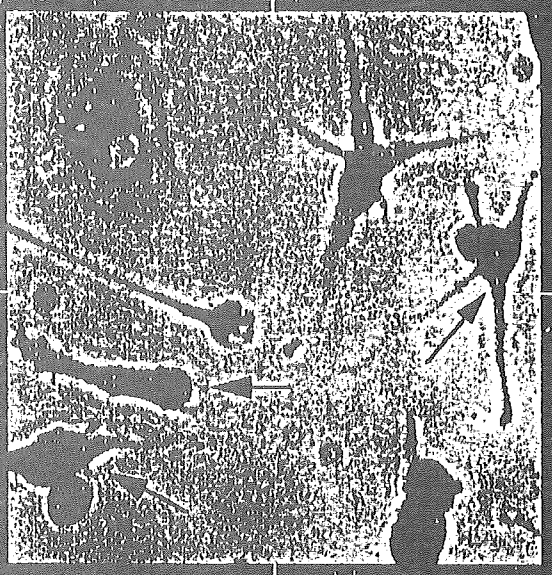

H. Mori • H. Matsuda (Eds.)

Cardiovascular Regeneration Therapies Using Tissue Engineering Approaches

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Closing Remarks

Recent advances in molecular biology have been remarkably effective in elucidating the pathophysiology and mechanisms of various cardiovascular diseases. New techniques in genetic, cellular, and tissue engineering have had significant impact and sparked a revolution in therapy for severe diseases.

In the heart, cardiac myocytes have long been considered terminal differentiated cells without the potential to multiply and participate in tissue repair. This is in contrast to hepatocytes, which can regenerate when injured. However, developments in molecular cardiology and angiology have raised the possibility of neovascularization as well as the regeneration of myocardium.

Japanese scientists working in the field of cardiovascular disease have been at the forefront of research into the regeneration of impaired heart tissue using methods as diverse as angiogenesis, myogenesis, and tissue engineering. These studies, featuring both experimental and clinical assessment, are gaining momentum rapidly and drawing attention to translational research. As shown in this publication, angiogenic cytokines, cardiovascular stem cells, and tissue engineering tools are substantially contributing to this revolution. Another highlight has been the development in many leading centers and hospitals in Japan of therapeutic angiogenesis and vasculogenesis for the treatment of ischemic diseases of the myocardium and the limbs. We expect that the developments summarized in the book may have a substantial impact on the progress of regeneration medicine.

Finally, we express thanks for the following research grants: Cardiovascular Disease (13C-1 and 16C-6) and Health and Labor Sciences Research Grants (Saisei-003 and Cardiovascular Res-001). These grants have facilitated significant progress in the field of regeneration therapy in cardiovascular disease.

October 20, 2004

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Clinical Survey of Cell Therapy in Japan

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Kurume University School of Medicine, and Collaborating Institutions of Cardiovascular Disease Grant from MHLW (13C-1)

Summary. Background: Therapeutic angiogenesis by using cells is being performed in Japan. However, it is unknown in how many centers and how these therapies are performed. The efficacy and side effects are also unknown. Thus, we conducted the survey by mailing questionnaire within Japan. Methods and results: Two surveys were performed in 2003. The first survey unveiled that cell therapy was performed in 32 facilities until October 2003. The second survey unveiled the followings. (1) The total number of performed cases was 221. 153 patients (69.2%) had arterio-sclerosis obliterance (ASO), 56 patients (25.3%) thromboangitis obliterans (TAO, Burger's disease), and 12 patients (5.4%) other conditions. (2) The sources of cells were bone marrow-mononuclear cells (61.5%), peripheral-mononuclear cells (9.5%), and peripheral $CD34^+$ cells (22.1%). A few patients (6.7%) were treated with a cytokine only (granulocyte-colony stimulating factor: G-CSF). (3) Inclusion criteria were the same for most facilities, such as patients with PAOD, especially with critical limb ischemia with rest pain, non-healing ischemic ulcers and non-candidates for non-surgical or surgical revascularization. All facilities excluded patients with histories of malignant disorder during the past 5 years, proliferative diabetic retinopathy, pregnancy, proliferative blood disease, uncontrolled ischemic heart disease, rheumatic arthritis, or psychiatric disease. (4) Subjective improvement was observed in 138 of 199 patients (69%). Objective improvements for ABI, TcO2 or angiographic

findings were observed in 98 of 182 patients (53.8%). (6) Three of 221 patients (1.4%) died after cell therapy. One died from cerebro-vascular attack (thrombo-embolizm), and two died from acute myocardial infarction (AMI). Conclusion: Our clinical survey has shown that cell therapy is being performed in many medical centers in Japan. It seems to be safe and effective for patients with PAOD with no surgical options

Key Words. Cell therapy, Clinical survey, Angiogenesis, PAOD

Introduction

Recently, it has been reported that treatment with angiogenic growth factors, such as vascular endothelial growth factor, for patients with peripheral artery obstructive disease (PAOD) could increase collateral blood flow in ischemic limbs (Asahara T and Isner M 1999). Therapeutic angiogenesis can be achieved by use of not only growth factors (Baumgartner I, et al. 1998) but also cells, such as bone marrow cells (Shintani S, et al. 2001), peripheral cells (Kalka C, et al. 2000) and colony stimulating factor (CSF)-induced stem cells (Seiler C, et al. 2001). Preclinical studies have shown that such cell therapy increased collateral flow of ischemic limbs, and also could relieve ischemic pain and ulcers (Tateishi-Yuyama E, et al. 2002). Because cell therapies use their own cells (autologous transplantation), they are associated with fewer problems, such as immunological complications and ethical issues. However, it is unknown in how many centers and how these therapies are performed. The efficacy and side effects are also unknown. Thus, we conducted the survey by mailing questionnaires in Japan as a project funded by Japanese Ministry of Health, Labor, and Welfare Grant-in-Aid for Scientific Research in Japan.

Methods

Two steps of survey of cell therapy were performed in 2003. The first survey was performed to examine whether cell therapy was performed for patients with PAOD. With the permission of the Japanese Circulation So-

ciety questionnaire were mailed to 190 facilities throughout Japan (125 Internal Medicine or Cardiology Department of universities, 65 national hospitals). The basic data such as names and addresses of facilities performing cell therapy were obtained.

Then, in order to examine the following issues, the questionnaire was mailed to the facilities in which cell therapy was performed (second survey): (1) Cause of chronic limb ischemia, (2) modes of cell therapy, (3) number of patients, (4) inclusion and exclusion criteria, (5) efficacy and (6) complications. The survey was co-performed with TACT (Therapeutic Angiogenesis by Cell Transplantation) group.

Table 1. Etiologies of PAOD performed cell therapy

Etiology of PAOD	No of patients
Total	221
ASO	153 (69.2%)
TAO	56 (25.3%)
Others	12 (5.4%)

ASO: arteriosclerosis obliterans, TAO: thromboangitis obliterans

Results

In the first survey, we obtained responses from 146 of 190 facilities (76%). The first survey unveiled that cell therapy was performed in 32 facilities until October 2003. In the second survey, we obtained response from 28 of 32 facilities (88%). The second survey unveiled the following.

Cause of chronic limb ischemia treated with cell therapy:

Etiologies of PAOD are shown in **Table 1**. The total number of cases performed was 221. 153 patients (69.2%) had arterio-sclerosis obliterance

(ASO), 56 patients (25.3%) thromboangitis obliterans (TAO, Burger's disease), and 12 patients (5.4%) had other conditions.

Modes of cell therapy performed:

The sources of cells were bone marrow-mononuclear cells (136 cases, 61.5%), peripheral-mononuclear cells (21 cases, 9.5%), and peripheral $CD34^+$ cells (49 cases, 22.1%). A few patients (6.7%) were treated with a cytokine only (granulocyte-colony stimulating factor: G-CSF). (Table 2).

Table 2. Modes of cell therapy

Source of cells using cell therapy	No. of patients
Total	221
Bone marrow- mononuclear cells	136 (61.5%)
Peripheral-mononuclear cells	21 (9.5%)
Peripheral stem cells ($CD34^+$)	49 (22.2%)
Administration of G-CSF only	15 (6.8%)

G-CSF: granulocyte-colony stimulating factor

Inclusion or exclusion criteria of cell therapy:

Inclusion criteria were similar for most facilities. They included patients with PAOD, especially with critical limb ischemia with rest pain, non-healing ischemic ulcers and non-candidates for non-surgical or surgical revascularization. Fontaine's classification was used in 31 of 32 facilities (96.9%). In most facilities (81.3%), Fontaine's classification more than III was indicated. All facilities excluded patients with a history of malignant disorders during the past 5 years, proliferative diabetic retinopathy, pregnancy, proliferative blood disease, uncontrolled ischemic heart disease, rheumatic arthritis, and psychiatric disease. To exclude malignant disorders, occult blood in stool, measurement of tumor maker, such as CEA or SCC, and brain CT were performed. Upper gastro-intestinal fiber, total colon fiber, computed tomography of chest or abdominal area, and

upper abdominal echo were also performed as necessarily. Written informed consent was mandatory in all facilities.

Assessments of efficacy:

To evaluate the subjective efficacy, the severity of pain was assessed by pain scale or visual analogue scale in all facilities. To assess the objective changes, the following parameters were monitored: the size of ischemic ulcer and ankle-brachial index (ABI) in all facilities, and laser doppler flow ratio or transcutaneous oxygen pressure (TcO2) in some facilities. Angiography, including digital subtraction angiography, was performed in all facilities.

Efficacy:

The data for subjective outcomes were not obtained in 22 of 221 patients (10.0%). Subjective improvement was observed in 138 of 199 patients (69%). The objective outcomes could not be evaluated in 32 of 221 patients(14.5%). Objective improvement of at least one parameter was observed in 98 of 182 patients (53.8%).

Adverse events or complications:

Three of 221 patients (1.4%) died after cell therapy. One died from cerebro-vascular attack (thrombo-embolizm), and two died from acute myocardial infarction (AMI) after bone marrow cell transplantation. In the patients with AMI, autopsy showed coronary restenosis of previous percutaneous coronary intervention (Tateishi-Yuyama E, et al. 2002). No relation of death to cell therapy was demonstrated in this survey. No embolic episode was reported for G-CSF treated patients. Although, various lineages of cells, such as fibroblasts, osteoblasts, and endothelial cells, may have been implanted with endothelial progenitor cells, angioma, bone formation or increased fibrosis was not reported. Minor edema or pain of the iliac crest was reported in some patients with bone marrow puncture. No other complications were reported in this survey.

Discussion

This clinical survey disclosed two important information of cell therapy performed in Japan. First, over 200 patients were treated by cell therapy in a few years. The most common etiology of chronic limb ischemia was ASO, and the next one was TAO. The types of cell used were bone marrow cells, peripheral cells and G-CSF stimulated cells. Bone marrow cells were most frequently used and then peripheral cells.

Second, this survey disclosed the efficacy and safety of cell therapy. Subjective improvement was observed in 69%, and objective improvement was observed in 53.8% of all cases. Most facilities extensively monitored adverse outcomes, and no major adverse effect was reported. In conclusion, cell therapy seems to be safe and effective for patients with PAOD with no surgical options.

Study limitations

First, the inclusion or exclusion criteria were not strictly uniform among facilities and various etiologies of PAOD might have been included. Second, subjective and objective outcomes were not assessed using the same criteria. Third, information on the detailed method of cell therapy was lacking. These limitations may have resulted in some differences in the assessment of the effects of cell therapies among facilities. In order to determine the efficacy and safety of cell therapies, a double-blind placebo controlled trial with the uniform protocol is desired.

References

Baumgartner I, Pieczek A, Manor O, et al.(1998) Constitutive expression of ph VEGF 165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. Circulation 97: 1114-23

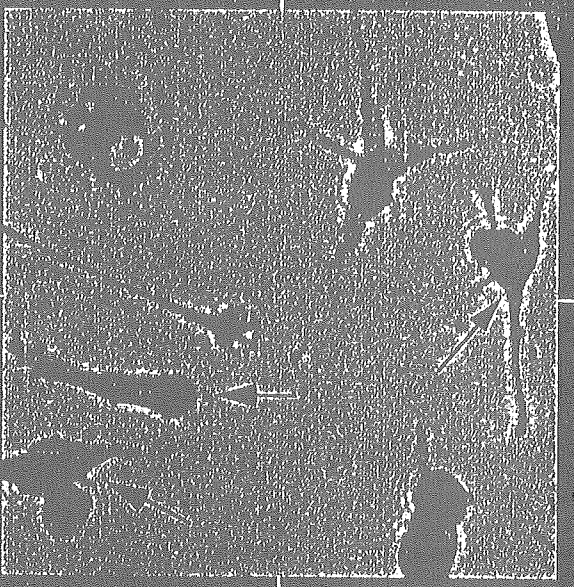
Isner M, Asahara T (1999) Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. J Clin Invest 103: 1231-36

Kalka C, Masuda H, Takahashi T, et al.(2000) Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci USA 97:3422-27

Seiler C, Pohl T, Wustmann K, et al.(2001) Promotion of collateral growth by granulocyte-macrophage colony-stimulating factor in patients with coronary artery disease. A randomized, double blind, placebo-controlled study. Circulation 104; 2012-17

Shintani S, Murohara T, Ikeda H, et al.(2001) Augmentation of postnatal neovascularization with autologous bone marrow transplantation. Circulation 103: 897-95

Tateishi-Yuyama E, Matsubara H, Murohara T, et al.(2002) Therapeutic angiogenesis for patients with limb ischemia by autologous transplantation of bone-marrow cells: a pilot study and a randomized controlled trial. Lancet 360: 427-35



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Cardiovascular Regeneration Therapies Using Tissue Engineering Approaches

Chapter 4: NEW ASPECTS OF ANGIOGENESIS

Chapter 5: CLINICAL RESULTS OF THERÁPEUTIC ANGIOGENESIS AND VASCULOGENESIS

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Off-Pump Coronary Artery Bypass Grafting Using Only Arterial Grafts in Elderly Patients

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Background. This study aimed to elucidate the safety and feasibility of off-pump coronary artery bypass grafting with only arterial grafts for elderly patients.

Methods. Of 653 patients who underwent off-pump coronary artery bypass grafting from April 2000 to December 2003, 581 patients did so with only arterial grafts. The average age was 66.9 ± 9.3 years. The patients were divided into the elder group E (75 years old or more: 111 cases) or the younger group Y (younger than 75 years old: 470 cases). The mean follow-up term was 21 ± 12 months.

Results. Additive and logistic EuroSCOREs of group E were significantly higher than those of group Y ($p < 0.0001$). The number of bypass grafts was 3.3 ± 0.9 in group E and 3.3 ± 1.1 in group Y ($p = 0.43$). The proportion of total revascularization was 74% (82 of 111) in group E and 80% (377 of 470) in group Y ($p = 0.15$). The number of bilateral implementations of internal thoracic artery was 10 (9.0%) in group E and 196 (42%) in group Y ($p < 0.0001$). The graft patency rate was 98.7% in group E and 97.8% in group Y ($p = 0.96$). Hospital mortality was 2.7% (3 of 111) in group E and 0.2% (1 of 470) in group Y ($p = 0.095$). The causes of death were unrelated to cardiac events. Major adverse cardiac events occurred in 5 patients (5.1%) in group E and in 24 patients (5.6%) in group Y ($p > 0.99$).

Conclusions. Off-pump coronary artery bypass grafting using only arterial grafts in elderly patients is as safe and feasible as in young patients.

(Ann Thorac Surg 2005;80:144–8)

Off-pump coronary artery bypass grafting (OPCAB) has been widely adopted in response to the suspected hazards of cardiopulmonary bypass and, possibly, because it offers an attractive alternative to percutaneous catheter intervention in coronary artery disease. Recently, OPCAB has become a standard surgical option for coronary artery disease owing to the development of such equipment as the stabilizer and associated techniques. Furthermore, the less invasive nature and the provision of satisfactory outcome of OPCAB have expanded the operative indications to high-risk cases such as elderly patients [1–11]. On the other hand, the selection of graft material, with or without cardiopulmonary bypass, has been a controversial topic for many years. Although it is common knowledge that the internal thoracic artery (ITA) yields the most reliable graft, not a few papers have suggested that other arterial grafts are advantageous, with respect to venous grafts, in terms of long-term graft patency [12–19]. Therefore, we have adopted OPCAB using only arterial grafts (ie, "total arterial OPCAB") in elderly patients, with positive results so far. The objective of this study is to elucidate the safety and feasibility of total arterial OPCAB in elderly patients.

Material and Methods

Between April 2000 and December 2003, 653 consecutive patients underwent isolated OPCAB (98% of all isolated coronary artery bypass grafting cases in this period). Of these, total arterial OPCAB was performed in 581 patients. The mean age was 66.9 ± 9.3 years, and 80.2% (n = 466) were male. The mean follow-up term was 21 ± 12 months. The patients were divided into the elderly group (group E; n = 111), in which age was 75 years or more, and the young group (group Y; n = 470), in which age was less than 75 years, at the time of operation. These different age groups did not differ in operative indications. Operative indication was determined by the angiographic findings whether the patients were symptomatic or not. Advanced age was not a contraindication to the operation, and no patients were excluded from the operation because of comorbid diseases. The number of patients who had stents placed during the same period was 2,545. Details of our basic strategy and surgical procedure of OPCAB were reported previously [20, 21]. All patients underwent OPCAB except those patients who required conversion to cardiopulmonary bypass because of hemodynamic instability. Arterial grafts were used in all patients except when arterial grafts were not available. We prefer multiple and complete coronary

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Table 1. Preoperative Variables

Variable	Group E	Group Y	*p* Value
Female (%)	39 (35.1%)	76 (16.2%)	<0.0001
Age (y)	77.8 ± 2.6	63.5 ± 7.9	<0.0001
Preoperative hemoglobin (g/dL)	12.2 ± 1.5	13.4 ± 1.7	<0.0001
Body mass index (kg/m^2)	23.0 ± 3.1	23.9 ± 3.1	0.013
CTR (%)	53.2 ± 5.6	49.8 ± 5.4	<0.0001
LVEDVI	84.7 ± 27.6	88.3 ± 30.3	0.32
LVESVI	45.3 ± 20.5	46.6 ± 25.1	0.67
EF	0.467 ± 0.122	0.490 ± 0.117	0.11

All continuous values are expressed as mean ± standard deviation.

CTR = cardiothoracic ratio; EF = ejection fraction; LVEDVI = left ventricular end-diastolic volume index; LVESVI = left ventricular end-systolic volume index.

revascularization with composite or sequential grafting using all available arterial grafts, especially in situ arterial grafts. Aortic no-touch technique is part of the basic philosophy. The flow volume and pattern were measured after the accomplishment of the anastomosis intraoperatively. To prevent arterial spasm, continuous intravenous infusion of diltiazem (0.5 to 1.0 μg/kg) or nicardipine (0.1 to 0.2 μg) was used intraoperatively and during the first 16 hours after the operation. Diltiazem (100 to 200 mg/day) or amlodipine (2.5 to 5.0 mg/day) was then prescribed for oral administration in conjunction with aspirin (162 mg/day), beginning the next morning. Coronary and graft angiography was performed at 10 to 21 days (mean, 14 days) after OPCAB. Graft patency was independently assessed by the interventional cardiologists.

The European System for Cardiac Operative Risk Evaluation (EuroSCORE) was applied as a risk stratification model to score the risk value of our patient groups for mortality [22, 23]. Institutional approval of the study was obtained, and each patients within the study gave informed consent for serving as a subject.

All data were reviewed retrospectively. All continuous values are expressed as mean ± standard deviation. A comparative analysis was performed between the different patient groups. Differences were analyzed with univariate analysis (χ^2 test, two-tailed Student's *t* test, and Mann-Whitney *U* test, as appropriate). A value of *p* less than 0.05 was used to indicate significance.

Results

The proportion of 75 years or more was not different between the patients who underwent OPCAB and catheter intervention. Preoperative variables are shown in Table 1. The proportion of female patients was significantly greater in group E (n = 39; 35.1%) than in group Y (n = 76; 16.2%; $p < 0.0001$). Preoperative hemoglobin level was less in group E (12.2 ± 1.5 g/dL) than in group Y (13.4 ± 1.7 g/dL; $p < 0.0001$). The body mass index was smaller in group E (23.0 ± 3.1 kg/m^2) than in group Y (23.9 ± 3.1 kg/m^2; $p < 0.013$). Preoperative cardiothoracic ratio was greater in group E (53.2% ± 5.6%) than in group Y (49.8% ± 5.4%).

Preoperative combined diseases are displayed in Table 2. The percentage with percutaneous catheter intervention history was not different between the two groups. Chronic obstructive pulmonary disease was present in 7.2% (n = 8) of group E and in 1.7% (n = 8) of group Y ($p = 0.005$). The proportion with acute myocardial infarction as a preoperative condition was significantly greater in group E (9.9%; n = 11) than in group Y (3.8%; n = 18; $p = 0.014$).

Table 2. Preoperative Combined Diseases

Variable	Group E	Group Y	*p* Value
PCI history	23 (20.7%)	93 (19.8%)	0.79
Hypertension	67 (60.4%)	240 (51.1%)	0.09
Hyperlipidemia	54 (48.6%)	243 (51.7%)	0.6
Diabetes mellitus	41 (36.9%)	194 (41.3%)	0.45
COPD	8 (7.2%)	8 (1.7%)	0.005
Craniocervical vascular disease	32 (28.8%)	109 (23.2%)	0.22
Renal failure	5 (4.5%)	29 (6.2%)	0.65
Preoperative IABP	5 (4.5%)	13 (2.8%)	0.36
Peripheral artery disease	9 (8.1%)	34 (7.2%)	0.69
Acute myocardial infarction	11 (9.9%)	18 (3.8%)	0.014

COPD = chronic obstructive pulmonary disease; IABP = intraaortic balloon pump; PCI = percutaneous catheter intervention.

Table 3. Perioperative Variables

Variable	Group E	Group Y	*p* Value
Operation time (min)	279 ± 71	320 ± 84	0.0002
No. of anastomosis	3.25 ± 0.90	3.33 ± 1.05	0.43
Redo	3 (2.7%)	12 (2.6%)	>0.99
Emergent	7 (6.3%)	16 (3.4%)	0.18
Use of bilateral ITA	10 (9.0%)	196 (41.7%)	<0.0001
Complete revascularization	82 (73.9%)	377 (80.2%)	0.15
Blood transfusion	46 (41.4%)	103 (21.9%)	<0.0001
Gastroepiploic artery	0 (0%)	20 (4.3%)	0.03

All continuous values are expressed as mean ± standard deviation.

ITA = internal thoracic artery.

The perioperative variables are shown in Table 3. The operation time was shorter in group E (279 ± 71 minutes) than in group Y (320 ± 84 minutes; $p = 0.0002$). The number of anastomoses was 3.3 ± 0.9 in group E and 3.3 ± 1.0 in group Y ($p = 0.43$). The percentage with use of bilateral ITA was significantly less in group E (9.0%; 10 patients) than in group Y (41.7%; 196 patients; $p < 0.0001$). The complete revascularization rate was 73.9% (82 of 111 patients) in group E and 80.2% (377 of 470 patients) in group Y ($p = 0.15$). Blood transfusion was required by more patients in group E (46.9%; 46 patients) than in group Y (24%; 103 patients; $p < 0.0001$).

The EuroSCORE is displayed in Table 4. The additive EuroSCORE of group E was significantly greater than that of group Y. Age is recognized as one of the important risk factors in the EuroSCORE. Therefore, the EuroSCORE excluding the age-related score, which was calculated by subtracting the contribution of age from EuroSCORE, is shown simultaneously. The score of group E remained significantly higher than that of group Y ($p < 0.0001$). Logistic EuroSCORE, which shows the predictive mortality rate, is also shown in Table 4.

Overall hospital mortality was 0.68% (n = 4). Hospital death was 2.7% (n = 3) in group E and 0.2% (n = 1) in group Y ($p = 0.095$). None of the hospital deaths were related to cardiac disease; the causes were bowel ischemia, mediastinitis, cerebral hemorrhage, and interstitial pneumonia. As compared with logistic EuroSCORE, overall actuarial hospital mortality was significantly lower than predictive mortality rate. The operative outcome is shown in Table 5. The incidence of major adverse cardiac events, perioperative myocardial infarction, perioperative stroke, and wound dehiscence was not different in the two groups ($p > 0.99$). Atrial fibrillation occurred more often in group E (20.4%; n = 20) than in group Y (11.9%; n = 51; $p = 0.03$).

Postoperative angiography was performed in 525 patients (90.4%). The definition of patency was based on the grading by FitzGibbon and colleagues [24]. The early graft patency rate was 97.8% in group Y and 98.7% in group E. There was no significant difference between group E and group Y ($p > 0.99$).

Comment

The number of elderly patients is growing exponentially, and cardiovascular disease, such as coronary artery disease, is strongly associated with death in this population. Many studies have examined the feasibility and efficacy of surgical intervention for cardiac disease in this population, and several reports have indicated that coronary artery revascularization can be performed with an acceptable mortality in octogenarians [25–27]. Now, efforts are aimed at decreasing morbidity and mortality after coronary artery bypass grafting in this population. Peterson and associates [28] reported the largest study of the outcome of coronary artery bypass grafting in octogenarians. This revealed longer hospital stays, larger hospital costs, and worse short-term mortality in comparison with the younger population. However, the long-term mortality of the patients in this population was similar to that of the general octogenarian population.

Recently, OPCAB has undergone widespread acceptance because of its cost-effectiveness and the commercial availability of stabilizing devices, not to mention the development of the operative techniques. And, worthy of

Table 4. EuroSCORE and Actuarial Survival Rate

	Whole		Group E		Group Y		*p* Value
Variable	ave ± SD	95% CI	ave ± SD	95% CI	ave ± SD	95% CI	(E vs Y)
Additive EuroSCORE	4.2 ± 2.8	4.0–4.5	7.2 ± 2.5		3.5 ± 2.3		<0.0001
EuroSCORE excluding age-related score	2.2 ± 2.2	2.0–2.4	3.0 ± 2.3		2.0 ± 2.0		<0.0001
Logistic EuroSCORE	4.9 ± 5.7	4.4–5.4	10.5 ± 8.5		3.6 ± 39		<0.0001
Actuarial hospital mortality (%)	0.7	0.2–1.8	2.7	0.6–7.7	0.2	0.0–1.2	0.02

Table 5. Operative Outcome

Variable	Group E	Group Y	*p* Value
MACE	5 (4.5%)	24 (5.1%)	>0.99
PMI (CKMB > 100)	2 (1.8%)	15 (3.2%)	0.75
Perioperative stroke	1 (0.9%)	3 (0.6%)	0.57
Postoperative renal failure	0 (0%)	5 (1.1%)	0.59
Mediastinitis	0 (0%)	4 (0.9%)	0.6
Wound dehiscence	2 (1.8%)	5 (1.1%)	0.62
Atrial fibrillation	20 (18.0%)	51 (10.9%)	0.03
Postoperative ICU stay (days)	2.08 ± 1.78	1.90 ± 2.53	0.55

CKMB = creatine kinase-MB; ICU = intensive care unit; MACE = major adverse cardiac event; PMI = perioperative myocardial infarction.

special mention is that the indication for OPCAB has been expanded to include elderly patients and higher-risk patients, because of its reduced invasiveness. Ricci and associates [29] retrospectively reviewed the results of myocardial revascularization with or without cardiopulmonary bypass in octogenarians. The risk of cerebrovascular disease was found to be diminished by the use of OPCAB. Yokoyama and colleagues [3] reported that OPCAB for elderly patients could obviate the incidence of postoperative complications, whereas Beauford and colleagues [4] also reported that OPCAB for octogenarians could decrease the incidence of postoperative complications other than atrial fibrillation, compared with younger patients.

Meanwhile, the influence of the selection of graft material on late outcome has been a matter of controversy for many years, aside from the issue of whether or not to use cardiopulmonary bypass [12–19]. Previous reports had found the ITA to have the most favorable late patency owing to a biochemical effect [26]. Furthermore, although the conclusion was not reached in this matter, a number of papers have suggested that other arterial grafts have advantages with respect to vein grafts [12–14]. Muneretto and colleagues [13, 17, 19] and Osswald and associates [30] recently reported the late outcome of off-pump total arterial revascularization in elderly patients. These reports claimed that off-pump total arterial revascularization in the elderly could provide better outcome than could coronary artery bypass grafting with vein grafts, and that vein graft use was a risk factor for recurrent angina or graft failure [13, 17, 19]. Therefore, we adopted the use of OPCAB with arterial grafts only (ie, total arterial OPCAB) for our elderly patients, with positive results so far. Furthermore, concerning the use of the radial artery, there is a discussion of whether the composite graft is superior. Maniar and colleagues [16] reported that the composite graft was superior from the viewpoint of length saving and the benefits of aorta no-touch techniques.

The characteristics of the elderly patients in our study population were as follows: female dominant, anemic, small body size, and cardiomegaly. The proportion of acute myocardial infarction was greater in the elderly population. The reason why the operation time was shorter in the elder group was that we commonly use the bilateral ITA for the younger group. The blood transfusion rate was greater in the elder group.

Predicted risk was measured by the EuroSCORE in each group. This score was devised to construct a stratification system for the assessment of the quality of cardiac surgical care in Europe [22, 23]. It is now regarded as one of the most reliable risk stratification scores for mortality prediction. In the present study, the EuroSCORE was significantly higher in the elder group. However, because the EuroSCORE includes age as one of the risk factors, we subtracted the contribution that pertained to age from the total EuroSCORE. The adjusted score was also significantly larger in the elderly group. This shows that, preoperatively, the older patients have a high-risk background that is independent of age.

Although the hospital mortality rate was higher in group E, none of the causes of deaths were related to cardiac events, and it was much lower than the predicted mortality. Also, the incidences of perioperative myocardial infarction and major adverse cardiac events in the follow-up period did not differ between the two groups. Nor were the incidences of perioperative stroke, postoperative renal failure, mediastinitis, or wound dehiscence different between the two groups. Atrial fibrillation, however, occurred more often in the elderly group. This finding reveals that total arterial OPCAB is as safe for elderly patients as for young patients, in terms of morbidity.

As Morris and colleagues [26] described, ITA is most reliable graft even for the elderly patients. We preferred the bilateral use of ITA. However, for the high-risk patients, to reduce the operation time and the amount of bleeding, we used unilateral ITA and radial artery. This is because bilateral ITA was used for fewer patients in the elderly group. Arteries other than ITA or radial artery were not generally used. As the left anterior descending coronary artery is the most important target, we used the ITA for this lesion. Another matter of concern is the quality of the bypass graft. The number of anastomoses and the complete revascularization rate were not different between the two groups. Meanwhile, the graft patency rate was not different between the two age groups. Furthermore, total revascularization rate is more important. Osswald and colleagues [30] reported that incomplete revascularization is a risk factor for early death, in a

retrospective study. In the present study, the total revascularization rate was not different between the two groups.

This study has several limitations, including its small sample size and its retrospective nature. Although arterial grafting is thought to be superior to venous grafting in terms of long-term patency, the follow-up period of this study was too short to make a conclusive determination. We could only confirm that elderly patients can undergo total arterial OPCAB as safely as can younger patients; the superiority of total arterial OPCAB will remain unknown until long-term results are available.

The results of this study demonstrate that total arterial OPCAB for elderly patients provides excellent early outcomes. Although extended follow-up is mandatory to confirm the superiority of arterial grafts to vein grafts, we preferentially approach all patients as potential candidates for total arterial OPCAB.

References

1. Samoa SC, Danas G, Ludlum MKC, et al. Beating heart surgery in octogenarians: Preoperative outcome and comparison with younger age groups. Ann Thorac Surg 2000;69: 1140–5.
2. Craver JM, Puskas JD, Weintraub WW, et al. 601 octogenarians undergoing cardiac surgery: outcome and comparison with younger age groups. Ann Thorac Surg 1999;67:1104–10.
3. Yokoyama T, Baumgartner FJ, Gheissari A, Capouya ER, Panagiotides GP, Declusin RJ. Off-pump versus on-pump coronary bypass in high risk subgroups. Ann Thorac Surg 2000;70:1546–50.
4. Beauford RB, Goldstein DJ, Sardari FF, et al. Multivessel off-pump revascularization in octogenarians: early and mid-term outcomes. Ann Thorac Surg 2003;76:12–7.
5. Boyed WD, Desai ND, Del Rizzo DF, Novick RJ, McKenzie N, Menkis AH. Off-pump surgery decreases postoperative complications and resource utilization in the elderly. Ann Thorac Surg 1999;68:1490–3.
6. Koutlas TC, Elbeery JR, Williams JM, Moran JF, Francalancia NA, Chitwood WR Jr. Myocardial revascularization in the elderly using beating heart coronary artery bypass surgery. Ann Thorac Surg 2000;69:1042–7.
7. Pfister AJ, Zaki MS, Garcia JM, et al. Coronary artery bypass without cardiopulmonary bypass. Ann Thorac Surg 1992;54: 1085–92.
8. Ascione R, Rees K, Santo K, et al. Coronary artery bypass grafting in patients over 70 years old: the influence of age and surgical technique on early and mid-term clinical outcomes. Eur J Cardiothorac Surg 2002;22:124–8.
9. Demers P, Cartier R. Multiple off-pump coronary artery bypass surgery in the elderly. Eur J Cardiothorac Surg 2001;20:908–12.
10. Hirose H, Amano A, Takahashi A. Off-pump coronary artery bypass grafting for elderly patients. Ann Thorac Surg 2001; 72:2013–9.
11. Demaria RG, Carrier M, Fortier S, et al. Reduced mortality and strokes with off-pump coronary artery bypass grafting surgery in octogenarians. Circulation 2002;106(Suppl 1):I-5–10.
12. Zacharias A, Habib RH, Schwann TA, Riordan CJ, Durham SJ, Sharh A. Improved survival with radial artery versus vein conduits in coronary bypass surgery with left internal thoracic artery to left anterior descending artery grafting. Circulation 2004;109:1489–96.
13. Muneretto C, Bisleri G, Negri A, et al. Left internal thoracic artery-radial artery composite grafts as the technique of choice for myocardial revascularization in elderly patients: a prospective randomized evaluation. J Thorac Cardiovasc Surg 2004;127:179–84.
14. Legare JF, Buth KJ, Sullivan JA, Hirsch GM. Composite arterial grafts versus conventional grafting for coronary artery bypass grafting. J Thorac Cardiovasc Surg 2004;127: 160–6.
15. Kouchoukos NT, Karp RB, Oberman A, Russell RO Jr, Alison HW, Holt JH Jr. Long term patency of saphenous veins for coronary bypass grafting. Circulation 1978;58(Suppl):I-96–9.
16. Maniar HS, Barner HB, Bailey MS, et al. Radial artery patency: are aortocoronary conduits superior to composite grafting? Ann Thorac Surg 2003;76:1498–503.
17. Muneretto C, Bisleri G, Negri A, et al. Total arterial myocardial revascularization with composite grafts improves results of coronary surgery in elderly: a prospective randomized comparison with conventional coronary artery bypass surgery. Circulation 2003;108(Suppl 1):II-29–33.
18. Kurlansky PA, Williams DB, Traad EA, et al. Arterial grafting results in reduced operative mortality and enhanced long-term quality of life in octogenarians. Ann Thorac Surg 2003;76:418–26.
19. Muneretto C, Negri A, Bisleri G, et al. Is total arterial myocardial revascularization with composite grafts a safe and useful procedure in the elderly? Eur J Cardiothorac Surg 2003;23:657–64.
20. Kobayashi J, Sasako Y, Bando K, et al. Multiple off-pump coronary revascularization with "aorta no-touch" technique using composite and sequential methods. Heart Surg Form 2002;5:114–8.
21. Kobayashi J, Tagusari O, Bando K, et al. Total arterial coronary revascularization with only internal thoracic artery and composite radial artery grafts. Heart Surg Forum 2002; 6:30–7.
22. Nashef SAM, Roques F, Gauducheau ME, Lemeshow S, Slamon LR. European system for cardiac operative risk evaluation (Euro SCORE). Eur J Cardiothorac Surg 1999;16: 9–13.
23. Roques F, Nashef SAM, Michel P, et al. Risk factors and outcome in European cardiac surgery: analysis of the Euro SCORE multinational database of 19030 patients. Eur J Cardiothorac Surg 1999;15:816–23.
24. FitzGibbon GM, Burton JR, Leach AJ. Coronary bypass graft fate: angiographic grading of 1400 consecutive grafts early after operation and of 1132 after one year. Circulation 1978;57:1070–4.
25. Mullany CJ, Darling GE, Pluth JR, et al. Early and late results after isolated coronary artery bypass surgery in 159 patients aged 80 year and older. Circulation 1990;82(Suppl 4):229–36.
26. Morris R, Strong MD, Grunewald KE, et al. Internal thoracic artery for coronary artery grafting in octogenarians. Ann Thorac Surg 1996;62:16–22.
27. Williams DB, Carillo RG, Traad EA, et al. Determinants of operative mortality in octogenarians undergoing coronary bypass. Ann Thorac Surg 1995;60:1038–43.
28. Peterson ED, Cowper PA, Jollis JG, et al. Outcomes of coronary artery bypass graft surgery in 24461 patients aged 80 years or older. Circulation 1995;92(Suppl 2):II-85–91.
29. Ricci M, Karamanoukian HL, Abraham R, et al. Stroke in octogenarians undergoing coronary artery surgery with or without cardiopulmonary bypass. Ann Thorac Surg 2000;69: 1471–5.
30. Osswald BR, Blackstone EH, Tochtermann U, et al. Does the completeness of revascularization affect early survival after coronary artery bypass grafting in elderly patients? Eur J Thorac Cardiovasc Surg 2001;20:120–6.

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Adrenomedullin enhances therapeutic potency of bone marrow transplantation for myocardial infarction in rats

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Departments of [1]Cardiac Physiology, [2]Regenerative Medicine and Tissue Engineering, [3]Internal Medicine, [4]Biochemistry, and [5]Pathology, National Cardiovascular Center, Osaka; and [6]Department of Cardiovascular Surgery, Okayama University Medical School, Okayama, Japan

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Fujii, Takafumi, Noritoshi Nagaya, Takashi Iwase, Shinsuke Murakami, Yoshinori Miyahara, Kazuhiro Nishigami, Hatsue Ishibashi-Ueda, Mikiyasu Shirai, Takefumi Itoh, Kozo Ishino, Shunji Sano, Kenji Kangawa, and Hidezo Mori. Adrenomedullin enhances therapeutic potency of bone marrow transplantation for myocardial infarction in rats. *Am J Physiol Heart Circ Physiol* 288: H1444–H1450, 2005. First published November 11, 2004; doi: 10.1152/ajpheart.00266.2004.—Adrenomedullin (AM), a potent vasodilator, induces angiogenesis and inhibits cell apoptosis through the phosphatidylinositol 3-kinase/Akt pathway. Transplantation of bone marrow-derived mononuclear cells (MNC) induces angiogenesis. We investigated whether infusion of AM enhances the therapeutic potency of MNC transplantation in a rat model of myocardial infarction. Immediately after coronary ligation, bone marrow-derived MNC (5×10^6 cells) were injected into the ischemic myocardium, followed by subcutaneous administration of 0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$ AM (AM-MNC group) or saline (MNC group) for 3 days. Another two groups of rats received subcutaneous administration of AM alone (AM group) or saline (control group). Hemodynamic and histological analyses were performed 4 wk after treatment. Cardiac infarct size was significantly smaller in the MNC and AM groups than in the control group. A combination of AM infusion and MNC transplantation demonstrated a further decrease in infarct size. Left ventricular (LV) maximum change in pressure over time and LV fractional shortening were significantly improved only in the AM-MNC group. AM significantly increased capillary density in ischemic myocardium, suggesting the angiogenic potency of AM. AM infusion plus MNC transplantation demonstrated a further increase in capillary density compared with AM or MNC alone. Although MNC apoptosis was frequently observed 72 h after transplantation, AM markedly decreased the number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells among the transplanted MNC. In conclusion, AM enhanced the angiogenic potency of MNC transplantation and improved cardiac function in rats with myocardial infarction. This beneficial effect may be mediated partly by the angiogenic property of AM itself and by its antiapoptotic effect on MNC.

angiogenesis; apoptosis; mononuclear cell

DESPITE THE RECENT REMARKABLE progress in medical and surgical treatment for ischemic heart disease, this disease remains a major cause of death worldwide (5). Bone marrow-derived mononuclear cells (MNC) contain various kinds of cell lineages and numerous cytokines that contribute to neovascularization (1, 15). In fact, autologous transplantation of bone marrow cells has been shown to enhance angiogenesis and improve cardiac function in an animal model of cardiac ischemia (6, 9, 10). Recent human studies have demonstrated beneficial effects of transplanted MNC in patients with ischemic heart disease (23, 25). However, some patients fail to respond to this cell therapy. Thus a novel therapeutic strategy to enhance the angiogenic property of MNC is desirable.

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma (8). We have shown that infusion of AM has beneficial hemodynamic and renal effects in patients with heart failure (17). On the other hand, AM has been shown to activate the phosphatidylinositol 3-kinase (PI3-kinase)/Akt-dependent pathway in vascular endothelial cells, which is considered to regulate multiple critical steps in angiogenesis including endothelial cell proliferation, migration, and capillary-like formation (14, 22). In fact, we have shown that AM gene transfer induces therapeutic angiogenesis in a rabbit model of hindlimb ischemia via activation of Akt (24). These findings suggest that AM may play an important role in the regulation of vascular regeneration. In addition, AM has been shown to exert an antiapoptotic effect on a variety of cells including vascular endothelial cells (7, 20). Taking these findings together, combination therapy with MNC transplantation and AM infusion may have additional or synergetic effects on therapeutic angiogenesis for the treatment of ischemic heart disease.

Thus the purposes of this study were *1*) to investigate whether infusion of AM enhances the angiogenic potency of MNC transplantation in a rat model of myocardial infarction, and *2*) to investigate the effects of AM on survival and differentiation of the transplanted MNC to examine the underlying mechanisms of the effects induced by AM.

MATERIALS AND METHODS

Animal model. Myocardial infarction was produced in male Lewis rats weighing 200–220 g by left coronary ligation. In brief, after rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg body wt), they were ventilated artificially. The heart was exposed via left thoracotomy, and the left coronary artery was ligated 2–3 mm from its origin between the pulmonary artery conus and the left atrium using a 6-0 proline suture. Finally, the heart was restored to its normal position, and the chest was closed. The Animal Care Committee of the National Cardiovascular Center approved this experimental protocol.

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Preparation of MNC. After Lewis rats were killed, bone marrow from the femur and tibia was collected and put in PBS. Marrow cells were loaded on a 1.077 gradient of Ficoll (Lymphoprep; Nycomed Pharma, Oslo, Norway) and centrifuged at 1,500 rpm for 20 min. The cells were then washed with 10 ml PBS to remove the Ficoll and centrifuged at 2,000 rpm for 10 min. The cells were finally suspended in PBS at a concentration of 5×10^6 cells in 50 μl PBS for transplantation. Fluorescence-activated cell sorting analysis demonstrated that 22 ± 1% of MNC were positive for lectin from ulex europaeus (UEA)-1 lectin (Sigma, St. Louis, MO).

MNC transplantation and AM infusion. Transplantation of bone marrow-derived MNC and/or 3-day infusion of AM was performed immediately after coronary ligation. MNC (5×10^6 cells in 50 μl PBS) were injected into the myocardium at five points in the border zone surrounding the infarct by using a 27-gauge needle. Recombinant human AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) was subcutaneously administered by using an osmotic minipump (model 2004; Alza, Palo Alto, CA) for 3 days. The pump was positioned in a pocket constructed in the subcutaneous tissue just below the subscapular region. For control, 5% glucose was infused in a similar manner in the rats receiving coronary ligation. This protocol resulted in the creation of four groups: *1*) AM infusion plus MNC transplantation (AM-MNC group, $n = 15$), *2*) vehicle infusion plus MNC transplantation (MNC group, $n = 14$), *3*) AM infusion plus PBS injection (AM group, $n = 14$), and *4*) vehicle infusion plus PBS injection (control group, $n = 13$).

Echocardiographic studies. Echocardiographic studies were performed 4 wk after surgery using a 7.5-MHz phased-array transducer (model HP SONOS 5500; Hewlett-Packard, Andover, MA). Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg body wt) as a supplement to maintain mild anesthesia. M-mode tracings were obtained at the level of the papillary muscles. Anterior and posterior end-diastolic wall thickness, left ventricular (LV) end-diastolic and end-systolic dimension, and LV fractional shortening were measured from three consecutive cardiac cycles by the American Society for Echocardiology leading-edge method (21).

Cardiac catheterization. Cardiac catheterization was performed 4 wk after surgery. Rats were anesthetized with intraperitoneal pentobarbital and placed on a heating pad to maintain body temperature at 37–38°C throughout the study. A 1.5 Fr micronanometer-tipped catheter was inserted in the right carotid artery for measurement of heart rate and mean arterial pressure. The catheter was then advanced into the LV for measurement of LV end-diastolic pressure and then replaced with a thermomicroprobe for measurements of cardiac output. These hemodynamic variables were measured with a pressure transducer (UFI, Morro Bay, CA) connected to a polygraph and recorded with a thermal recorder (model 7758 B system; Hewlett-Packard).

Infarct size measurement. After completion of hemodynamic measurements, the heart was arrested by an injection of 2 mmol KCl through the carotid artery, and the cardiac ventricles were excised. The size of myocardial infarction was determined by a previously described method (2). In brief, incisions were made in the LV so that the tissue could be pressed flat. The circumference of the entire flat LV and the visualized infarcted area, as judged from both the epicardial and endocardial sides, was outlined on a clear plastic sheet. The difference in weight between the two marked areas on the sheet was used to determine infarction size and was expressed as a percentage of LV surface area.

Histological analysis of microvessel density. LV myocardium was fixed in 10% formalin. Three cross sections of the LV, cut from apex to base, were obtained from individual rats for comparison among four groups ($n = 5$ each). They were embedded in paraffin and stained with Masson's trichrome for measurement of interstitial fibrosis. In other rats ($n = 5$ each), LV myocardium was embedded in optimum cutting temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan), snap frozen in liquid nitrogen, and cut into 5-μm-thick sections. Tissue sections were stained for alkaline phosphatase with an indoxyltetrazolium method to detect capillary endothelial cells ($n = 5$ in each group). The number of capillary vessels was counted in the peri-infarct area (a 1.0-mm band next to the scar) excluding scar region using a light microscope at a magnification of ×200. The numbers in five high-power fields in each rat were averaged and expressed as the number of capillary vessels. These morphometric studies were performed by two examiners who were blinded to treatment.

Detection of MNC apoptosis. To examine the antiapoptotic effect of AM on transplanted MNC, red fluorescence-labeled MNC were transplanted into ischemic myocardium in rats with ($n = 5$) and without ($n = 5$) AM infusion. Before implantation into the ischemic heart, suspended MNC were labeled with fluorescent dyes with a PKH26 (Red Fluorescent Cell Linker Kit; Sigma), as reported previously (13). AM was subcutaneously administered by using a minipump for 3 days. Rats were killed 72 h after MNC transplantation. The LV was enucleated, and muscle samples were embedded in OCT compound and snap frozen in liquid nitrogen for the detection of apoptosis. Serial sections of the heart were stained by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) for apoptosis using an in situ apoptosis detection kit (model S7111 Apoptag Fluorescein Kit; Intergen). Apoptosis of transplanted MNC was also evaluated by the detection of cleaved caspase-3-positive cells. In brief, the frozen tissue sections were incubated with anticleaved caspase-3 antibody (Cell Signaling), followed by incubation with FITC-conjugated IgG antibody (BD Pharmingen, San Diego, CA). The number of TUNEL/PKH26 double-positive cells and caspase-3/PKH26 double-positive cells was counted in 10 fields of each rat using a confocal microscopy (Fluoview model 500; Olympus, Tokyo, Japan).

The antiapoptotic effect of AM on MNC was also evaluated by in vitro TUNEL assay. MNC were plated on 12-well plates (1×10^6 cells per well) and cultured in serum-free medium for 24 h with control buffer, AM (1×10^{-7} M), or AM plus wortmannin, a PI3-kinase inhibitor (50 nM). Randomly selected microscopic fields ($n = 10$) were evaluated for calculating the ratio of TUNEL-positive cells to total cells.

Monitoring of implanted MNC in ischemic heart. Additional rats were used to examine whether transplanted MNC differentiate into endothelial cells, cardiomyocytes, vascular smooth muscle cells, or macrophages in the ischemic heart. PKH26 (red fluorescence)-labeled MNC were injected into the ischemic heart in rats with ($n = 8$) and without ($n = 8$) AM infusion. These subgroups of rats were killed 4 wk after coronary ligation. To identify vascular endothelial cells in vivo, FITC-labeled UEA-1 lectin was intravenously administered 30 min before the rats were killed ($n = 5$ in each group). The LV was enucleated, and muscle samples were then embedded in OCT compound, snap frozen in liquid nitrogen, and cut into sections. Sections were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) to detect nuclei. The number of DAPI/PKH26 double-positive cells and lectin-positive cells in the peri-infarct area was counted in 10 fields of each rat using a confocal microscopy. Frozen sections from other rats ($n = 3$ in each group) were incubated with mouse anticardiac troponin T (Novocastra, Newcastle, UK), anti-α-smooth muscle actin antibody (Dako, Copenhagen, Denmark), and anti-ED1 antibody (Serotec, Oxford, UK), followed by incubation with FITC-conjugated IgG antibody. In other rats (MNC group, $n = 5$; AM-MNC group, $n = 5$), the cardiac muscle from base to apex was transversely cut into 6-μm slices to calculate the number of transplanted MNC present within the heart 4 wk after transplantation. These morphometric studies were performed by two examiners who were blinded to treatment.

Statistical analysis. Numerical values were expressed as means ± SE. Comparisons of parameters among the four groups were performed by one-way ANOVA, followed by Newman-Keuls test for unpaired data. Comparisons of parameters between two groups were made by unpaired Student's *t*-test. A value of $P < 0.05$ was considered significant.