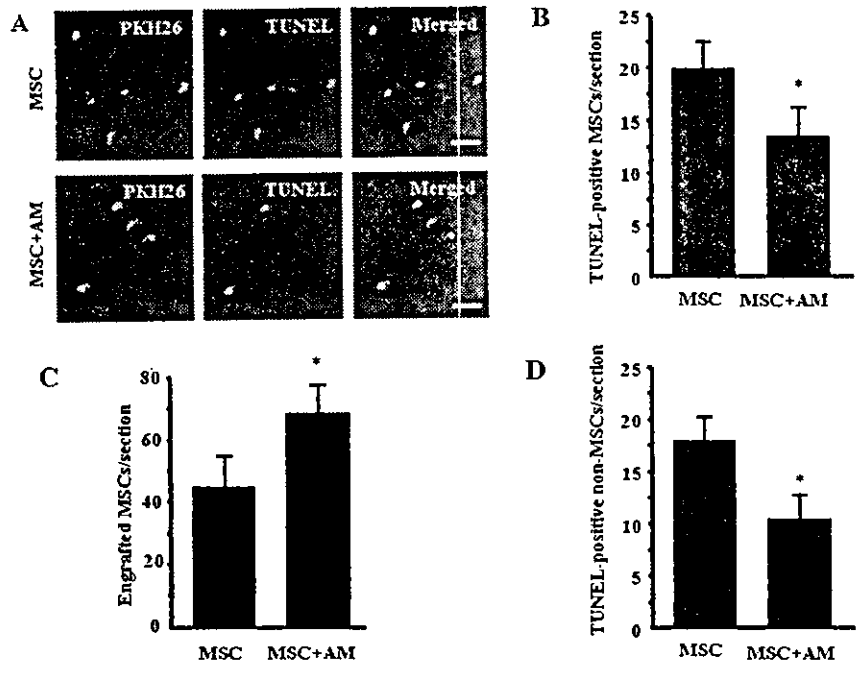

Figure 5. A, Representative photomicrographs of MSC apoptosis after transplantation. Transplanted MSCs were labeled with PKH26. TUNEL-positive cells (green) were frequently observed in ischemic penumbra. Infusion of AM decreased TUNEL-positive MSCs (double-positive cells, merged). B, Quantitative analysis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

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

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

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

Acknowledgments

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Effect of cardiopulmonary bypass on pulmonary clearance of adrenomedullin in humans

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

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Key words: adrenomedullin; cardiopulmonary bypass; pulmonary clearance.

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

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

Material and method

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