Adrenomedullin Infusion Attenuates Myocardial Ischemia/Reperfusion Injury Through the Phosphatidylinositol 3-Kinase/Akt-Dependent Pathway

Hiroyuki Okumura, MD; Noritoshi Nagaya, MD; Takefumi Itoh, MD; Ichiro Okano, PhD; Jun Hino, PhD; Kenji Mori, PhD; Yoshitane Tsukamoto, MD; Hatsue Ishibashi-Ueda, MD; Senri Miwa, MD; Keiichi Tambara, MD; Shinya Toyokuni, MD; Chikao Yutani, MD; Kenji Kangawa, PhD

Background—Infusion of adrenomedullin (AM) has beneficial hemodynamic effects in patients with heart failure. However, the effect of AM on myocardial ischemia/reperfusion remains unknown.

Methods and Results—Male Sprague-Dawley rats were exposed to a 30-minute period of ischemia induced by ligation of the left coronary artery. They were randomized to receive AM, AM plus wortmannin (a phosphatidylinositol 3-kinase [PI3K] inhibitor), or saline for 60 minutes after coronary ligation. Hemodynamics and infarct size were examined 24 hours after reperfusion. Myocardial apoptosis was also examined 6 hours after reperfusion. The effect of AM on Akt phosphorylation in cardiac tissues was examined by Western blotting. Intravenous administration of AM significantly reduced myocardial infarct size (28±4% to 16±1%, $P<0.01$), left ventricular end-diastolic pressure (19±2 to 8±2 mm Hg, $P<0.05$), and myocardial apoptotic death (19±2% to 9±4%, $P<0.05$). Western blot analysis showed that AM infusion accelerated Akt phosphorylation in cardiac tissues and that pretreatment with wortmannin significantly attenuated AM-induced Akt phosphorylation. Moreover, pretreatment with wortmannin abolished the beneficial effects of AM: a reduction of infarct size, a decrease in left ventricular end-diastolic pressure, and inhibition of myocardial apoptosis after ischemia/reperfusion.

Conclusions—Short-term infusion of AM significantly attenuated myocardial ischemia/reperfusion injury. These cardioprotective effects are attributed mainly to antiapoptotic effects of AM via a PI3K/Akt-dependent pathway. (***Circulation.*** **2004;109:242-248.)**

Key Words: peptides ■ reperfusion ■ apoptosis ■ myocardial infarction ■ hemodynamics

Coronary revascularization has been established as the most effective treatment for coronary artery disease. However, reperfusion can elicit a number of adverse reactions that may limit its beneficial actions. Although it has been attempted to reduce ischemia/reperfusion injury in many basic or clinical studies, few agents are clinically available for ischemia/reperfusion injury.

Adrenomedullin (AM) is a potent vasodilatory peptide that was originally isolated from human pheochromocytoma.[1] We have shown that AM peptide and mRNA are distributed in the heart[2,3] and that plasma and cardiac AM markedly increase after acute myocardial infarction.[4,5] AM has been shown to be a possible endogenous suppressor of myocyte hypertrophy[6] and fibroblast proliferation.[7] In addition, intravenous infusion of AM has beneficial hemodynamic effects in patients with heart failure.[8] These findings suggest that AM induces cardioprotective effects not only as a circulating factor but also as a paracrine and/or autocrine factor.

Recently, AM has been shown to activate the Akt pathway in vascular endothelial cells.[9] Interestingly, the Akt activation has been reported to lead to the prevention of myocardial injury after transient ischemia in vivo through antiapoptotic effects.[10] However, whether AM, a potent Akt activator, attenuates myocardial ischemia/reperfusion injury remains unknown.

Thus, the purposes of this study were (1) to investigate whether short-term infusion of AM reduces myocardial infarct size, inhibits myocyte apoptosis, and thereby improves cardiac function after ischemia/reperfusion and (2) to determine whether the underlying mechanisms are associated with

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From the Department of Biochemistry (H.O., I.O., J.H., K.M., K.K.), National Cardiovascular Center Research Institute, Osaka, Japan; Department of Internal Medicine (N.N., T.I.) and Department of Pathology (Y.T., H.I.-U., C.Y.), National Cardiovascular Center, Osaka, Japan; and Department of Cardiovascular Surgery (S.M., K.T.) and Department of Pathology and Biology of Diseases (S.T.), Graduate School of Medicine, Kyoto University, Kyoto, Japan.

Correspondence to Noritoshi Nagaya, MD, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. E-mail nagayann@hsp.ncvc.go.jp

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the phosphatidylinositol 3-kinase (PI3K)/Akt-dependent pathway.

Methods

Reperfusion Model

We used male Sprague-Dawley rats (Japan SLC Inc, Hamamatsu, Japan) weighing 180 to 220 g. Ligation of the left coronary artery was performed as described previously.[11] In brief, under anesthesia with pentobarbital sodium (30 mg/kg) and artificial ventilation, the heart was exposed via left thoracotomy, and the left coronary artery was ligated 2 to 3 mm from its origin between the pulmonary artery conus and the left atrium with a 6-0 Prolene suture. The heart was subjected to regional ischemia for 30 minutes, followed by coronary reperfusion through release of the tie. After ligation of the left coronary artery, AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$), AM plus wortmannin (16 μg/kg intravenous injection 15 minutes before AM infusion; a PI3K inhibitor),[12] or placebo (0.9% saline) was administered for 60 minutes through a catheter inserted into the left jugular vein. Sham-operated rats only underwent left thoracotomy. The chest wall was then closed, and the animal was allowed to recover. This protocol resulted in the creation of 4 groups: sham-operated rats (sham group, n=12), placebo-treated rats with ischemia/reperfusion (I/R-placebo group, n=19), AM-treated rats with ischemia/reperfusion (I/R-AM group, n=19) and AM plus wortmannin–treated rats with ischemia/reperfusion (I/R-Wo+AM group, n=15).

All animal experiments were conducted in accordance with the principles and procedures outlined in the *National Cardiovascular Center Guide for the Care and Use of Laboratory Animals*, which adheres strictly to the National Institutes of Health animal experimental guidelines, with the approval of the National Cardiovascular Center Animal Experimental Committee.

Hemodynamic Studies

We performed hemodynamic measurements 24 hours after ischemia/reperfusion. A 1.5F micromanometer-tipped catheter was advanced into the left ventricle through the right carotid artery, and a polyethylene catheter (PE-50) was advanced into the right ventricle through the right jugular vein to measure right ventricular pressure. Heart rate was also monitored with an ECG.

Measurement of Plasma AM Level

Blood samples were obtained from the right carotid artery during 0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$ AM infusion. Plasma AM level was measured by immunoradiometric assay, as described previously.[8,11]

Assessment of Infarct Size

After hemodynamic measurements, the heart was removed and perfused with a Langendorff apparatus for 10 minutes to wash out the blood and then fixed with 10% neutral buffered formalin. The heart was sliced transversely from the apex to the atrioventricular groove in 2.5-mm thicknesses and weighed separately. Within 24 hours after fixation, each section was embedded in paraffin. Serial 5-μm myocardial sections were cut with microtome and mounted on siliconized slides. After Masson trichrome staining, infarct size of each slice was analyzed by microscopy. Myocardial coagulation necrosis could be distinguished from viable myocardium as a definite alteration of staining, and then the infarct area was outlined and measured by planimetry. Infarct weight was determined with the following equation: % infarct area × weight of each slice, as described previously.[13] Finally, we determined percent infarct size as total infarct weight divided by total left ventricular (LV) weight.

TUNEL Staining

Hearts were isolated from each group (n=5) 6 hours after reperfusion for the terminal dUTP nick-end labeling (TUNEL) assay. After the blood and the fixation were washed out, the heart was also sliced transversely in 2.5-mm thicknesses. Paraffin-embedded, 5-μm-thick myocardial sections were used as described previously.[14] In brief, after deparaffinization and enzyme-mediated antigen retrieval, TUNEL staining was performed with a commercially available kit (Apop Tag Plus, Intergen). Samples were incubated with monoclonal anti-desmin antibody (Sigma) followed by tetramethylrhodamine isothiocyanate-conjugated rabbit anti-mouse antibody (DAKO). Counterstaining was performed with propidium iodide. Finally, these slides were mounted with Vector Shield (Vector Laboratories) containing an antifade reagent. We measured the number of TUNEL-positive nuclei in myocytes by means of confocal microscopy (Olympus, Fluoview 500). Quantitative analysis was performed on 60 high-power fields (magnification ×600) with at least 10 randomly selected fields used per section. We counted the number of cardiomyocytes at least $>10^4$ cells per heart.

DNA Ladder Assay

We used 10 additional rats for the DNA ladder assay (sham group, n=2; I/R-placebo group, n=4; I/R-AM group, n=4). Rats were killed, and the heart was excised 24 hours after ischemia/reperfusion. Immediately before heart isolation, 1% Evans blue was infused slowly into the left ventricle to delineate the risk area after coronary religation. Then, 40 mg of myocardium in the posterolateral border zone between the nonrisk area and the risk area was resected. Each specimen was frozen in liquid nitrogen and stored at −80°C until DNA extraction. DNA extraction and electrophoresis were performed with a commercially available kit (Apoptosis Ladder Detection Kit, WAKO).

Immunohistochemical Analysis

To assess localization of calcitonin receptor-like receptor (CRLR), a receptor for AM, in cardiac tissues, we performed immunohistochemical analysis using rabbit anti-rat CRLR antibody (Zymed). Localization of Akt phosphorylation was examined with rabbit anti-rat phospho-Akt antibody (Cell Signaling).

Western Blot Analysis

To identify Akt phosphorylation in myocardial tissues after AM infusion, Western blotting was performed with a commercially available kit (PhosphoPlus Akt [Ser 473] antibody kit, Cell Signaling). Myocardial tissues were obtained from rats treated with intravenous AM (0.01, 0.05, and 0.25 $\mu g \cdot kg^{-1} \cdot min^{-1}$), AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) plus wortmannin (16 μg/kg intravenous injection 15 minutes before AM infusion), or saline for 60 minutes during ischemia/reperfusion. These samples were homogenized on ice in a 0.1% Tween 20 homogenization buffer with a protease inhibitor (Complete, Roche). After centrifugation for 20 minutes at 4°C, the clear supernatant was used for Western blot analysis. Protein concentration was measured by Bradford's method (Bio-Rad). Fifty micrograms of each protein extract were transferred in sample buffer, loaded on 7.5% SDS-polyacrylamide gel, and blotted onto nitrocellulose membrane (Bio-Rad) with a wet blotting system. After being blocked for 60 minutes, the membranes were incubated with primary antibodies in blocking buffer (1:500) at 4°C overnight. Antibodies were used at the manufacturer's recommended dilution (Cell Signaling). The membranes were incubated with secondary antibodies, which were conjugated with horseradish peroxidase (Cell Signaling), at a final dilution of 1:2000. Signals were detected with LumiGLO chemiluminescence reagents (Cell Signaling).

Statistical Analysis

All data are expressed as mean±SEM unless otherwise indicated. Comparisons of parameters among the 3 or 4 groups were made by 1-way ANOVA for repeated measures, followed by Scheffé test. A probability value <0.05 was considered to indicate statistical significance.

Results

Reduction of Myocardial Infarct Size After AM Infusion

Moderate to large infarcts were observed in Masson trichrome–stained myocardial sections 24 hours after ische-

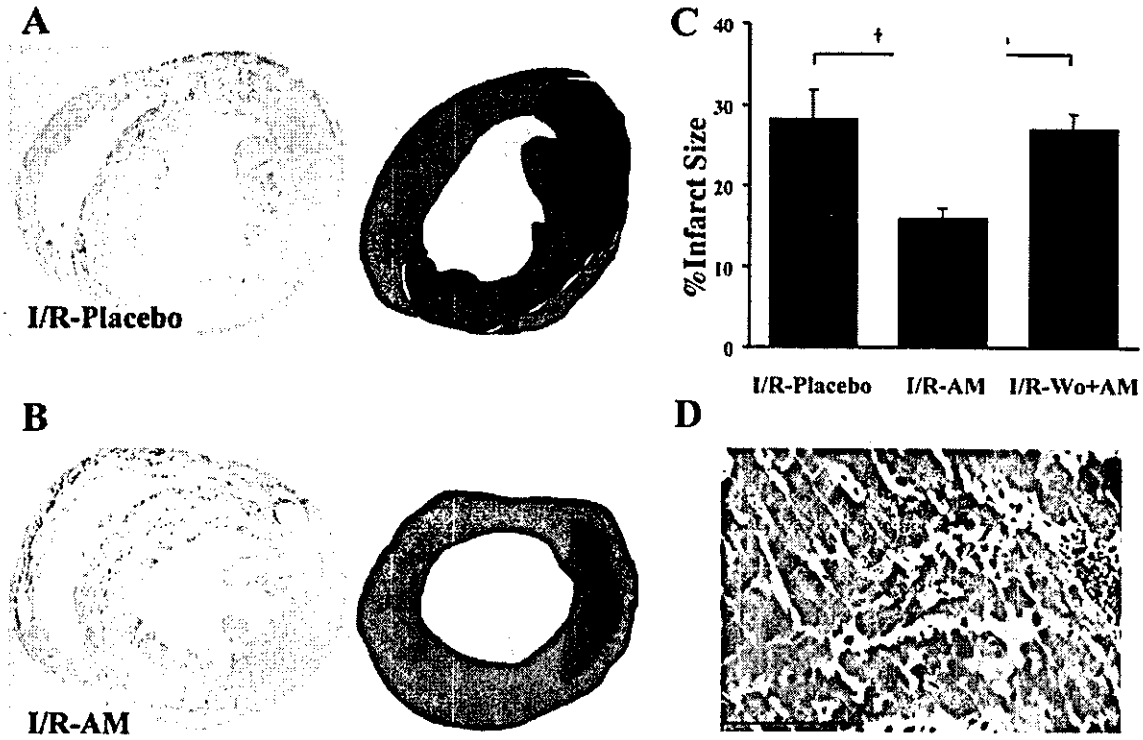

Figure 1. Effect of AM on myocardial infarct size 24 hours after ischemia/reperfusion. A and B, Photomicrographs show representative myocardial sections stained with Masson trichrome in I/R-placebo (A) and I/R-AM groups (B). Light red area indicates coagulation necrosis (right). C, Quantitative analysis demonstrated that AM infusion decreased infarct size after ischemia/reperfusion. However, pretreatment with wortmannin attenuated effect of AM. D, Typical reperfusion injury was observed in all groups on high-power field. Bar=100 μm. Data are mean±SEM. *P<0.05, †P<0.01.

mia/reperfusion (Figures 1A and 1B). Quantitative analysis revealed that 60-minute infusion of AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) significantly reduced myocardial infarct size compared with placebo infusion (16±1 versus 28±4%, P<0.01; Figure 1C). Infusion of AM markedly increased plasma AM level (from 10±2 fmol/mL at baseline to 96±13 fmol/mL at 60 minutes), which suggests that the plasma AM level was pharmacologically high. Pretreatment with wortmannin reversed the reducing effects of AM on myocardial infarct size (from 16±1% to 27±2%, P<0.05 versus I/R-AM group; Figure 1D). Although typical reperfusion injury, including contraction bands, hemorrhage, myocardial cell coagulation, and inflammatory cell infiltration, was observed after ischemia/reperfusion (Figure 1D), there were no histological differences among the 3 groups.

Hemodynamic Effects of AM

Twenty-four hours after ischemia/reperfusion, LV end-diastolic pressure (LVEDP) showed a marked elevation in the I/R-placebo group (19±2 mm Hg); the elevation was significantly attenuated in the I/R-AM group (8±2 mm Hg, P<0.05; Figure 2A). Pretreatment with wortmannin attenuated the reducing effects of AM on LVEDP (from 8±2 to 17±2 mm Hg, P<0.05 versus I/R-AM group; Figure 2A) 24 hours after ischemia/reperfusion. LV dP/dt_{max} tended to be higher in the I/R-AM group than in the I/R-placebo group (5285±285 versus 4524±247 mm Hg/s), and LV dP/dt_{min} tended to be lower in the I/R-AM group than in the I/R-placebo group (−4700±303 versus −3695±165 mm Hg/s; Figure 2B). Furthermore, pretreatment with wortmannin reversed the effects of AM on LV dP/dt_{max} and LV dP/dt_{min} after ischemia/reperfusion (5285±285 to 4570±239 mm Hg/s, −4700±303 to −3843±227 mm Hg/s, respectively; Figure 2B). These results suggest that AM infusion improved LV systolic and diastolic function after ischemia/reperfusion through the PI3K pathway. Interestingly, heart rate was significantly higher in the I/R-placebo and I/R-AM groups than in the sham group (Table). Although mean aortic pressure was significantly lower in the I/R-placebo group than in the sham group, a significant decrease in mean aortic pressure was not observed in the I/R-AM group. Right ventricular systolic pressure was significantly lower in the I/R-AM group than in the I/R-placebo group.

Antiapoptotic Effect of AM in Cardiomyocytes

Representative photomicrographs showed that TUNEL-positive myocytes were more frequently observed in the I/R-placebo group than in the sham group. However, TUNEL-positive myocytes were less frequently observed in the I/R-AM group than in the I/R-placebo group (Figure 3). Although a typical DNA ladder indicating fragmented DNA in cardiomyocytes was also observed in the I/R-placebo group, it was attenuated in the I/R-AM group (Figure 4). Quantitative analyses demonstrated that the number of TUNEL-positive cardiomyocytes was significantly smaller in the I/R-AM group than in the I/R-placebo group (9±4%

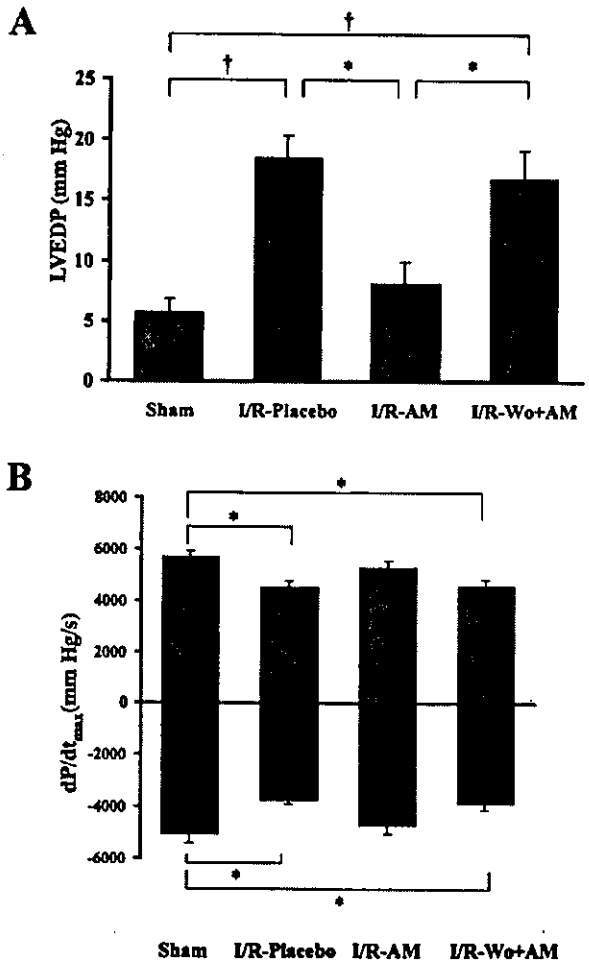

Figure 2. Effects of AM on LVEDP (A) and LV dP/dt (B) 24 hours after ischemia/reperfusion. AM infusion significantly inhibited increase in LVEDP compared with placebo infusion. AM infusion also improved LV dP/dt 24 hours after ischemia/reperfusion. Pretreatment with wortmannin attenuated effects of AM on LVEDP and LV dP/dt. Data are mean±SEM. *P<0.05; †P<0.01.

versus 19±2%, P<0.05; Figure 5). Furthermore, pretreatment with wortmannin abolished the AM-induced antiapoptotic effect in cardiomyocytes (from 9±4% to 20±1%, P<0.05; Figure 5). These results suggest that AM exerted antiapoptotic effects through the PI3K-dependent signal.

Summary of Hemodynamic Studies

	Sham (n=5)	I/R-Placebo (n=8)	I/R-AM (n=8)	I/R-Wo+AM (n=10)
Body weight, g	184±10	184±9	183±7	195±6
Heart rate, bpm	450±10	501±5*	494±9*	488±4
MAP, mm Hg	120±3	97±3*	105±4	99±7*
RAP, mm Hg	3±1	5±2	4±1	3±1
RVSP, mm Hg	32±1	47±1†	43±2†‡	48±2†

MAP indicates mean aortic pressure; RAP, right atrial pressure; and RVSP, right ventricular systolic pressure. Data are mean±SEM.
*P<0.05 vs sham group.
†P<0.01 vs Sham group.
‡P<0.01 vs I/R-placebo group.

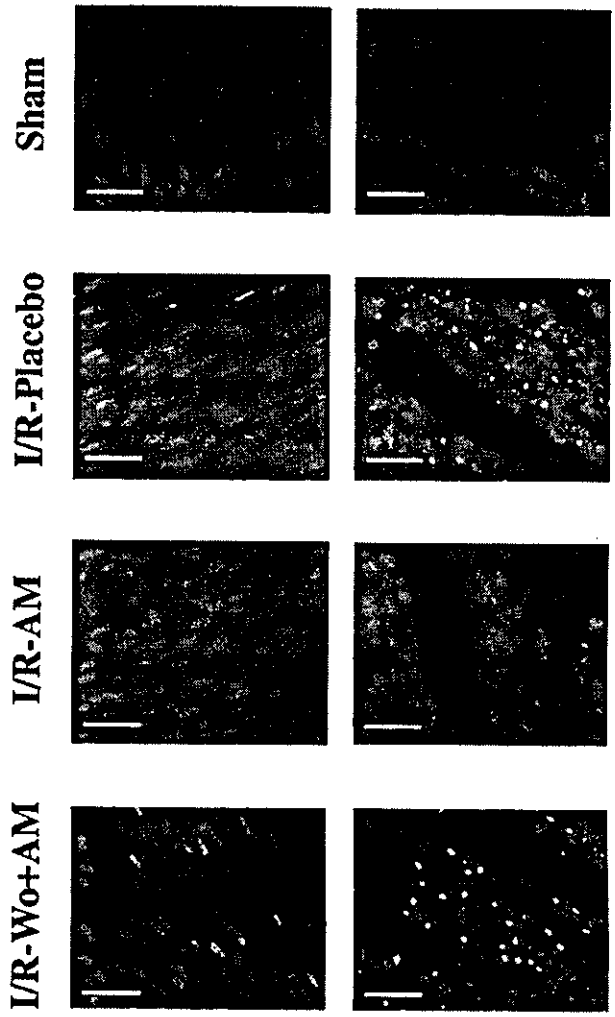

Figure 3. Representative photomicrographs of immunofluorescent staining for TUNEL-positive nuclei in sham, I/R-placebo, I/R-AM, and I/R-Wo+AM groups. Each left panel shows longitudinal myocytes, and each right panel shows short-axial myocytes. Yellow nuclei with red-stained myofilaments indicate TUNEL-positive myocytes. TUNEL-positive myocytes were less frequently observed in I/R-AM group than in I/R-placebo group. Pretreatment with wortmannin increased number of TUNEL-positive nuclei despite receipt of AM. Original magnification ×600. Bar=20 μm.

Akt Phosphorylation Induced by AM Infusion in Cardiac Tissue

Immunohistochemical analysis revealed that CRLR, a receptor for AM, was localized in cardiomyocytes and vascular endothelial cells (Figure 6). After 60-minute infusion of AM, Akt phosphorylation was detected in the nuclei of cardiomyocytes and vascular endothelial cells (Figures 7A and 7B). Western blot analyses also revealed that AM at 0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$ significantly phosphorylated Akt in cardiac tissue that was exposed to ischemia/reperfusion (Figure 7C). The effect of AM on Akt was inhibited by pretreatment with wortmannin. These results suggest that AM acts directly on myocardium and induces cardioprotective effects through the activation of PI3K/Akt-pathway.

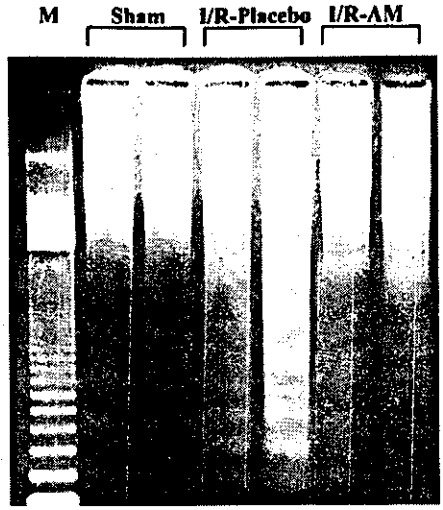

Figure 4. DNA ladder in sham, I/R-placebo, and I/R-AM groups. Although typical DNA ladder indicating fragmented DNA in cardiomyocytes was observed in I/R-placebo group, it was attenuated in I/R-AM group. M indicates molecular marker.

Discussion

In the present study, we demonstrated that short-term infusion of AM during the early phase of ischemia/reperfusion significantly reduced myocardial infarct size and inhibited myocyte apoptosis, and AM significantly decreased LVEDP and tended to improve LV dP/dt_{max} and dP/dt_{min}. We also demonstrated that AM enhanced Akt phosphorylation in cardiac tissue and that pretreatment with a PI3K inhibitor attenuated AM-induced cardioprotective effects against ischemia/reperfusion and inhibited AM-induced Akt phosphorylation.

Intravenous infusion of AM has beneficial hemodynamic and renal effects in patients with heart failure.[8] However, whether AM has direct cardioprotective effects in vivo remains unclear. In the present study, we demonstrated that short-term infusion of AM during the early phase of ischemia/reperfusion markedly reduced myocardial infarct size. Cardiomyocyte apoptosis is one of the major contributors to the development of myocardial infarcts,[15,16] which is related to the pathogenesis of heart failure. Thus, we examined whether AM has antiapoptotic effects in cardiomyocytes. Interestingly, short-term infusion of AM significantly reduced myocyte apoptosis after ischemia/reperfusion. This is the first study to demonstrate antiapoptotic effects of AM against myocardial ischemia/reperfusion injury, although AM has been shown to have antiapoptotic effects in vascular endothelial cells.[17,18] Given that cardiomyocyte apoptosis rather than necrosis contributes to myocyte death after ischemia/reperfusion, the antiapoptotic effects of AM may result in the reduced infarct size after ischemia/reperfusion.

In the present study, 60-minute infusion of AM improved cardiac function after ischemia/reperfusion, as indicated by a significant decrease in LVEDP and a tendency for an increase in LV dP/dt_{max} and a decrease in LV dP/dt_{min}. Previous studies have shown that the susceptibility to cardiac dysfunction

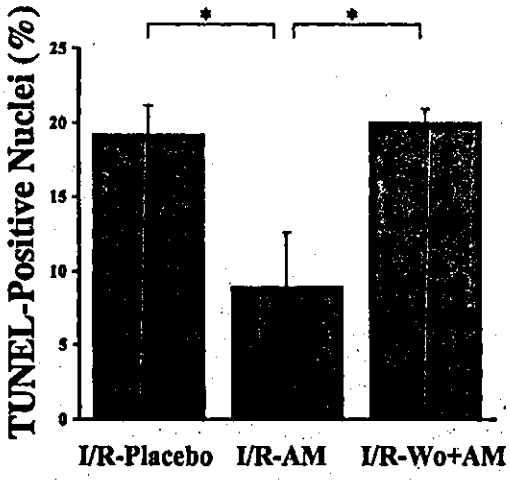

Figure 5. Quantitative analysis of TUNEL-positive nuclei in myocytes. Number of TUNEL-positive myocytes was lower in I/R-AM group than in I/R-placebo group. However, number of TUNEL-positive myocytes in I/R-Wo+AM group was as large as in I/R-placebo group. Data are mean±SEM. *$P<0.05$.

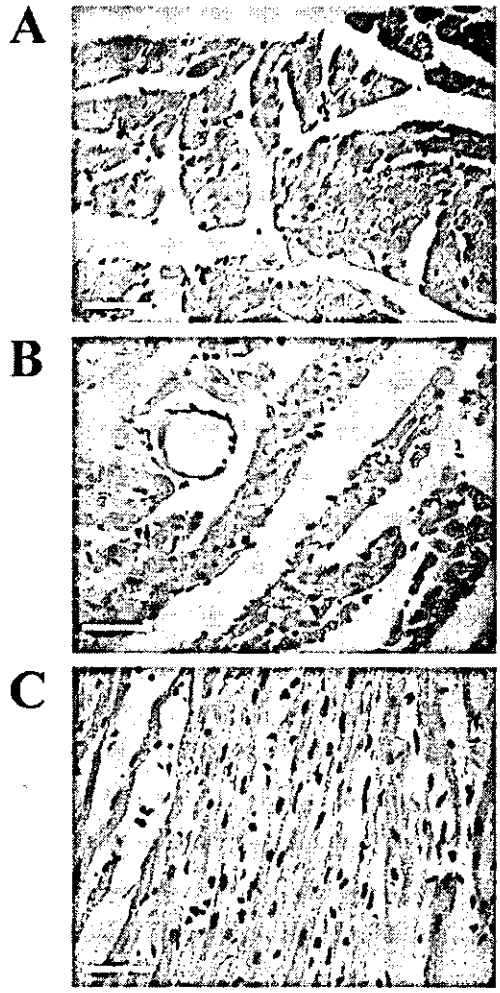

Figure 6. Immunohistochemistry for CRLR in rat cardiac tissue. Representative photomicrographs revealed that CRLR was localized in cardiomyocytes (A) and vascular endothelial cells (B). Negative control study (using mouse IgG) showed no positive staining in cardiac tissue (C). Original magnification ×400. Bar=20 μm.

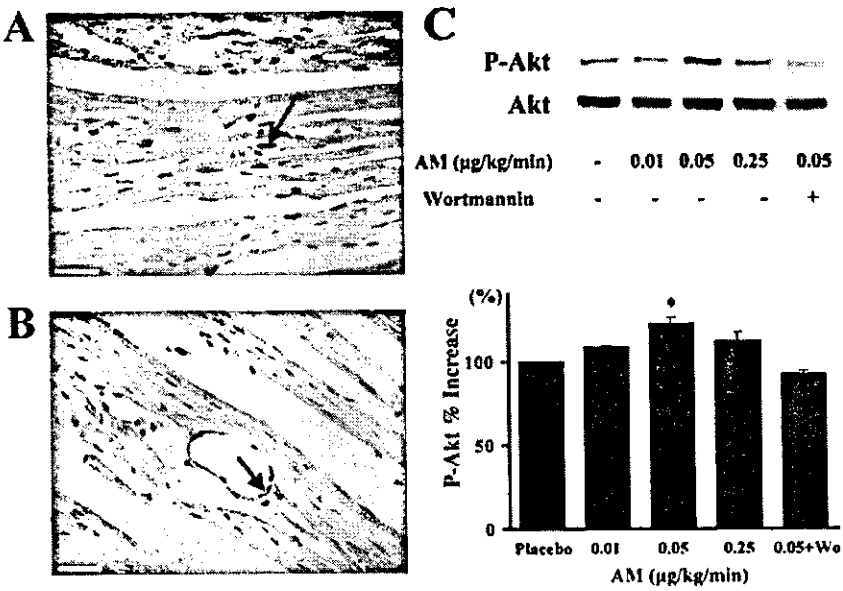

Figure 7. A and B, Immunohistochemistry for Akt phosphorylation in rat cardiac tissue. Infusion of AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) phosphorylated Akt predominantly in nuclei of cardiomyocytes (A, B) and vascular endothelial cells (B). Arrow indicates nuclei of cardiomyocytes with positive staining for P-Akt antibody. Arrowhead indicates nuclei of endothelium with positive staining for P-Akt antibody. Original magnification ×400. Bar=20 μm. C, Western blot analysis of AM-induced Akt phosphorylation in cardiac tissues. Infusion of AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) activated Akt in myocardial tissues exposed to ischemia/reperfusion. Pretreatment with wortmannin significantly inhibited AM-induced Akt phosphorylation. P-Akt indicates phosphorylated Akt; Wo, wortmannin. Data are mean±SEM. *$P<0.05$ vs placebo.

depends on the degree of myocyte apoptosis within 24 hours after ischemia/reperfusion.[19] Thus, the early prevention of myocyte apoptosis and the resultant reduced infarct size by AM may contribute to the hemodynamic improvement after ischemia/reperfusion. AM infusion reduced right ventricular systolic pressure, which may be attributable not only to the potent vasodilatory effects of AM but also to improvement in cardiac function.

Recently, Akt activation has been shown to reduce myocyte apoptosis and thereby prevent myocardial injury after transient ischemia.[10] Akt is the downstream effector molecule for signal transduction initiated by cardioprotective hormones such as insulin-like growth factor I.[20] Thus, Akt is considered to be a powerful survival signal in myocytes.[21] More recently, AM has been shown to activate the PI3K/Akt-pathway in vascular endothelial cells.[9] However, localization of AM-specific receptors in cardiac tissue had been unknown. The present study demonstrated that CRLR was present in rat cardiomyocytes and vascular endothelial cells and that AM infusion accelerated Akt phosphorylation in nuclei of cardiomyocytes and vascular endothelial cells. Furthermore, Western blot analyses demonstrated that AM 0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$ significantly increased phosphorylated Akt in cardiac tissue compared with placebo treatment and that pretreatment with wortmannin significantly inhibited Akt phosphorylation. Interestingly, pretreatment with wortmannin attenuated the AM-induced beneficial effects, such as reduction of infarct size, hemodynamic improvements, and inhibition of apoptosis. These findings suggest that AM infusion directly induces cardioprotective effects through the PI3K/Akt-dependent pathway.

In the present study, plasma AM level during infusion was much higher than baseline plasma level in rats, plasma level in normal human subjects (≈10 fmol/mL),[8] and plasma level in patients with acute myocardial infarction (≈14 fmol/mL).[22] These findings suggest that exogenously administered AM functions at pharmacological levels.

Preclinical studies have demonstrated that a variety of antioxidative or antiapoptotic agents reduce myocardial infarct size after ischemia/reperfusion.[23,24] However, few agents are clinically available for patients with coronary artery disease. In contrast, the safety and hemodynamic benefits of short-term treatment with intravenous AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) have been demonstrated in patients with heart failure[8] and patients with myocardial infarction.[25] Given the results of the present study, a prospective, randomized, placebo-controlled clinical trial should be planned.

Conclusions

Short-term infusion of AM significantly attenuated myocardial ischemia/reperfusion injury. These cardioprotective effects were attributed mainly to the antiapoptotic effects of AM via a PI3K/Akt-dependent pathway.

Acknowledgments

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

Takashi Iwase, MD; Noritoshi Nagaya, MD; Takafumi Fujii, MD; Takefumi Itoh, MD; Hatsue Ishibashi-Ueda, MD; Masakazu Yamagishi, MD; Kunio Miyatake, MD; Toshio Matsumoto, MD; Soichiro Kitamura, MD; Kenji Kangawa, PhD

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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From the Departments of Regenerative Medicine and Tissue Engineering (T. Iwase, N.N., T. Itoh), Cardiac Physiology (T.F.), and Biochemistry (K.K.), National Cardiovascular Center Research Institute, Osaka, Japan; Departments of Internal Medicine (N.N., M.Y., K.M.), Pathology (H.I.-U.), and Cardiovascular Surgery (S.K.), National Cardiovascular Center, Osaka, Japan; and Department of Medicine and Bioregulatory Sciences (T. Iwase, T.M.), University of Tokushima Graduate School of Medicine, Tokushima, Japan.

Reprint requests to Noritoshi Nagaya, MD, Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. E-mail nagayann@hsp.ncvc.go.jp

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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 LI33.1, 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

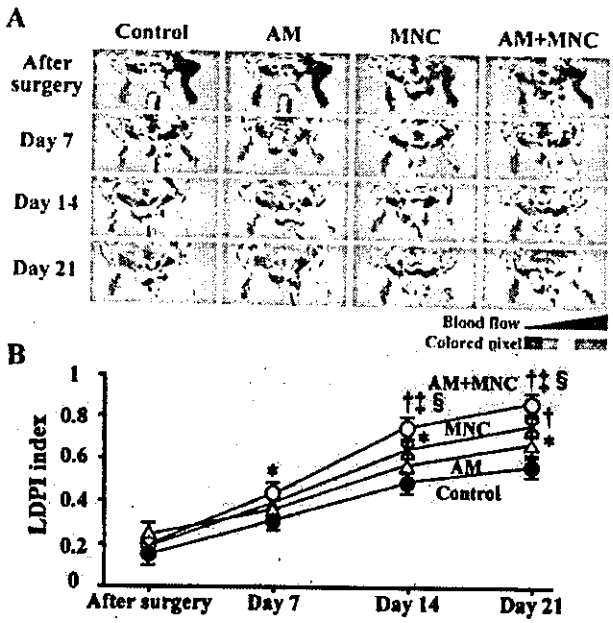

Figure 1. A, Representative examples of serial laser Doppler perfusion images. Blood perfusion of ischemic hindlimb increased notably in AM+MNC group (red to yellow). B, Quantitative analysis of hindlimb blood perfusion with LDPI index, ratio of ischemic to nonischemic hindlimb blood perfusion. Data 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).

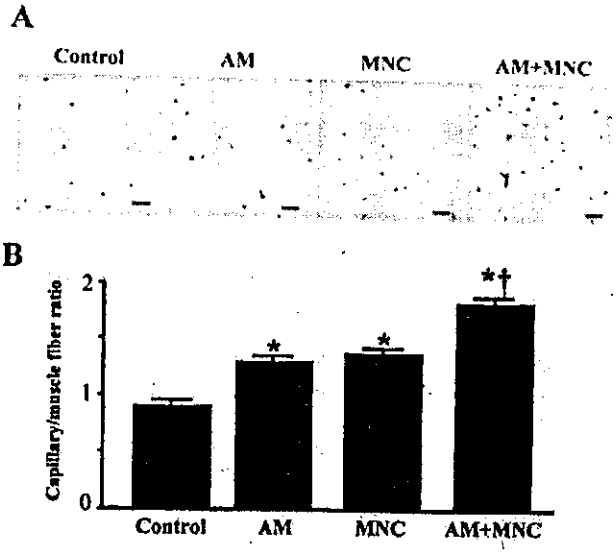

Figure 2. A, Representative photographs of alkaline phosphatase staining in ischemic hindlimb muscles. Capillary density in AM+MNC group was markedly higher than that in other groups. B, Quantitative analysis of capillary density in ischemic hindlimb muscles. Data are mean±SEM. *P<0.01 vs control; †P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

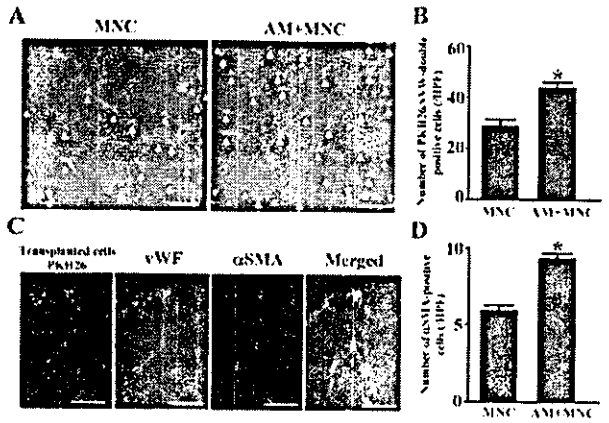

Figure 3. In vivo differentiation of transplanted MNCs. A, Representative photographs of MNC-derived vascular structures in MNC and AM+MNC groups. Red fluorescence (PKH26)–labeled MNCs were transplanted into ischemic thigh muscle. PKH26 (red)/vWF (blue) double-positive cells (pink, arrows) were frequently 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.

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

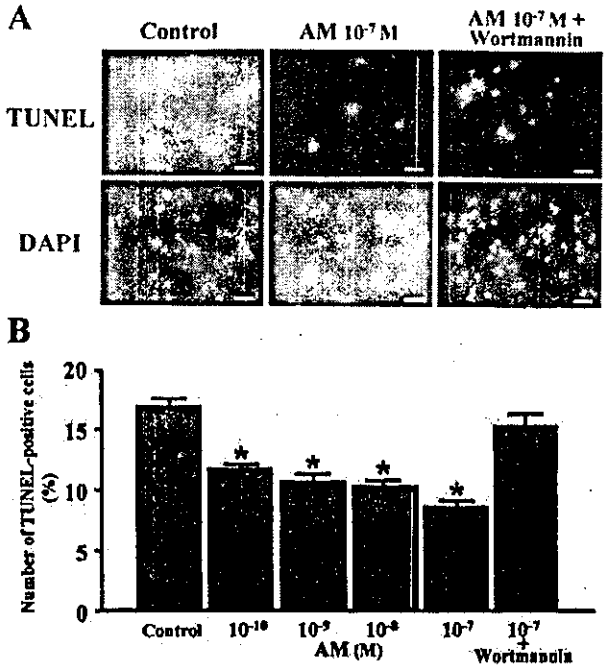

Figure 4. Apoptosis assay. A, Apoptosis of MNC was detected by TUNEL assay (green). Nuclei of MNC were stained with DAPI (blue). AM inhibited MNC apoptosis in serum-free medium. B, Quantitative analysis. AM decreased percentage of TUNEL-positive cells in dose-dependent manner. Pretreatment with wortmannin,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

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.

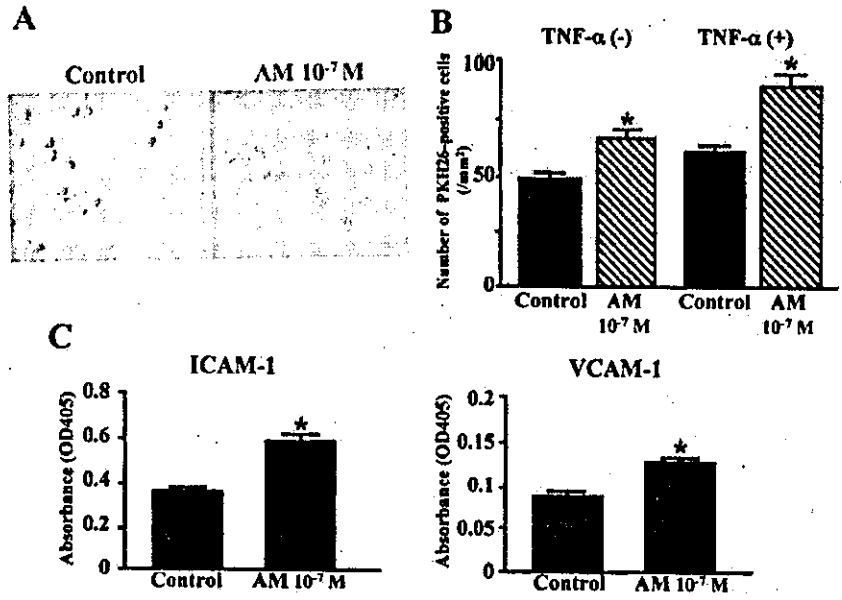

Figure 5. A and B, Adhesion assay. Representative photographs of red fluorescence–labeled MNC adhesion to HUVEC monolayer with and without AM (A). Quantitative analysis of MNC adhesion (B). Bars: 50 μm. C, Surface expression of ICAM-1 and VCAM-1 in HUVECs with or without AM. Data are mean±SEM. TNF indicates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

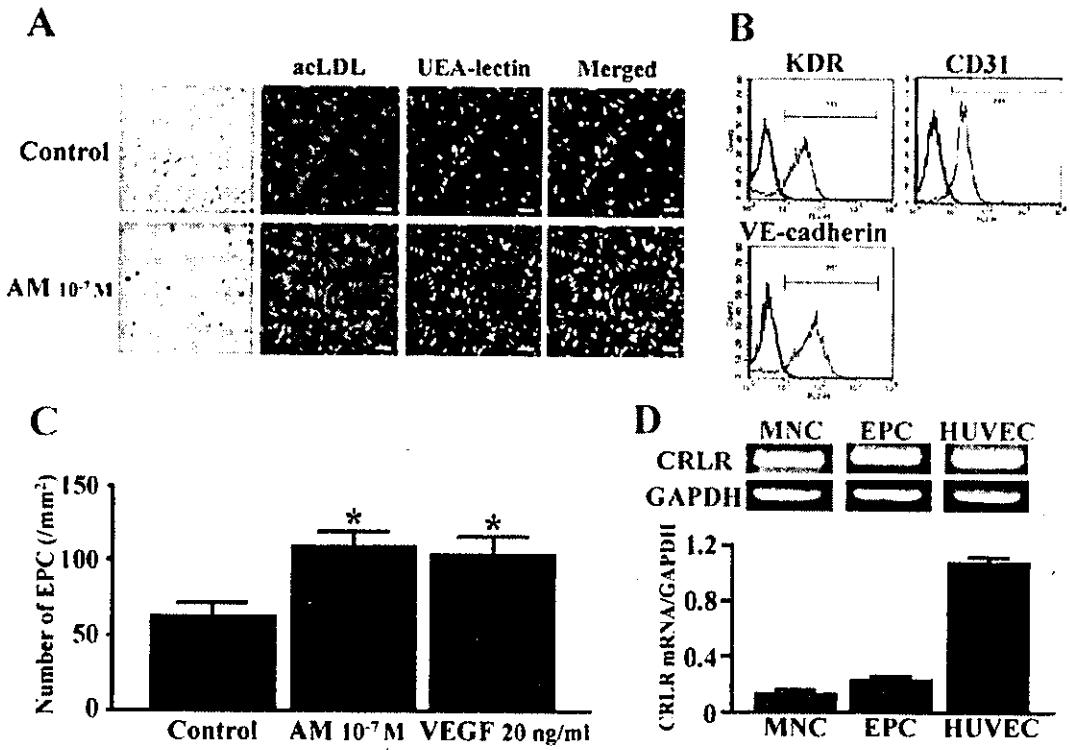

Figure 6. A through C, EPC culture assay. Cultured adherent cells took up DiI-acLDL (red) and FITC-labeled lectin (green) in same fields (A). Fluorescence-activated cell sorting analyses revealed that most adherent cells expressed KDR, VE cadherin, and CD31 (B). Culture of MNCs with AM significantly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

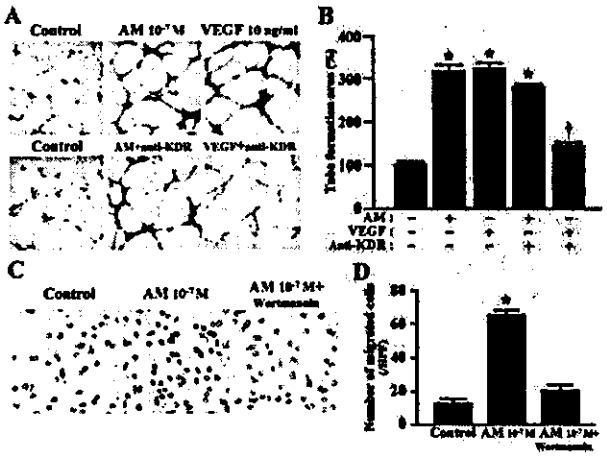

Figure 7. A and B, Matrigel assay. Representative photographs of tube formation (A). Quantitative analysis of tube formation area (B). Data are mean±SEM. *P<0.01 vs control; †P<0.01 vs VEGF. Bars: 20 μm. C and D, Migration assay. Representative photographs of migrated SMCs (C). Quantitative analyses 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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細胞シート工学を利用した組織再構築

■清水　達也・岡野　光夫
東京女子医科大学 先端生命医科学研究所

清水　達也
1992年東京大学医学部医学科卒業。99年同大学大学院医学系研究科修了，同年東京女子医科大学先端生命医科学研究所助手，2003年同講師，現在に至る。研究テーマは組織工学的手法による心筋組織再生。趣味は旅行。

Key words：再生医療，組織工学，細胞シート

はじめに

近年，欠損部あるいは機能不全に陥った組織・臓器に対する新たな治療法として再生医療が注目を集め，下肢虚血に対する骨髄単核球細胞移植など細胞を使ったいくつかの治療法が臨床応用されるに至っている。再生医療には細胞を注射針などで不全部に注入する細胞移植療法と組織工学 (tissue engineering) 的手法により細胞から組織を再構築したうえで移植する方法がある。後者はこれまでの医学だけでは実現が困難であった研究領域であり工学的な技術との融合により急速に進歩しつつある。ここでは，組織工学を基盤とした再生医療の現状について概説し，当研究所が開発した独自の組織工学的手法『細胞シート工学』による種々の組織再構築に関する研究を紹介する。

1．組織工学 (tissue engineering)

組織工学は1993年，工学者であるLangerおよび外科医であるVacantiが提唱した学際的な学問である。細胞から組織を再構築するという研究は皮膚などに関してそれ以前より行われていたが，彼らはマウスの背中で耳を再生させたことにより組織工学を世界に知らしめた。組織の再生には細胞，細胞の足場となる細胞外マトリックス (ECM)，細胞の分化・増殖のためのサイトカインが必要であるとし，その足場として3次元の生体吸収性材料を用いた。細胞を3次元の支持体に播種・培養後，生体内に移植すると，生体吸収性の支持体が徐々に分解，細胞が産生するECMと置換され生体に類似した組織が再構築されるという手法である。組織工学において組織再生の足場として用いられる生体材料の多くは生体吸収性の高分子である。これには天然高分子と合成高分子がある。いずれも酵素分解あるいは加水分解によって高分子主鎖が切断され吸収される。天然高分子の中でよく利用されているのは生体のECMの主成分であるコラーゲンである。一方，合成高分子としては，ポリ乳

Cell sheet engineering for tissue reconstruction：Tatsuya Shimizu, Teruo Okano, Institute of Advanced Biomedical Engineering and science, Tokyo Women's Medical University

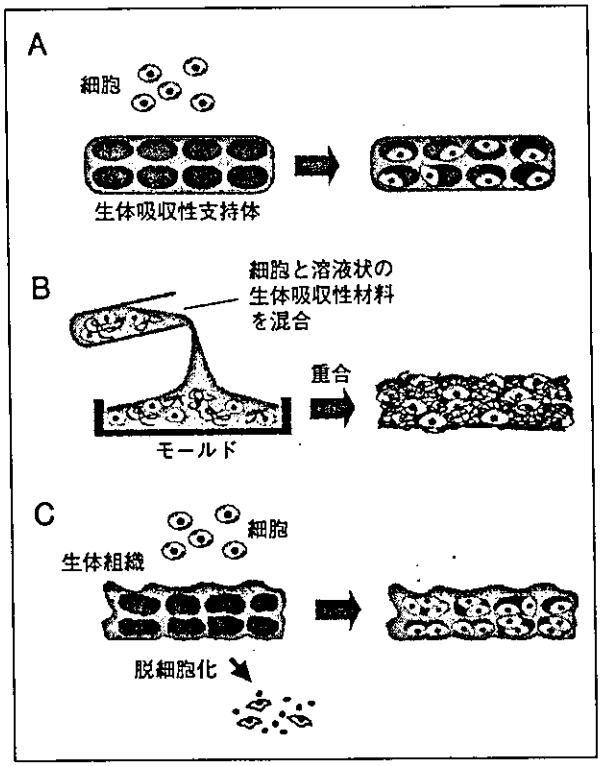

図1　生体吸収性支持体を用いた組織工学的手法
A. 生体吸収性高分子からなる支持体を作成し，それを足場として細胞を播種する手法。
B. 溶液状の支持材料と細胞を混合したのち重合する手法。
C. 生体由来の組織を脱細胞化したのちにそれを支持体として細胞を播種する方法。

酸 (PLA)，ポリグリコール酸 (PGA)，およびそれらの共重合体が最も盛んに使用されている。また，生体由来の組織が支持体として用いられる場合もある。具体的な方法としては①生体吸収性高分子からなる多孔性の3次元支持体を作製，それを足場として細胞を播種する方法（図1 A），②溶液状の生体吸収性材料と細胞を混合したのちモールドに流し込み，重合させることで細胞を3次元化する方法（図1 B），③生体組織を界面活性剤やマイクロウェーブを用いて脱細胞化した結合組織を支持体として細胞を播種し培養する方法（図1 C）がある。それぞれ支持体は生体が産生するECMと置換されて組織が再生される。

これら生体吸収性の3次元生体材料を細胞の足場として用いた組織工学の研究は殆どすべての組織に対して行われている。既に組織工学的に製造された軟骨・皮膚は商品化されており，また血管に関しても臨床応用されている。

2．細胞シート工学による組織再構築

生体吸収性の支持体を用いる組織工学的手法においては支持体内へ十分な細胞を播種することが困難なことやまた移植後支持体の分解に伴い炎症反応が生じることが問題となっている。また骨，軟骨，血管，弁など細胞が疎な組織の作製には適しているが心筋，肝臓など細胞の密な組織を作製するには新たな技術開発が必要となっている。そこで我々は最初から3次元的な足場を整えることにより組織を再生するという既存のコンセプトに対し，温度変化のみで細胞の接着・脱着を制御できる培養基材を用い，細胞をシート状に回収，この細胞シートを一つの組織として移植したり，積層化により3次元組織を再構築したうえで移植するという独自のコンセプト「細胞シート工学」を提唱し新たな治療法の確立に取り組んでいる。

この培養基材は通常のポリスチレン培養皿上に温度応答性高分子であるポリ（*N*-イソプロピルアクリルアミド）を電子線を用いて薄く（数十μm）共有結合させたもので，通常の培養温度である37℃では疎水性表面となり細胞接着性であるが，32℃以下では親水性表面に変化し細胞非接着性となる。この培養皿の使用により，接着した細胞をトリプシンやディスパーゼなどの蛋白分解酵素を用いること

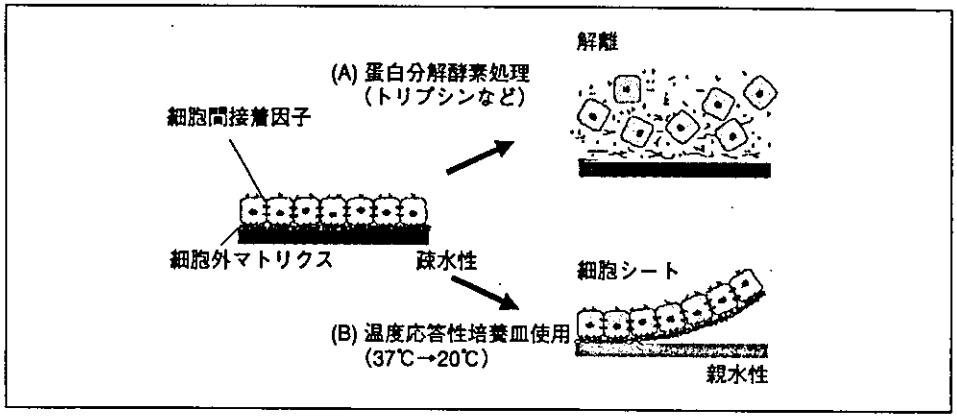

図2　温度応答性培養皿からの細胞シートの脱着

細胞を密に培養した場合は細胞と細胞が細胞間接着因子により互いに接着する。(A) 蛋白分解酵素を用いた場合は細胞と培養皿の接着が解離するとともに，細胞間接着も破壊されるため，それぞれの細胞は解離して浮遊することになる。(B) これに対し，温度応答性培養皿を使った低温処理においてはこの細胞間接着は全く影響を受けずに，シート下面のECMと培養皿表面の接着のみ解離するため細胞がシート状に脱着する。

なく脱着させることが可能である。さらに，細胞を密に培養し細胞が互いに接着した状態では，温度を降下させることにより細胞がその下面の接着因子・ECMとともに培養皿から脱着するものの，細胞間の結合は全く解離せず維持されるため蛋白分解酵素を用いたときのように細胞をばらばらにすることなく，シート状に回収できる（図2）。また細胞シート下面の接着因子が新たな培養基材や別の細胞シート上への移動時に糊の役目を果たすため速やかな接着・積層化が可能である。既にこの培養皿を用い，種々の細胞シートの回収が可能となっている。

細胞シートの再生医療への応用としては①単層シートの移植 ②同一細胞シートの積層化により構築した均一な組織の移植 ③数種の細胞シートの積層化により構築した層状構造を呈する組織の移植がある（図3）。それぞれ対象組織・臓器として ①角膜，膀胱（上皮），歯周組織，②心筋，③肝臓などが挙げられる。以下に，それぞれについてのこれまでの研究成果を示す。

3．単層シート

(1) 角　膜

角膜移植に関しては，ドナー角膜の不足が問題となっており，組織工学的手法による角膜再生が追求されてきた。角膜は組織構造が比較的単純な層構造を呈し，細胞シートによる再生医療のもっとも良い応用例の一つである。我々は，自己の健常側角膜輪部より採取した角膜上皮の幹細胞を温度応答性培養表面上で培養して細胞シートを作製，これを損傷した病変角膜部に移植する治療法を開発した。さらに，両眼性疾患に対しては，自己の口腔粘膜から採取した細胞を用いて細胞シートを作製し移植する方法も確立した。既に本法のヒトへの臨床応用を開始，視力の回復を確認している（大阪大学眼科との共同研究）。

(2) 膀　胱

現在，膀胱機能不全や悪性腫瘍などによる膀胱の欠損に対しては自己の消化管を使用した再建が行われているが，消化管粘膜の残存

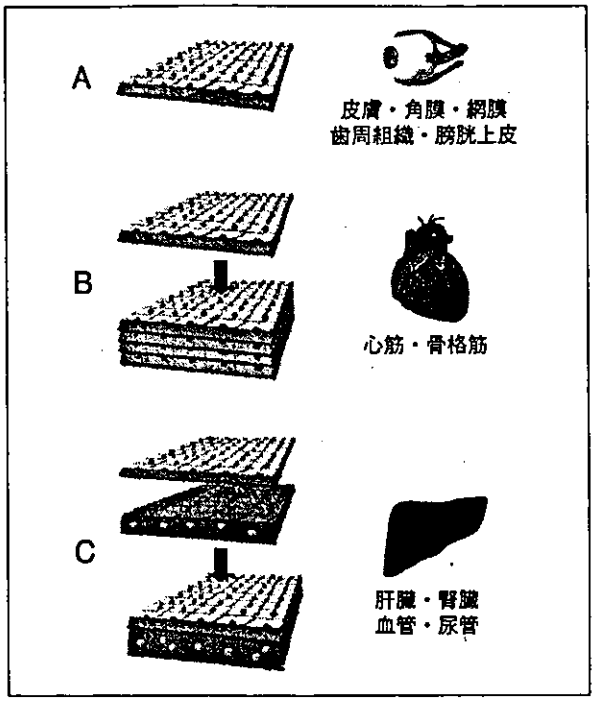

図3　細胞シートの再生医療への応用

温度応答性培養皿から回収した細胞シートの再生医療への応用として3つのコンセプトがある。単層シートの移植(A)。同一の細胞シートを重層化した均一な組織の移植(B)。異なる細胞シートの重層化による層状組織の移植(C)。

に起因する電解質異常，結石の形成などの合併症が大きな問題となっている。これらの問題を解決するために尿路上皮細胞シートを作製し，消化管粘膜を切除した消化管平滑筋層上に移植した。尿路上皮シートは消化管平滑筋層上に生着し，膀胱組織と同様の尿路上皮組織層が再生された。この粘膜置換型フラップは従来の手術方法に細胞シート技術を融合した新規の手法であり，早期の臨床応用が期待されている。

(3) 歯周組織

歯周病は，成人の8割以上が患っているといわれており，歯根膜を中心とする歯周組織の炎症により，放置すれば歯の脱落を招く。従来の治療法では失われた歯周組織は再生することなく，高齢化社会におけるQOL (Quality of Life) の低下の大きな要因といえる。そこで，我々は歯根膜組織由来細胞シートを歯周組織欠損部位へ移植することで，きわめて効果的に歯根組織膜が再生することを明らかとした(東京医科歯科大学歯学部との共同研究)。この技術は歯周組織の再生治療や歯根膜を持った次世代の人工歯根の開発に大きく貢献するものと考えている。

4．同一細胞シートの積層化

心　筋

心筋組織に対する再生医療としては，心筋細胞の代替として自己の筋芽細胞や骨髄由来の細胞を用いた浮遊液注入による細胞移植治療が既に臨床応用されている。一方，組織工学的なアプローチとしてコラーゲンゲル，ポリ乳酸，アルギン酸あるいはゼラチンからなる3次元の支持体を用いた心筋細胞の3次元培養の研究が報告されている。しかし，3次元支持体を用い心筋細胞培養を行うことは細胞の密な接着や自由な収縮弛緩の妨げになると考えられる。そこで我々は心筋細胞シートを重層化することで支持体を用いない心筋組織の再生を追求してきた。重層化した心筋細胞シート間には電気的にも形態的にも結合が生じ，組織全体が同期して拍動することが確認され，*in vitro*で4枚まで積層化したところ肉眼レベルで拍動する心筋組織が構築された。さらにヌードラット背部皮下組織への移植実験を行ったところホストの心電図とは異なるグラフト由来の電位が確認された。また心筋グラフトの肉眼レベルでの拍動が確認さ

れるとともに組織切片上，多数の新生血管を認め，心筋様組織が再生されていた。既に移植後1年まで，心筋グラフトが拍動を維持したまま生着しうることを確認している。さらに重層化心筋細胞シートの心筋梗塞モデルへの移植実験により心機能が改善することが確認されている（大阪大学臓器機能制御外科との共同研究)。将来，幹細胞生物学の発展によりヒトに移植可能な心筋細胞の分化誘導法が確立されれば体外で再生した心筋組織を不全心に移植することも可能となることが予測される。

5．異なる細胞シートの積層化

肝　臓

肝臓は生体内である程度の再生能をもつものの，その形態・機能の複雑さ故に組織工学的手法での再生が困難な組織のひとつである。肝組織は肝実質細胞と血管内皮細胞が層状に配列し，生体内における合成，代謝を効率的に行っている。肝実質細胞は，通常の培養環境ではその分化機能を著しく低下させ，長期培養も困難であることが知られている。そこで肝細胞シートと内皮細胞シートの重層化共培養を行ったところ，アルブミン合成能を維持した2ヶ月以上にわたる肝細胞の長期培養が可能となっている。これは，肝細胞と内皮細胞との層状の接着により生体に似た環境を再現し，細胞相互間のコミニュケーションを可能にしたことによるものと考えられる。この手法を基盤として，3次元的な肝組織を再生・移植するための技術開発を行っている。

以上に挙げた組織・臓器の他，皮膚・網膜・尿管・血管・腎臓などに関しても細胞シートの技術を使った研究を進めている。

おわりに

再生医療は今まで治療が困難であった疾患に対する新たな治療法として期待され，種々のアプローチで開発が行われ，研究者人口も増大している。その中で温度応答性培養皿およびそれを使った細胞シート工学は既存の培養技術では不可能であったシート状の細胞の回収・移動・重層化を実現し世界的にも注目されており，再生医療分野をさらに発展させる新技術として貢献するものと考える。

＜BIO Information＞

第18回 日本臨床内科医学会 in 岡山のお知らせ

会　期：2004年9月19日（日）～20日（月・祝）
会　場：岡山衛生会館・おかやま三光荘・岡山プラザホテル
テーマ：21世紀の理想の医療を目指して
　　～良質な医療提供とかかりつけ医の役割～
会　長：亀山一郎（亀山内科院長）

主なプログラム：基調講演　医療保険制度と診療報酬体系（講師　遠藤久夫・学習院大学経済学部教授）
　　インフルエンザの展望（講師　柏木征三郎・福岡日赤血液センター所長）
　　良質な医療提供とかかりつけ医の役割（講師　伴信太郎・名古屋大学医学部教授）

教育講演／ワークショップ／シンポジウム／市民公開講座　他

組織工学の心血管病への応用

清水達也*
SHIMIZU Tatsuya
*東京女子医科大学先端生命医科学研究所

SUMMARY

近年，欠損部あるいは機能不全に陥った組織・臓器に対する新たな治療法として再生医療が注目を集め，循環器領域においても組織工学的手法により細胞から血管，弁，心筋組織を再構築する研究が加速している．世界的には生体吸収性の支持体を細胞の足場とする手法が主流であるが，われわれは支持体を用いることなくシート状の細胞を積層化することにより3次元組織を再構築する独自の手法『細胞シート工学』を確立した．すでに肉眼レベルで拍動する心筋組織の構築に成功している．今後より高機能な組織の作製には，医学と工学の融合による新たな技術開発が必須である．

POINTS

■組織工学は医学と工学の融合による新たな学問である．
■生体吸収性高分子を細胞の足場とするのが組織工学の主流である．
■シート状の細胞の積層化により3次元組織の構築が可能である．
■細胞シート工学により肉眼レベルで拍動する心筋組織の再構築が可能である．

KEY WORDS

組織工学，生体吸収性高分子，細胞シート

はじめに

近年，欠損部あるいは機能不全に陥った組織・臓器に対する新たな治療法として再生医療が注目を集め，循環器領域においても細胞を使ったいくつかの治療法が臨床応用されるに至っている．再生医療には回収した細胞を注射針などで不全部に注入する細胞移植療法と組織工学的手法により細胞から組織を再構築したうえで移植する方法がある．後者はこれまでの医学だけでは実現が困難であった研究領域であり，工学的な技術との融合により急速に進歩しつつある．ここでは，血管，弁，心筋に関する組織工学の近年の動向を概説するとともに，当研究所が開発した独自の組織工学的手法『細胞シート工学』による3次元心筋組織の再構築に関する研究を紹介する．