control group in the present study was similar to that in the control group of the CAVEAT trial (56% vs 57%)[29]

Mechanism of Restenosis and Thrombin Activation

The mechanism of restenosis after PCI is considered to be a healing process after a balloon injury. Immediately after arterial injury with a balloon catheter, many factors lead to the activation of medial smooth muscle cells (SMC), but there are 3 major ones. First, elastic recoil is a pivotal factors after mechanical trauma to the abnormal vessel wall and stretching of the normal vessel wall (ie, arterial remodeling). Second, the formation of thrombus on the intimal surface and inside the disrupted plaque is an important part of the restenosis process. The intensity of thrombus formation could serve to reduce the initial gain in lumen both by adding to the plaque mass and by elaborating more growth factors[30] Third, the most intense interest has been on the impact of mitogenic factors (basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), and SMC-derived growth factor (SDGF)) released by platelets, monocytes, and by components of the intact parts of the vascular wall, including the SMC. In vitro and in vivo studies have shown that injury to the endothelium and the vessel wall causes increased thrombin production[31] Thrombin, in particular, may play a significant role in the initiation of the restenosis process, because the regulation of these growth factors has been reported to be modulated by thrombin via a thrombin receptor[32,33] Moreover, thrombin activates a variety of vascular and inflammatory cell types that promote wound healing[10,32] Thus, the inhibition of the initial thrombin activation may exert a potent preventative effect on restenosis after POBA.

Direct Thrombin Inhibitors and Restenosis

The direct thrombin inhibitors, such as r-hirudin, hirulog, hirugen and D-Phe-Pro-Arg-chloromethylketone (PPACK), are expected to reduce the restenosis rate after PCI[11,34] and several relevant experimental studies have been performed in recent years. Rogasta et al reported that the 2-h systemic infusion of hirudin failed to reduce cell proliferation within the first 7 days, whereas the 2-h infusion of hirulog improved the late angiographic luminal dimensions and reduced the cross-sectional area narrowing by plaque in rabbits compared with heparin controls after angioplasty[12] They suggested that (1) hirudin inhibits cellular migration rather than proliferation, and (2) hirudin reduces mural thrombosis, resulting in less thrombus incorporation into the plaque. However, Serruys et al reported that the systemic administration of r-hirudin failed to reduce restenosis in a clinical study (HELVETICA study)[35] This discrepancy between the experimental study (Rogasta et al[12]) and the clinical study (Serruys et al[35]) may be explained by a difference in the local concentration of hirudin at the target lesion. Accordingly, it is expected that the local delivery of a high concentration of a direct thrombin inhibitor using a drug delivery device would reduce restenosis without increasing adverse effects in the clinical setting. However, there has not a previous clinical prospective randomized trial using a direct thrombin inhibitor and a local delivery device for preventing restenosis after angioplasty.

The present study has demonstrated that the intracoronary local delivery of argatroban, in addition to a 4-h intravenous infusion, prevents restenosis following POBA. Argatroban has been reported to inhibit platelet activation by fibrin- or clot-incorporated thrombin more effectively than does hirudin[16] The reason that both local delivery and continuous intravenous infusion of argatroban were used in the present study was to inhibit thrombin activity, which may increase immediately after angioplasty before the local delivery of argatroban, because there was a time delay (approximately 10 min) between the first balloon inflation and the local delivery of argatroban (thrombin receptors have been reported to appear on a SMC within a few min after balloon injury[12]). The present findings, together with the report of Rogasta et al[13] suggest that direct thrombin inhibition may successfully inhibit cell migration in the initiation of restenosis in human patients.

Local Drug Delivery Device

Several local delivery balloon catheters have been designed. The double-balloon catheter was the first percutaneous drug delivery device. Other drug delivery devices such as the Wolinsky perforated-balloon catheter, a microporous balloon, a channel catheter, and the Transport coronary angioplasty catheter have been developed since then. More recently, the drug delivery devices known as the Infusasleeve™, a hydrogel-coated balloon, and the Dispatch™ catheter have become available[36] The hydrogel-coated balloon does not have a perfusion port to support distal blood flow during balloon inflation. Imanishi et al reported that the local delivery of argatroban using a hydrogel-coated balloon reduced intimal thickening after balloon injury in an experimental study[37] The Dispatch™ catheter consists of an over-the-wire, non-dilatation catheter with a spiral inflation coil and a perfusion port on its distal tip. There are several advantages of this system for the drug delivery. First, this device allows distal coronary perfusion during balloon inflation for a sufficiently longer time compared with other drug delivery catheters. Second, this system makes it easier to deliver the drug than a hydrogel-coated balloon catheter, because in the case of the hydrogel-coated balloon, the drug must first penetrate the hydrogel-balloon surface. Third, the pharmacokinetic validity of the local delivery of argatroban using a Dispatch™ catheter has been established. Anabuki et al confirmed that the local delivery of argatroban using a Dispatch™ catheter resulted in the intramural deposition of high concentration argatroban without any arterial damage[38] This new device has been used for the prevention of reocclusion after revascularization in patients with acute myocardial infarction and unstable angina pectoris[18,39] However, there are no other clinical reports on the prevention of restenosis using the Dispatch™ catheter except for one small non-randomized trial[10] Thus, this is the first prospective randomized controlled trial using the Dispatch™ catheter and argatroban to prevent restenosis following POBA. Moreover, it is expected that these local delivery devices may be available not only for direct thrombin inhibitor but also gene therapy in the future[40]

Study Limitations

First, it is unclear whether the local delivery of argatroban using a Dispatch™ catheter is effective in patients with small vessels (<2.5 mm). Second, this study was designed as an open-label randomized trial in the light of safety concerns. Although a double-blind design may be better, it is not easy to use a specific device such as the Dispatch™ catheter in a double-blind manner. Because no

obvious benefit of long-term inflation in preventing restenosis was found in a previous study[41] the long-term inflation (20 min) with the Dispatch™ catheter is unlikely to be responsible for the significant reduction of restenosis in the present study. Third, this trial was performed at a single center, with a small number of patients. Further study is necessary with a larger number of patients in a double-blind, randomized, multicenter trial with a placebo group (local delivery of normal saline using a Dispatch™ catheter). We are now planning to conduct such a trial in Japan. Fourth, the effect of the exclusively local delivery of argatroban remains undetermined, because postoperative intravenous infusion of argatroban was combined with the intracoronary local delivery in the present study. Further study is necessary to assess the 'pure' efficacy of the local delivery of argatroban. Moreover, further study is necessary to assess the efficacy for stenting lesions in the present stenting era. Final, the present study did not evaluate local delivery direct pressure, although it is reported that high, local delivery pressure is a key determinant of vascular damage and intimal thickening.[42] Further study is needed to examine the local drug delivery pressure during infusion of argatroban in the clinical setting.

Conclusions

The local delivery of argatroban using a Dispatch™ catheter was observed to be safe and effective in preventing restenosis after balloon angioplasty.

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Pediatric Cardiology

Total Anomalous Pulmonary Venous Return with the Circular Pulmonary Venous Connection: Outcome of Common Pulmonary Venous Agenesis

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Abstract. A rare case of total anomalous pulmonary venous return, in which the right and left peripheral pulmonary veins connected circularly and there was no central part of the pulmonary vein or the common pulmonary vein, is presented. To our knowledge, total anomalous pulmonary venous return with circular pulmonary venous connection has not been reported previously in the literature. It is thought that the complex connection between peripheral pulmonary veins with the absence of the central part of the pulmonary vein as well as the common pulmonary vein results from common pulmonary venous agenesis.

Keywords: Total anomalous pulmonary venous return — Common pulmonary vein — Asplenia

Case Report

A female neonate, born at 33 weeks of gestation and weighing 2640 g, received mechanical ventilation due to persistent severe cyanosis. Echocardiography showed asplenia, atrioventricular septal defect, common atrium, left ventricular hypoplasia, double-outlet right ventricle, subpulmonary stenosis, and bilateral superior vena cava (SVC). Two abnormal transverse vessels, one of which drained into the left SVC and the other into the right SVC, were found but neither the central part of the pulmonary vein (CPPV) nor the common pulmonary vein (CPV) were detected. Pulmonary arteriography revealed the absence of the CPPV as well as the CPV and the circular connection between right and left peripheral pulmonary veins that drained into both SVCs through three separate bridging vessels (two right vessels and one left vessel). Each bridging vein seemed stenotic at its junction with the SVC (Fig. 1).

A procedure to relieve the pulmonary venous obstruction was performed. The junction of the left bridging vein and the left SVC was enlarged using a Gore-Tex patch and the right midportion of the pulmonary venous circle was directly anastomosed to the common atrium. Although she recovered from critical hypoxia (PaO_2 elevated from 17 to 35 mmHg), she developed hypoxic spells caused by infundibular stenosis. On day 2 after the first operation a right modified Blalock–Taussig shunt was added, but 4 days later after the second operation she died due to decreasing ventricular contraction.

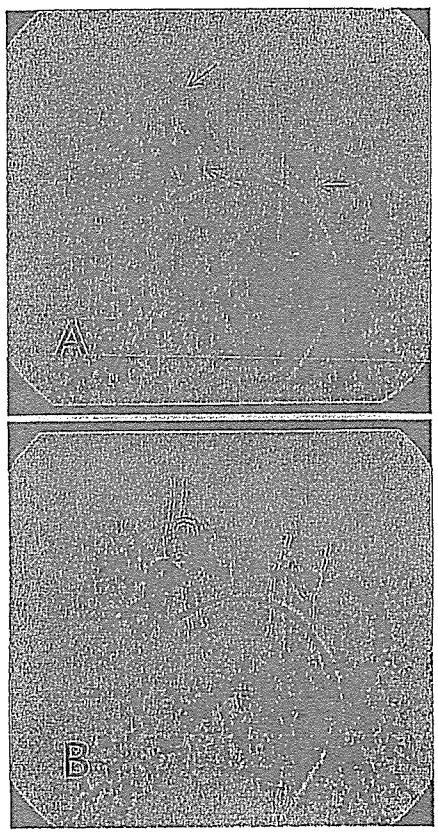

Fig. 1. (A) Angiogram showing the absence of any central part of the pulmonary vein as well as the common pulmonary vein and the circular connection between the right and left peripheral pulmonary veins that drain into both bilateral superior vena cava (*SVC*) through three separate bridging vessels. Each bridging vein seems stenotic at its junction with the SVC (*arrows*). (B) Broken lines and solid lines indicate bilateral SVC and bridging veins, respectively.

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Discussion

From the embryologic stand point, the pulmonary venous plexus engages with the splanchnic plexus and communicates with cardinal veins and the umbilicovitelline system. The CPV grows from the left atrium and connects with the pulmonary venous plexus. This direct connection allows atrophy of primitive complex systemic venous channels and provides normal pulmonary venous drainage to the left atrium through four central parts of the pulmonary vein (right upper, right lower, left upper, and left lower CPPV) [1, 2, 5]. It is believed that total anomalous pulmonary venous return results from abnormal development of the CPV and persistence of the embryologic pulmonary–systemic venous anastomosis [1].

In this case, the right and left peripheral pulmonary veins connected circularly and there was no CPPV or CPV. It is thought that the complex connection between peripheral pulmonary veins results from either agenesis of the CPV or early atrophy of the CPV connecting to the primitive pulmonary venous plexus, and it is also associated with asplenia. Sutherland et al. [4] reported a case of the latter with asplenia: an atretic strand extended from the common atrium to the CPPV, which directly connected to four other central parts of the pulmonary vein (right upper, right lower, left upper, and left lower CPPV). Then, the left lower CPPV connected to the right middle CPPV and drained into the SVC [4]. Ritter et al. [5] reported a case of the former in which there was no CPPV but there were complex connections between peripheral pulmonary veins [3]. We consider that our case is similar to the former one because of the absence of the CPPV; that is, the complex connection between peripheral pulmonary veins without forming the CPPV resulted from common pulmonary venous agenesis.

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SCIENTIFIC LETTER

p53Arg72Pro polymorphism of tumour suppressor protein is associated with luminal narrowing after coronary stent placement

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The cause of in-stent luminal narrowing has been primarily considered to be neointimal hyperplasia that is caused by proliferating vascular smooth muscle cells (VSMC). It has been recently reported that local drug delivery systems produce good results for the inhibition of VSMC proliferation.[1] The potential of suppressive agents in the treatment of in-stent luminal narrowing arises from basic studies according to cell cycle regulation and gene expression.[1]

p53 is a tumour suppressor protein involved in regulating the growth of VSMC. Loss of p53 activity results in the growth of VSMC, while increased concentrations of p53 result in apoptosis of VSMC. A common polymorphism in the p53 amino acid sequence which results in the presence of either arginine (Arg) or proline (Pro) at position 72 may influence the susceptibility to malignancy through its interaction with p73. The effects of this polymorphism on p53 function seem to be related to p73, and p53Arg was reported to be more susceptible to the inactivation of p73 than p53Pro alleles.[2]

It is conceivable that this common polymorphism of p53, Arg72Pro, may also influence VSMC proliferation after coronary stent implantation. In the present study, we tested this hypothesis in patients after coronary stent implantation using quantitative coronary angiography.

METHODS

The study population was selected from outpatients at the National Cardiovascular Center, Osaka, Japan, who underwent follow up coronary angiography after successful stent placement. This genetic study was approved by our institutional ethics committee and included 132 consecutive patients admitted between August and October 1999, from whom informed consent was obtained. Major adverse cardiac events (death, myocardial infarction, coronary artery bypass graft surgery, and repeat interventions) did not occur in any of the patients undergoing stent implantation, as assessed by follow up angiography.

Quantitative computer assisted angiographic measurements were performed on end diastolic frames using an automated edge detection system CMS (MEDIS Medical Imaging Systems, Leiden, The Netherlands). The minimal lumen diameter (MLD), reference lumen diameter (RLD), and per cent diameter stenosis were obtained using this system.

Genomic DNA was extracted from peripheral blood leucocytes. The p53 genotype was determined using the TaqMan system, which combines polymerase chain reaction amplification and detection in a single closed tube. The primers and probes were used for allelic discrimination of p53Arg72Pro polymorphism. The validity of the TaqMan system was confirmed using DNA samples of the three genotypes as confirmed by direct sequencing.

VSMC were prepared from the aorta of p53 knockout mice. Transient transfection of the p53 gene was performed using Lipofect Amin (Gibco BRL) according to the instructions of the manufacturer. VSMC were plated onto a 96 well multititre plate (10 000 cells/well) and cultured in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS) for 24 hours. After deprivation of serum for 24 hours, VSMC were transfected with wild/mutant p53 plasmids (n = 48). Transfected VSMC were cultured in DMEM and 2% FBS for 48 hours. Forty eight hours after the transfection, the cell number was counted with a WST (water soluble tetrazolium salt) cell counting kit (Wako). The expression construct of p53 was purchased from Invitrogen (GeneStorm expression-ready human clones). p53 cDNA is expressed under the control of a cytomegalovirus promoter. p53Pro was made from p53Arg by in vitro mutagenesis. The sequences of both constructs were confirmed by direct sequencing.

RESULTS

The 132 stenting lesions were divided into three groups according to the p53 genotype (47 Arg/Arg, 64 Arg/Pro, and 21 Pro/Pro). These genotype frequencies were compatible with the Hardy-Weinberg equilibrium. There were no significant differences in RLD among the three genotypes (3.00 (0.39) mm *v* 3.00 (0.44) mm *v* 3.05 (0.35) mm). MLD did not significantly differ before (0.46 (0.47) mm *v* 0.52 (0.51) mm *v* 0.48 (0.48) mm) or immediately after stent implantation (2.82 (0.47) mm *v* 2.74 (0.58) mm *v* 2.79 (0.42) mm) (fig 1A). However, MLD was significantly smaller (1.36 (0.86) mm *v* 1.83 (0.90) mm *v* 2.01 (0.92) mm, $p < 0.005$) and the per cent diameter stenosis was significantly greater (55 (27)% *v* 41 (26)% *v* 34 (25)%, $p < 0.005$) in the Arg/Arg genotype than in the other groups at follow up (fig 1B).

To assess the determinants of MLD at follow up, a multiple regression analysis with a backward elimination was performed. The results revealed that MLD at follow up was determined ($r = 0.531$, $p < 0.0001$) by the p53 genotype ($p = 0.001$), RLD ($p < 0.005$), diabetes mellitus ($p < 0.05$), and MLD immediately after stent implantation ($p < 0.05$).

To confirm the effect of the p53 genotype on VSMC we transfected Arg and Pro of p53 genes to aortic VSMC from p53 knockout mice. The cell count after transfection with p53Arg (25 423 (4022), n = 48) was greater than that after transfection with p53Pro (18 623 (2538), n = 48) ($p < 0.0001$).

Abbreviations: Arg, arginine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MLD, minimal lumen diameter; Pro, proline; RLD, reference lumen diameter; VSMC, vascular smooth muscle cells

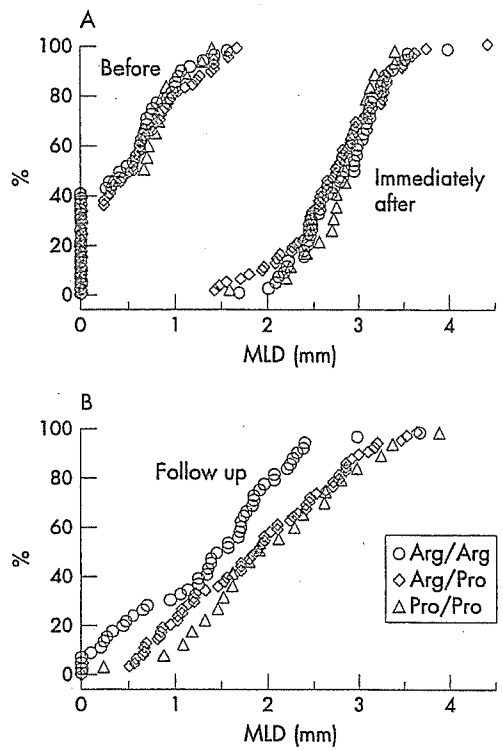

Figure 1 (A) Cumulative distribution curves of the MLD before and immediately after stent implantation for each genotype of p53. (B) Cumulative distribution curves of MLD at the time of follow up after stent implantation for each genotype of p53.

DISCUSSION

In the present study, we found that MLD at follow up angiography was significantly smaller in the Arg/Arg genotype than in the Arg/Pro and Pro/Pro genotypes of p53 in patients who underwent coronary stenting, and that MLD at follow up was determined by the p53 genotype, RLD, diabetes mellitus, and MLD immediately after stent implantation.

p53 has been shown to induce cell cycle arrest at the G_1/S boundary and also apoptosis. Increased concentrations of p53 result in apoptosis of VSMC, while loss of p53 activity results in the growth of VSMC. Guevara and co-workers reported the excessive proliferation of VSMC at S phase as a result of p53 inactivation in vivo.[3] Taken together, the present results suggest that p53Arg72Pro polymorphism is related to the functional activity of p53, which regulates VSMC proliferation leading to luminal narrowing after stent placement.

We also demonstrated that p53Arg has less of an inhibitory effect on VSMC proliferation than p53Pro. The functional difference between p53 variants was observed in a recent study which showed that the p53Arg variant is more efficient than p53Pro at inducing apoptosis, and that one mechanism underlying this greater efficiency is enhanced localisation of the p53Arg variant to mitochondria.[4] However, the localisation of p53 to mitochondria seems to occur only in tumour cells.[5] Therefore, this p53Arg induced apoptosis is unlikely to be the cause of our findings. On the other hand, the manner of p53 according to the codon 72 polymorphic variants may be deeply affected by p73. Marin and colleagues[2] recently reported that the Arg/Arg genotype in squamous cell tumours may reduce the inhibition of cell growth, possibly because the conformational p53 protein, consisting of the homozygote for p53Arg, can bind to the p73 protein, and neutralise p73-induced apoptosis. This indicates that interaction of the p53 protein with the p73 protein is influenced by a common polymorphism of p53 at amino acid residue 72. Consequently, VSMC proliferation may occur because p53Arg tends to block p73 function more effectively than p53Pro.

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Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis

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Departments of [1]Regenerative Medicine and Tissue Engineering, [3]Cardiac Physiology, and [6]Biochemistry, National Cardiovascular Center Research Institute, Osaka 565-8565; Departments of [2]Internal Medicine and [7]Cardiovascular Surgery, National Cardiovascular Center, Osaka; [4]Tissue Engineering Research Center, National Institute of Advanced Industrial Science and Technology, Hyogo; and [5]Cardiovascular Division, Kansai Rosai Hospital, Hyogo 660-8511, Japan

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Nagaya, Noritoshi, Takafumi Fujii, Takashi Iwase, Hajime Ohgushi, Takefumi Itoh, Masaaki Uematsu, Masakazu Yamagishi, Hidezo Mori, Kenji Kangawa, and Soichiro Kitamura. Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. *Am J Physiol Heart Circ Physiol* 287: H2670–H2676, 2004. First published July 29, 2004; doi:10.1152/ajpheart.01071.2003.—Mesenchymal stem cells (MSCs) are pluripotent cells that differentiate into a variety of cells, including cardiomyocytes and endothelial cells. However, little information is available regarding the therapeutic potency of systemically delivered MSCs for myocardial infarction. Accordingly, we investigated whether intravenously transplanted MSCs induce angiogenesis and myogenesis and improve cardiac function in rats with acute myocardial infarction. MSCs were isolated from bone marrow aspirates of isogenic adult rats and expanded ex vivo. At 3 h after coronary ligation, 5×10^6 MSCs (MSC group, $n = 12$) or vehicle (control group, $n = 12$) was intravenously administered to Lewis rats. Transplanted MSCs were preferentially attracted to the infarcted, but not the noninfarcted, myocardium. The engrafted MSCs were positive for cardiac markers: desmin, cardiac troponin T, and connexin43. On the other hand, some of the transplanted MSCs were positive for von Willebrand factor and formed vascular structures. Capillary density was markedly increased after MSC transplantation. Cardiac infarct size was significantly smaller in the MSC than in the control group (24 ± 2 vs. 33 ± 2%, $P < 0.05$). MSC transplantation decreased left ventricular end-diastolic pressure and increased left ventricular maximum dP/d*t* (both $P < 0.05$ vs. control). These results suggest that intravenous administration of MSCs improves cardiac function after acute myocardial infarction through enhancement of angiogenesis and myogenesis in the ischemic myocardium.

left ventricular end-diastolic pressure; cell transplantation; differentiation; homing

INTERRUPTION OF MYOCARDIAL blood flow leads to cardiomyocyte death (20). Although myocyte mitosis and the presence of cardiac precursor cells in adult hearts have recently been reported (6, 17), death of large numbers of cardiomyocytes results in the development of heart failure (16). Thus it would be desirable to induce angiogenesis and myogenesis for the treatment of ischemic heart disease.

Mesenchymal stem cells (MSCs) are pluripotent adult stem cells residing within the bone marrow microenvironment (11, 18). In contrast to their hematopoietic counterparts, MSCs have an adherent nature and are expandable in culture. MSCs can differentiate into not only osteoblasts, chondrocytes, neurons, and skeletal muscle cells but also vascular endothelial cells (19) and cardiomyocytes (23, 24). In vitro, MSCs have the potential to induce a neovascular response in murine Matrigel angiogenesis assay (2). In vivo, local MSC implantation induces therapeutic angiogenesis in a rat model of hindlimb ischemia (1). On the other hand, MSCs directly injected into the infarcted heart have been shown to induce myocardial regeneration and improve cardiac function (21). Stem or progenitor cells have been shown to circulate in peripheral blood and home to ischemic tissues (4). These results raise the possibility that intravenously administered MSCs participate in repair of the ischemic myocardium primarily by angiogenesis, which prevents apoptosis of native cardiomyocytes, and by direct regeneration of lost cardiomyocytes. However, little information is available regarding the therapeutic potential of systemically delivered MSCs for myocardial infarction.

Thus the purpose of this study was to investigate whether *1*) intravenously administered MSCs are able to engraft in the ischemic myocardium, *2*) transplanted MSCs induce angiogenesis and myogenesis after myocardial infarction, and *3*) transplantation of MSCs decreases infarct size and improves cardiac function.

METHODS

Animals. Male Lewis rats ($n = 70$) weighing 220–250 g were used in this study. These isogenic rats ($n = 8$) served as donors and recipients of MSCs to simulate autologous implantation. The Animal Care Committee of the National Cardiovascular Center approved the experimental protocol.

Model of myocardial infarction and cell transplantation. Fifty-one rats underwent ligation of the left coronary artery to produce myocardial infarction, as described previously (15). Briefly, after rats were anesthetized by injection of pentobarbital sodium (30 mg/kg body wt ip), they were artificially ventilated using a volume-regulated respirator. The heart was exposed via a 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 Prolene suture.

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At 3 h after coronary ligation, 40 rats survived (78% survival rate): 30 were randomized to receive an intravenous injection of MSCs (MSC group, $n = 14$) or PBS (control group, $n = 16$), and 10 received fluorescence-labeled MSCs for examination of MSC differentiation ($n = 5$) and incorporation ($n = 5$). Eleven rats underwent a sham operation consisting of thoracotomy and cardiac exposure but without coronary artery ligation. At 3 h after coronary ligation, we administered 5×10^6 MSCs/100 μl in PBS or PBS alone through a catheter inserted into the left jugular vein in ~30 s. The subsequent mortality for 4 wk was 25% in the control group and 14% in the MSC group. This protocol resulted in the creation of three groups: normal rats given PBS (sham group, $n = 11$), myocardial infarction rats given PBS (control group, $n = 12$), and myocardial infarction rats given MSCs (MSC group, $n = 12$).

Expansion of bone marrow MSCs. MSC expansion was performed according to previously described methods (18). Briefly, we killed the male Lewis rats and harvested the bone marrow by flushing the cavity of the femurs and tibias with PBS. Bone marrow cells were introduced into 100-mm dishes and cultured in α-MEM supplemented with 10% FBS and antibiotics. A small number of cells developed visible symmetrical colonies by *day 5–7*. Nonadherent hematopoietic cells were removed, and the medium was replaced. The adherent, spindle-shaped MSC population expanded to $>5 \times 10^7$ cells by approximately four to five passages after the cells were first cultured.

Flow cytometry. Adherent cells were analyzed by fluorescence-activated cell sorting (FACS SCAN flow cytometer, Becton Dickinson). Cells were incubated for 30 min at 4°C with the FITC-conjugated mouse monoclonal antibodies against rat CD34 (clone ICO-115, Santa Cruz Biotechnology) and CD45 and CD90 (clones OX-1 and OX-7, respectively, Becton Dickinson). FITC-conjugated hamster anti-rat CD29 monoclonal antibody (clone Ha2/5, Becton Dickinson) and rabbit anti-rat c-Kit polyclonal antibody (clone C-19, Santa Cruz Biotechnology) were used. Isotype-identical antibodies served as controls.

Echocardiographic studies. Echocardiographic studies were performed by an investigator blinded to treatment allocation 4 wk after coronary ligation. Two-dimensional targeted M-mode traces were obtained at the level of the papillary muscles using an echocardiographic system equipped with a 7.5-MHz phased-array transducer (SONOS 5500, Hewlett-Packard, Andover, MA). Anterior and posterior end-diastolic wall thickness and left ventricular (LV) end-diastolic and end-systolic dimensions were measured by the American Society for Echocardiology leading-edge method from at least three consecutive cardiac cycles. LV fractional shortening was calculated as follows: $(LVD_d - LVD_s)/LVD_d \times 100$, where LVD_d is LV diastolic dimension and LVD_s is LV systolic dimension. LV volume and ejection fraction were calculated on the basis of the Teichholtz formula.

Hemodynamic studies. Hemodynamic studies were performed 4 wk after coronary ligation. A 1.5-Fr micromanometer-tipped catheter (Millar Instruments) was inserted in the right carotid artery for measurement of mean arterial pressure. Then the catheter was advanced into the LV for measurement of LV pressure. Hemodynamic variables were measured using a pressure transducer (model P23 ID, Gould) connected to a polygraph. After completion of these measurements, the left and right ventricles were excised and weighed. Infarction size was determined as a percentage of the entire LV area, as reported previously (8). Briefly, 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 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 examination. To detect fibrosis in cardiac muscle, the LV myocardium ($n = 5$ each group) was fixed in 10% formalin, cut transversely, embedded in paraffin, and stained with Masson's trichrome. To detect capillary endothelial cells in the peri-infarct area, samples of the harvested muscle ($n = 5$ each) were embedded in OCT compound (Miles Scientific), snap frozen in liquid nitrogen, and cut into transverse sections. Tissue sections were stained for alkaline phosphatase with an indoxyltetrazolium method. The number of capillary vessels was counted in the peri-infarct area using a light microscope at ×200 magnification. The numbers in five high-power fields were averaged and expressed as the number of capillary vessels. These morphometric studies were performed by two examiners who were blinded to treatment.

An additional five rats were used to examine whether transplanted MSCs differentiated into cardiomyocytes or vascular endothelial cells. Suspended MSCs were labeled with fluorescent dyes with a PKH-26 red fluorescent cell linker kit (Sigma Chemical, St. Louis, MO) before implantation, as reported previously (13). Fluorescence-labeled MSCs were intravenously administered 3 h after coronary ligation. This subgroup of rats was killed 4 wk after coronary ligation. After the LV was excised and dissected free, muscle samples were embedded in OCT compound, snap frozen in liquid nitrogen, and cut into sections. Immunofluorescent staining for cardiac and endothelial cell markers was performed using monoclonal mouse antidesmin (Dako), anti-cardiac troponin T (Novo), anticonnexin43 (Sigma Chemical), and polyclonal rabbit anti-von Willebrand factor (Dako). FITC-conjugated IgG antibody (BD Pharmingen and Molecular Probes) was used as a secondary antibody.

At 24 h after intravenous administration of PKH-26-labeled MSCs, cardiac muscle was embedded in OCT compound and snap frozen in liquid nitrogen. Then the cardiac muscle from base to apex was

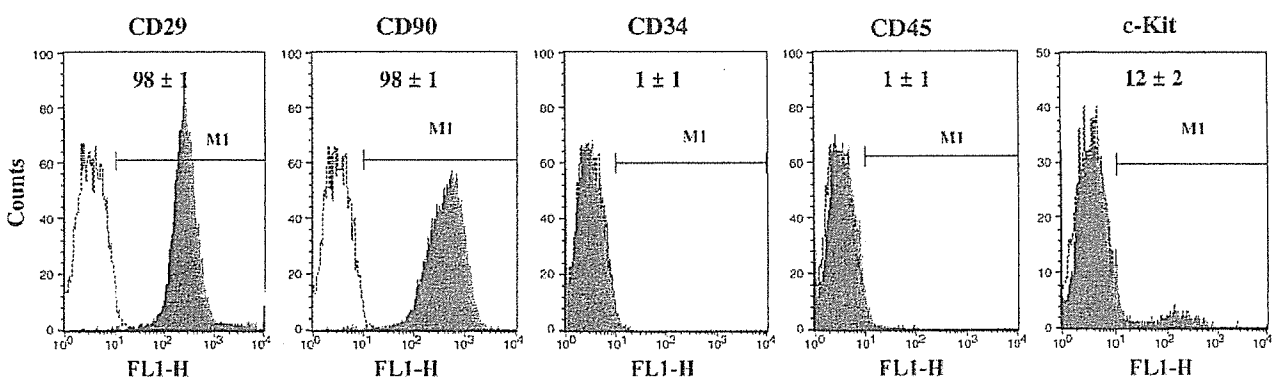

Fig. 1. Flow cytometric analysis of adherent, spindle-shaped mesenchymal stem cell (MSC) population expanded to 4–5 passages. Most of the cells expressed CD29 and CD90 but were negative for CD34 and CD45. Some cells were positive for c-Kit. MI, myocardial infarction.

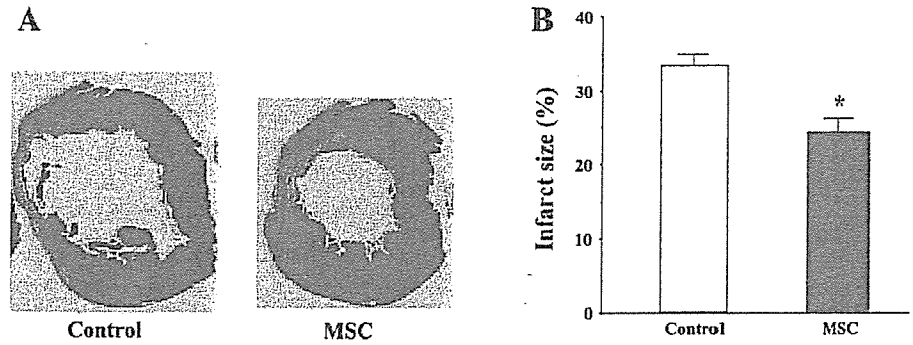

Fig. 2. Effect of MSC transplantation on myocardial infarct size 4 wk after myocardial infarction. *A*: representative Masson's trichrome-stained myocardial sections from control and MSC groups. *B*: quantitative analysis demonstrating that MSC transplantation significantly decreased infarct size. Values are means ± SE. $*P < 0.05$ vs. control.

transversely cut into 5-μm slices for calculation of the numbers of transplanted MSCs in the heart ($n = 5$).

Statistical analysis. Numerical values were expressed as means ± SE unless otherwise indicated. Comparisons of parameters among the three groups were made using one-way analysis of variance (ANOVA) followed by Scheffé's multiple comparison test. Comparisons of parameters between two groups were made by unpaired Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Characterization of cultured MSCs. Most of cultured adherent cells expressed CD29 and CD90 (Fig. 1). In contrast, a majority of adherent cells were negative for CD34 and CD45. A small fraction of the adherent cells expressed c-Kit. Thus we confirmed that the major population of adherent cells was MSCs.

Reduction of myocardial infarct size after MSC transplantation. Moderate-to-large infarcts were observed in Masson's trichrome-stained myocardial sections 4 wk after coronary ligation (control group; Fig. 2*A*). However, MSC transplantation markedly decreased the infarct size after myocardial infarction (MSC group). Quantitative analysis also demonstrated that cardiac infarct size was significantly smaller in the MSC than in the control group: 24 ± 2 vs. 33 ± 2% ($n = 12$ each, $P < 0.05$; Fig. 2*B*).

Hemodynamic effects of MSC transplantation. At 4 wk after coronary ligation, hemodynamic studies were performed in the sham ($n = 11$), control ($n = 12$), and MSC ($n = 12$) groups. LV end-diastolic pressure showed a marked elevation in the control group (18 ± 1 mmHg); the elevation was significantly attenuated in the MSC group (13 ± 1 mmHg, $P < 0.05$; Fig. 3*A*). LV maximum dP/d*t* was significantly higher in the MSC than in the control group (Fig. 3*B*). LV minimum dP/d*t* tended to be lower in the MSC than in the control group (Fig. 3*C*). Although mean arterial pressure was significantly lower in the control than in the sham group, no decrease was observed in the MSC group (Table 1). Heart rate did not significantly differ among the three groups.

LV diastolic dimension was significantly smaller in the MSC than in the control group (Table 2). Fractional shortening was significantly greater in the MSC than in the control group (Fig. 3*D*). LV ejection fraction was also higher in the MSC than in

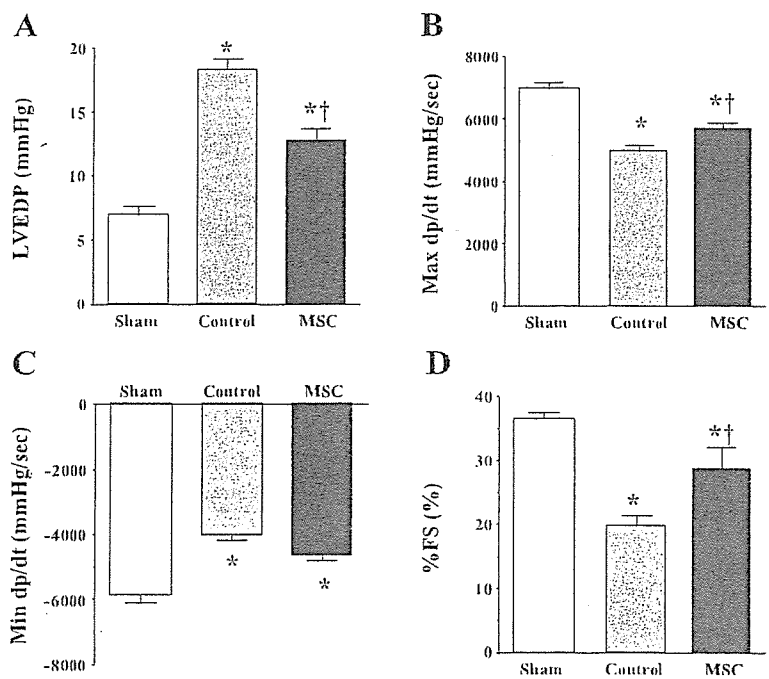

Fig. 3. Effects of MSC transplantation on hemodynamic parameters. LVEDP, LV end-diastolic pressure (*A*); max dP/d*t*, LV maximum dP/d*t* (*B*); Min dP/d*t*, LV minimum dP/d*t* (*C*); %FS, LV fractional shortening (*D*). Values are means ± SE. $*P < 0.05$ vs. sham. $\dagger P < 0.05$ vs. control.

Table 1. *Characterization of animals*

	Sham (n = 11)	Control (n = 12)	MSC (n = 12)
Body wt, g	331±4	301±7*	321±7†
LV wt/body wt, g/kg	1.83±0.11	2.22±0.10*	2.17±0.09*
RV wt/body wt, g/kg	0.55±0.02	0.83±0.04*	0.71±0.03*†
Heart rate, beats/min	404±15	428±17	418±15
Mean arterial pressure, mmHg	128±2	113±4*	119±3

Values are means ± SE. Sham, sham-operated rats given vehicle; control, myocardial infarction rats given vehicle; MSC, myocardial infarction rats given mesenchymal stem cells; LV, left ventricle; RV, right ventricle. *P < 0.05 vs. sham. †P < 0.05 vs. control.

Table 2. *Echocardiographic data*

	Sham	Control	MSC
LVD_d, mm	6.3±0.1	8.6±0.2*	7.5±0.3*†
LVD_s, mm	4.0±0.1	6.9±0.3*	5.5±0.5*†
%FS, %	37±1	20±2*	29±3*†
LVEF, %	65±1	39±3*	53±5*†
AWT diastole, mm	1.6±0.1	1.1±0.1*	1.4±0.1†
PWT diastole, mm	1.6±0.1	1.7±0.1	1.7±0.1

Values are means ± SE. LVD_d, LV diastolic dimension; LVD_s, LV systolic dimension; %FS, LV fractional shortening; LVEF, LV ejection fraction; AWT, anterior wall thickness; PWT, posterior wall thickness. *P < 0.05 vs. sham. †P < 0.05 vs. control.

the control group (Table 2). Diastolic anterior wall thickness was significantly attenuated in the MSC group compared with the control group.

Myogenesis and angiogenesis induced by MSCs. Red fluorescence-labeled MSCs were intravenously administered 3 h after coronary ligation (n = 5). Semiquantitative analysis demonstrated that ~3% of the transplanted MSCs were incorporated into the heart 24 h after transplantation. At 4 wk after transplantation (n = 5), MSCs were incorporated predominantly into the border zone of infarcts (Fig. 4), whereas few MSCs were detected in the noninfarcted myocardium. Immunofluorescence analyses demonstrated that the engrafted MSCs were positive for desmin (Fig. 4), cardiac troponin T (Fig. 5*A*), and connexin43 (Fig. 5*B*). These results suggest the ability of MSCs to engraft in the ischemic myocardium and differentiate into cardiomyocytes. On the other hand, some of the transplanted MSCs were positive for von Willebrand factor and formed vascular structures (Fig. 6). Alkaline phosphatase staining of the ischemic myocardium showed marked augmentation of neovascularization in the MSC group (Fig. 7*A*). Quantitative analysis demonstrated that capillary density was significantly higher in the MSC than in the control group (n = 5 each; Fig. 7*B*).

DISCUSSION

In the present study, we demonstrated that intravenously administered MSCs were capable of engraftment in the ischemic myocardium and that the engrafted MSCs differentiated into cardiomyocytes and vascular endothelial cells, resulting in myogenesis and angiogenesis. We also demonstrated that MSC transplantation decreased myocardial infarct size and improved cardiac function after acute myocardial infarction in rats.

Earlier studies showed that MSCs directly injected into the myocardium or those injected into coronary arteries improve cardiac function after myocardial infarction. However, little information is available regarding the therapeutic potential of systemically delivered MSCs for myocardial infarction. This study demonstrated that intravenous administration of MSCs

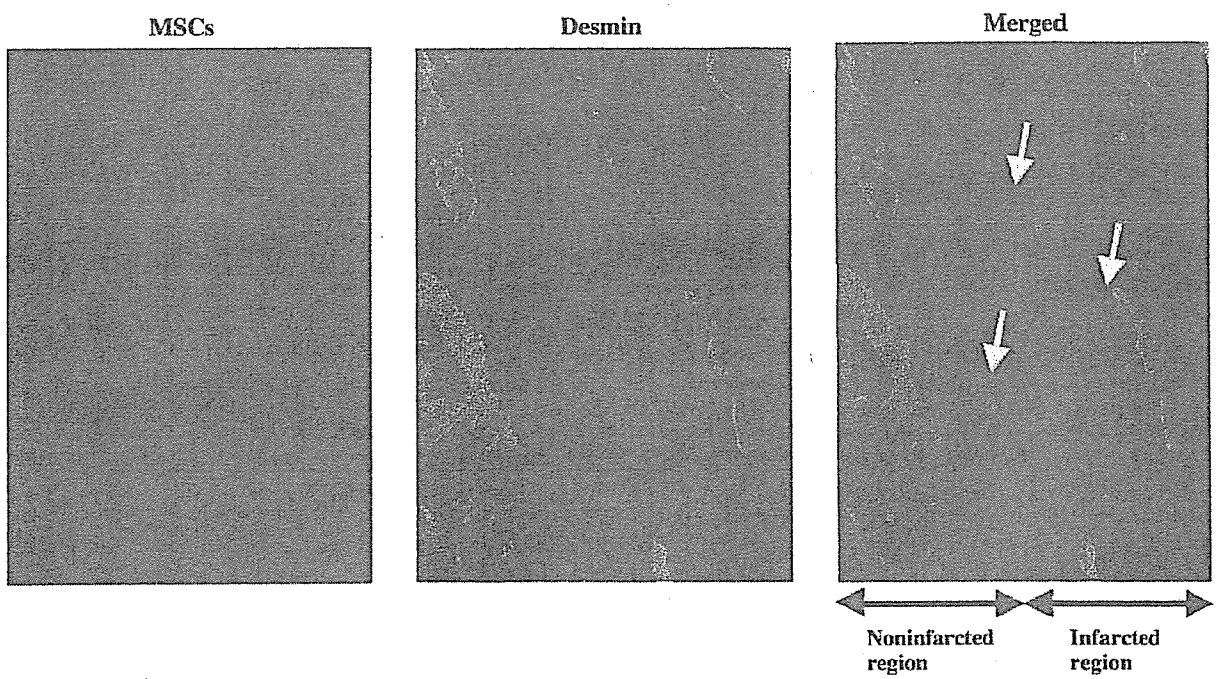

Fig. 4. Distribution of intravenously administered MSCs in myocardium after acute myocardial infarction. Red fluorescence-labeled MSCs were incorporated into ischemic boundary zone of the heart. These cells were positive for desmin (arrows), a cardiac marker. Magnification ×400.

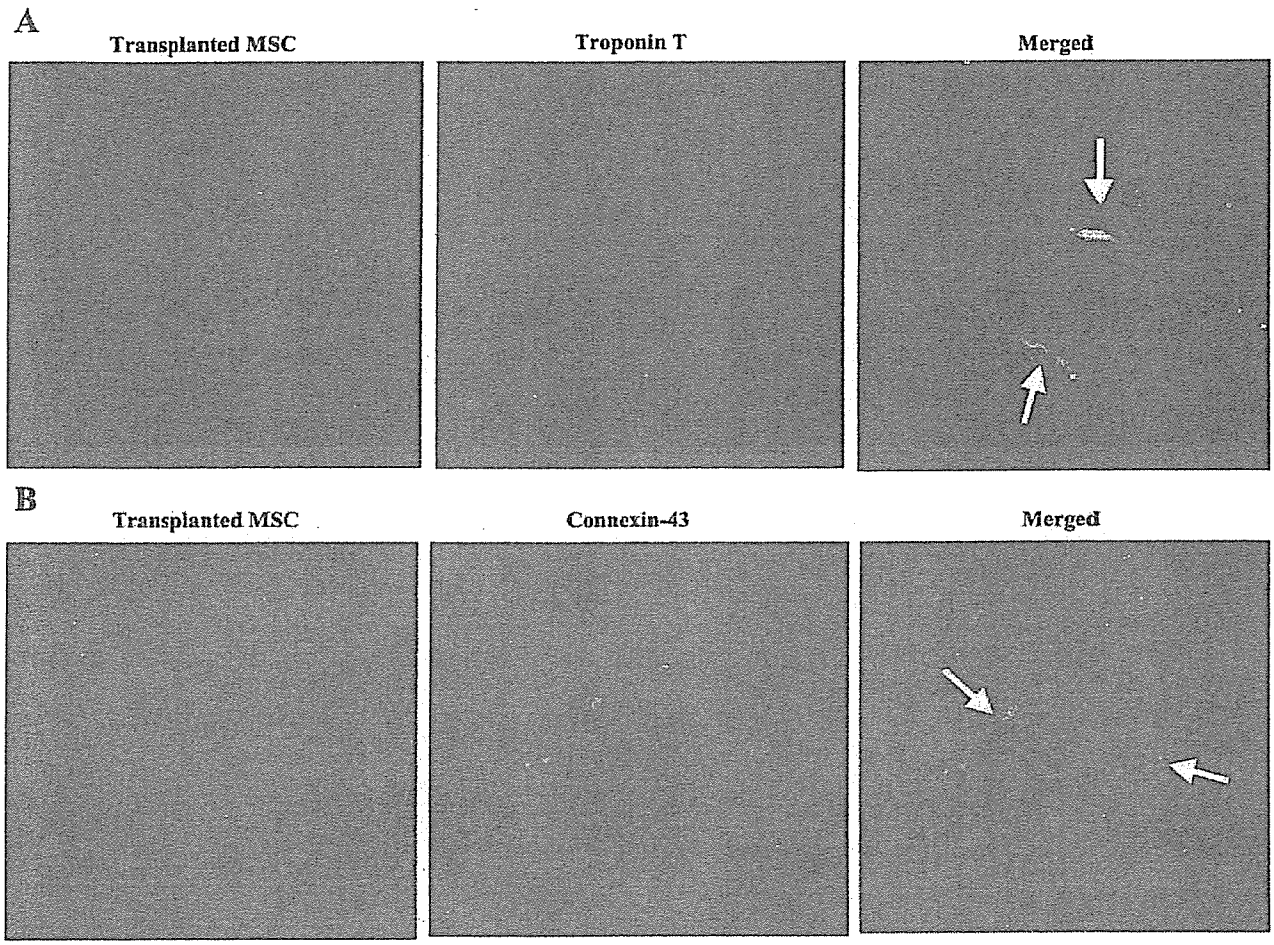

Fig. 5. Differentiation of transplanted MSCs in ischemic myocardium. Engrafted MSCs were positive (arrows) for cardiac troponin T (*A*) and connexin43 (*B*). Magnification ×400.

improves cardiac function after acute myocardial infarction through enhancement of angiogenesis and myogenesis in the ischemic myocardium.

Earlier studies showed that endothelial progenitor cells are mobilized from bone marrow into the peripheral blood in response to tissue ischemia and home to and incorporate into sites of neovascularization (21). Similar to epithelial progenitor cells, in the present study, transplanted MSCs were preferentially attracted to and retained in the border zone of infarcts. This is consistent with recent findings in the ischemic heart (5)

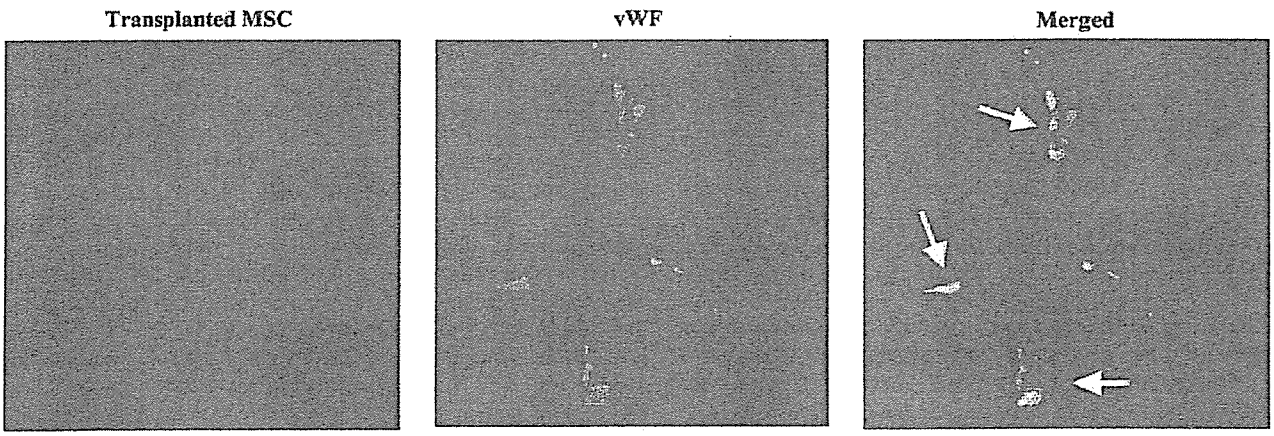

Fig. 6. Transplanted MSCs were positive for von Willebrand factor (vWF) and formed vascular structures. Magnification ×400.

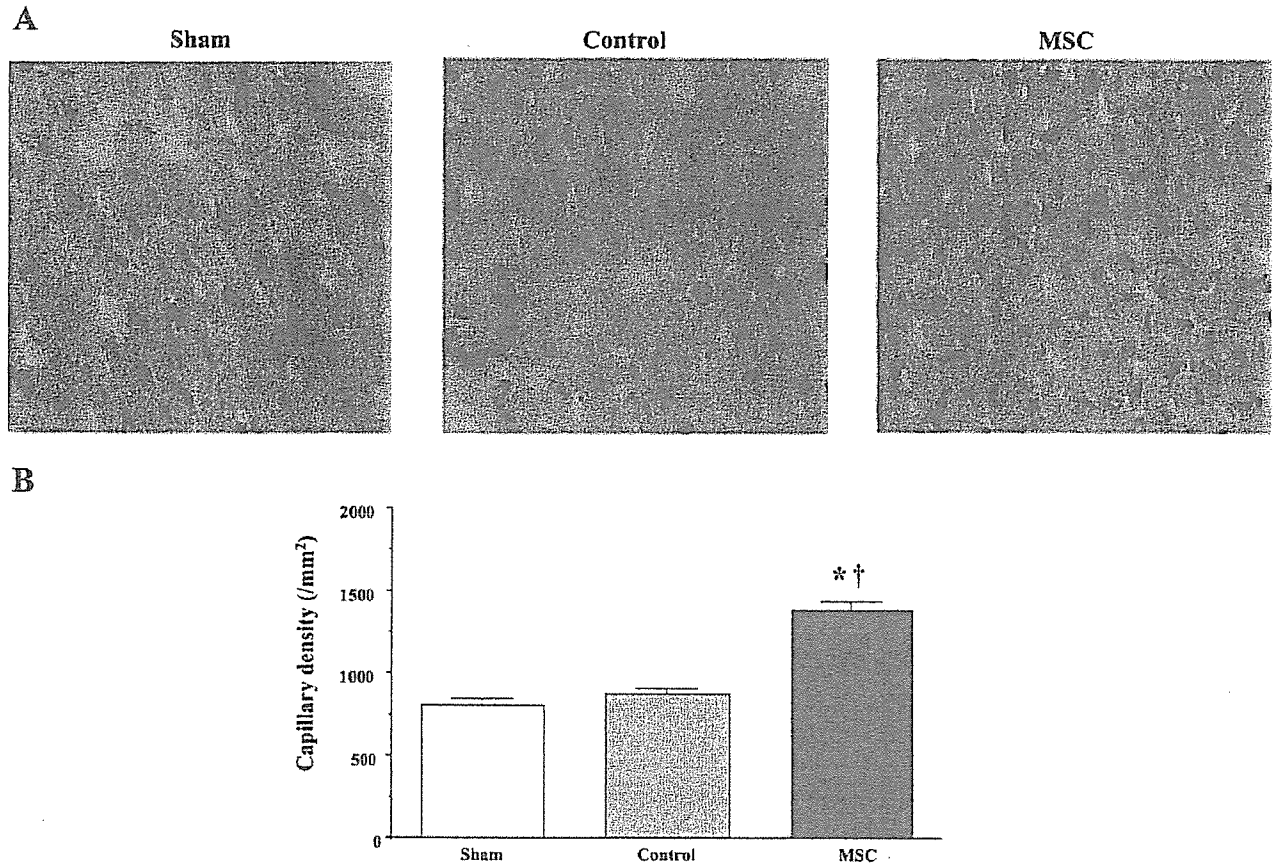

Fig. 7. *A*: representative samples of alkaline phosphatase staining in peri-infarct area. Magnification ×200. *B*: quantitative analysis of capillary density in peri-infarct area. Values are means ± SE. $*P < 0.05$ vs. sham. $\dagger P < 0.05$ vs. control.

or brain (7). Although the underlying mechanisms remain unclear, ischemic tissue may express specific receptors or ligands to facilitate trafficking, adhesion, and infiltration of MSCs to ischemic sites.

In the present study, some of the engrafted MSCs were stained by cardiac proteins such as desmin and cardiac troponin T. Transplanted MSCs also expressed connexin43, a gap junction protein, at contact points with native cardiomyocytes. These results suggest that MSCs differentiated into cardiomyocytes in the ischemic myocardium and formed connections with native cardiomyocytes. In contrast to skeletal myoblasts, which have been used as a tool for myocardial repair, MSCs may have the capacity for electromechanical coupling. Earlier studies demonstrated the importance of the microenvironment for cardiomyogenic differentiation. Possible factors might include direct cell-cell contact (9), electrical and mechanical stimulation (10), and unknown growth factors. On the other hand, recent studies showed that stem cells may fuse with existing native cells (22, 25). Although the mechanisms by which MSCs develop into cardiomyocyte-like cells remain unclear, it is possible that the direct attachment with host cardiomyocytes in the ischemic myocardium contributes to the cardiogenic differentiation of transplanted MSCs. Further studies are necessary to investigate whether engrafted MSCs are actually becoming contractile.

In the present study, some of the transplanted MSCs were positive for an endothelial cell marker and participated in vessel formation. MSC transplantation significantly increased the capillary density in ischemic myocardium. The recently reported phenotypic plasticity of MSCs to transform into endothelial-like cells provides a rationale for their potential role in neovascularization. Hypoxia has been shown to induce MSC migration and capillary-like structure formation by upregulation of membrane type 1 matrix metalloproteinase (3). MSC implantation has been shown to induce therapeutic angiogenesis in a rat model of chronic hindlimb ischemia (1). These findings support the theory that intravenously administered MSCs are able to differentiate into vascular endothelial cells in the ischemic myocardium. Interestingly, MSCs enhance angiogenesis partly by increasing endogenous levels of vascular endothelial growth factor and vascular endothelial growth factor type 2 receptor (7). Together, these findings suggest that MSCs may contribute to neovascularization in the ischemic myocardium not only through their ability to generate capillary-like structures and but also through growth factor-mediated paracrine regulation.

The present study showed that MSC transplantation significantly reduced infarct size and attenuated wall thinning after acute myocardial infarction. Cardiomyocyte apoptosis during ischemia is one of the major contributors to the development of myocardial infarcts (16, 20). It is possible that newly formed vessels after MSC transplantation improve tissue perfusion around the ischemic boundary zone, resulting in functional recovery after acute myocardial infarction. We also demonstrated that transplanted

MSCs differentiated into cardiomyocytes in the ischemic myocardium. These results suggest that the decrease in infarct size and the increase in wall thickness may be attributable not only to MSC-induced neovascularization but also to myocardial regeneration. In the present study, MSC transplantation improved cardiac function after acute myocardial infarction, as indicated by a significant decrease in LV end-diastolic pressure, a tendency for an increase in maximum LV dP/dt, and a decrease in minimum LV dP/dt. Thus MSC-induced angiogenesis and myogenesis and the resultant reduced infarct size may have contributed to the hemodynamic improvement after acute myocardial infarction.

The low percentage of MSC migration to the heart is in agreement with some previous studies (5, 14). The present study also showed that only a small percentage of transplanted MSCs were incorporated into the heart. This may be explained by MSC apoptosis (12), tracking in the lung (5), and a dilution of the fluorescent dyes as the cells reproduce. Nevertheless, when MSCs were intravenously administered in an acute phase of myocardial infarction, MSCs induced angiogenesis and myogenesis and modestly, but significantly, improved cardiac function. Thus systemic delivery of MSCs may be beneficial for the treatment of myocardial infarction.

A limitation of this study is that the cell population may be mixed, rather than limited to MSCs, although cell surface markers of cultured cells were consistent with those of previously reported MSCs (12, 18).

In conclusion, intravenously administered MSCs were preferentially attracted to the infarcted myocardium and differentiated into vascular endothelial cells and cardiomyocytes. MSC transplantation decreased the infarct size and improved cardiac function after acute myocardial infarction through enhancement of angiogenesis and myogenesis. Thus MSC transplantation may be a new therapeutic strategy for the treatment of myocardial infarction.

GRANTS

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Effects of Adrenomedullin Inhalation on Hemodynamics and Exercise Capacity in Patients With Idiopathic Pulmonary Arterial Hypertension

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Background—Adrenomedullin (AM) is a potent pulmonary vasodilator peptide. However, whether intratracheal delivery of aerosolized AM has beneficial effects in patients with idiopathic pulmonary arterial hypertension remains unknown. Accordingly, we investigated the effects of AM inhalation on pulmonary hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension.

Methods and Results—Acute hemodynamic responses to inhalation of aerosolized AM (10 μg/kg body wt) were examined in 11 patients with idiopathic pulmonary arterial hypertension during cardiac catheterization. Cardiopulmonary exercise testing was performed immediately after inhalation of aerosolized AM or placebo. The work rate was increased by 15 W/min until the symptom-limited maximum, with breath-by-breath gas analysis. Inhalation of AM produced a 13% decrease in mean pulmonary arterial pressure (54±3 to 47±3 mm Hg, $P<0.05$) and a 22% decrease in pulmonary vascular resistance (12.6±1.5 to 9.8±1.3 Wood units, $P<0.05$). However, neither systemic arterial pressure nor heart rate was altered. Inhalation of AM significantly increased peak oxygen consumption during exercise (peak $\dot{V}O_2$, 14.6±0.6 to 15.7±0.6 mL · kg^{-1} · min^{-1}, $P<0.05$) and the ratio of change in oxygen uptake to that in work rate ($\Delta\dot{V}O_2/\Delta W$ ratio, 6.3±0.4 to 7.0±0.5 mL · min^{-1} · W^{-1}, $P<0.05$). These parameters remained unchanged during placebo inhalation.

Conclusions—Inhalation of AM may have beneficial effects on pulmonary hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension. **(*Circulation*. 2004;109:351-356.)**

Key Words: peptides ■ hypertension, pulmonary ■ respiration ■ exercise ■ hemodynamics

Idiopathic pulmonary arterial hypertension is a rare but life-threatening disease characterized by progressive pulmonary hypertension, ultimately producing right heart failure and death.[1,2] Although a variety of vasodilators have been proposed as potential therapy for this disease over the past 30 years,[3–7] some patients ultimately require heart-lung or lung transplantation.[8,9] Thus, a novel therapeutic strategy is desirable.

Adrenomedullin (AM) is a potent, long-lasting vasodilator peptide that was originally isolated from human pheochromocytoma.[10] Immunoreactive AM has subsequently been detected in plasma and a variety of tissues, including blood vessels and lungs.[11,12] It has been reported that there are abundant binding sites for AM in the lungs.[13] We have shown that the plasma AM level increases in proportion to the severity of pulmonary hypertension and that circulating AM is partially metabolized in the lungs.[14,15] Interestingly, AM has been shown to inhibit the migration and proliferation of vascular smooth muscle cells.[16,17] These findings suggest that AM plays an important role in the regulation of pulmonary vascular tone and vascular remodeling. In fact, we have shown that short-term intravenous infusion of AM significantly decreases pulmonary vascular resistance in patients with congestive heart failure[18] or pulmonary arterial hypertension.[19] Unfortunately, however, intravenously administered AM induced systemic hypotension in such patients because of nonselective vasodilation in the pulmonary and systemic vascular beds.

More recently, inhalation of aerosolized prostacyclin and its analogue iloprost has been shown to cause pulmonary vasodilation without systemic hypotension in patients with idiopathic pulmonary arterial hypertension.[20,21] In addition, inhalant application of vasodilators does not impair gas exchange because the ventilation-matched deposition of drug

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TABLE 1. Baseline Characteristics of Patients With Idiopathic Pulmonary Arterial Hypertension

Demographics	
Age, y	39±3
Male/female, n	2/9
NYHA functional class, n	
III	10
IV	1
Baseline hemodynamics	
MPAP, mm Hg	54±3
CI, $L \cdot min^{-1} \cdot m^{-2}$	2.4±0.1
PVR, Wood units	12.6±1.5
RAP, mm Hg	7±1
PCWP, mm Hg	7±1
Pulmonary function	
Sao_2, %	94±3
Svo_2, %	63±4
FVC, % predicted	86±4
FEV_1, % predicted	75±1
6-Minute walk test, m	355±35
Medication use, n	
Anticoagulant agents	10
Diuretics	9
Digitalis	7
Oral prostacyclin analogue	6
Calcium antagonists	2

NYHA indicates New York Heart Association; MPAP, mean pulmonary arterial pressure; CI, cardiac index; PVR, pulmonary vascular resistance; RAP, mean right atrial pressure; PCWP, pulmonary capillary wedge pressure; Sao_2, arterial oxygen pressure; Svo_2, mixed venous oxygen saturation; FVC, forced vital capacity; and FEV_1, forced expiratory volume in 1 second. Data are mean±SEM.

in the alveoli causes pulmonary vasodilation matched to ventilated areas.[20] In clinical settings, inhalation therapy may be more simple, noninvasive, and comfortable than continuous intravenous infusion therapy. Thus, the purpose of the present study was to investigate the effects of AM inhalation on hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension.

Methods

Study Subjects

Eleven patients with idiopathic pulmonary arterial hypertension (9 women and 2 men; age, 39±3 years) were included in this study. Idiopathic pulmonary arterial hypertension was defined as pulmonary hypertension unexplained by any secondary cause, on the basis of the criteria of the National Institutes of Health registry.[1] Ten patients were classified as New York Heart Association (NYHA) functional class III and 1 as class IV (Table 1). Two of the 11 patients (18%) were acute responders who showed a significant decrease in mean pulmonary arterial pressure of ≥20% with a decrease in mean pulmonary arterial pressure to <35 mm Hg and no change or an increase in cardiac index during short-term infusion of epoprostenol. Long-term medication, including anticoagulant agents, digitalis, and diuretics, was kept constant. Vasodilator agents, such as oral prostacyclin analogue and calcium antagonists, were stopped ≥12 hours before the study procedure was begun. The ethics committee of the National Cardiovascular Center approved the study, and all patients gave written informed consent.

Preparation of Human AM

Human AM was dissolved in saline with 4% D-mannitol and sterilized by passage through a 0.22-μm filter (Millipore Co). At the time of dispensing, randomly selected vials were submitted for sterility and pyrogen testing. The chemical nature and content of the human AM in vials were verified by high-performance liquid chromatography and radioimmunoassay. All vials were stored frozen at −80°C from the time of dispensing until the time of preparation for administration.

Hemodynamic Studies

Acute hemodynamic responses to AM inhalation were assessed in all patients while they were in a stable condition during hospitalization. Hemodynamic variables, including pulmonary arterial pressure, right atrial pressure, pulmonary capillary wedge pressure, and cardiac output (in triplicate), were determined with a thermodilution catheter (TOO21H-7.5F, Baxter Co).[22] A 22-gauge cannula was inserted into a radial artery for hemodynamic measurements and blood sampling. After an equilibration period of 30 minutes, baseline hemodynamics were measured. Then, AM (10 μg/kg body wt) was inhaled as an aerosol with a jet nebulizer (Porta-Nebu, MEDIC-AID) for 15 minutes, which resulted in a cumulative dose of 400 to 600 μg AM. Hemodynamic parameters were measured at 15-minute intervals starting 15 minutes before AM inhalation until 60 minutes after inhalation. Blood samples for AM measurement were taken at 15-minute intervals from 15 minutes before inhalation until 60 minutes after the end of inhalation.

Cardiopulmonary Exercise Testing

The effects of AM inhalation on exercise capacity were examined in 10 of 11 patients; 1 patient with NYHA class IV underwent the 6-minute walk test according to decision of attending physicians. Cardiopulmonary exercise testing was performed immediately after inhalation of aerosolized AM (10 μg/kg body wt) or saline in a double-blind, randomized, crossover design. This study was performed on 2 separate days, 1 week apart. The first cardiopulmonary exercise testing was performed within 10 days after the cardiac catheterization. The patients performed exercise seated on a cycle ergometer. They first pedaled at 55 rpm without any added load for 1 minute. The work rate was then increased by 15 W/min up to the symptom-limited maximum. Breath-by-breath gas analysis was performed with an AE280 (Minato Medical Science) connected to a personal computer running analyzing software.[23] The ratio of change in oxygen uptake to that in work rate ($\Delta\dot{V}o_2/\Delta W$ ratio) was calculated as the slope of oxygen consumption per unit workload from 1 minute after the start of load addition until 85% maximal $\dot{V}o_2$. Exercise capacity was evaluated by peak oxygen consumption (peak $\dot{V}o_2$), which was defined as the value of averaged data during the final 15 seconds of exercise. Ventilatory efficiency during exercise was represented by the $\dot{V}E$-$\dot{V}co_2$ slope, which was determined as the linear regression slope of $\dot{V}E$ and $\dot{V}co_2$ from the start of exercise until the RC point (the time until which ventilation is stimulated by CO_2 output and end-tidal CO_2 tension begins to decrease).

Measurement of Plasma AM, cAMP, and cGMP

Blood samples were immediately transferred into chilled glass tubes containing disodium EDTA (1 mg/mL) and aprotinin (500 U/mL) and centrifuged immediately at 4°C, and the plasma was frozen and stored at −80°C until assayed. Plasma AM level was measured by a specific immunoradiometric assay kit (Shionogi Pharmaceutical Co Ltd).[24] Plasma cAMP and cGMP were determined with radioimmunoassay kits (cAMP assay kit, cGMP assay kit, Yamasa Shoyu).[18]

Statistical Analysis

All data were expressed as mean±SEM unless otherwise indicated. Changes in hemodynamic and hormonal parameters by AM inhalation were analyzed by 1-way ANOVA for repeated measures,

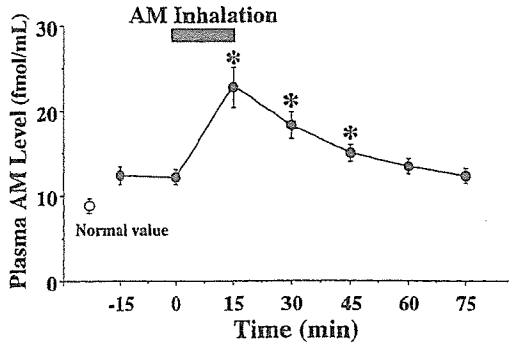

Figure 1. Changes in plasma AM level by inhalation of aerosolized AM in patients with idiopathic pulmonary arterial hypertension. Normal value indicates plasma AM level derived from 15 age-matched healthy subjects. Data are mean±SEM. *P<0.05 vs value at time 0.

followed by Newman-Keuls test. Comparisons of exercise parameters between the 2 groups were analyzed with paired Student's t test. A probability value of P<0.05 was considered statistically significant.

Results

All patients tolerated this study protocol. One patient developed a headache, and another patient had mild arterial hypoxemia during AM inhalation. None of them experienced other adverse effects, such as systemic hypotension, infection, or arrhythmia.

Plasma AM Level After Inhalation

Baseline plasma AM level in patients with idiopathic pulmonary arterial hypertension was significantly higher than the normal value, which was determined from pooled data of 15 age-matched healthy subjects (11.9±0.8 versus 9.3±0.1 fmol/mL, P<0.05). Inhalation of AM significantly increased the plasma AM level to 22.9±2.1 fmol/mL immediately after inhalation (Figure 1). The half-life of plasma AM after inhalation was approximately 20 minutes, and the elevation of AM lasted for >45 minutes. Plasma cAMP level increased significantly 30 minutes after the initiation of AM inhalation (10.8±0.7 to 12.0±0.6 pmol/mL, P<0.05), although plasma cGMP level was not significantly altered (6.5±1.0 to 6.8±1.0 pmol/mL, P=NS).

Hemodynamic Effects of AM Inhalation

Inhalation of AM significantly decreased mean pulmonary arterial pressure in patients with idiopathic pulmonary arterial hypertension (54±3 to 47±3 mm Hg, P<0.05) without a significant decrease in mean arterial pressure (85±4 to 83±4 mm Hg, P=NS) (Figure 2). AM inhalation slightly but significantly increased cardiac index by 12% (2.4±0.1 to 2.7±0.2 L · min^{-1} · m^{-2}, P<0.05). Thus, AM inhalation resulted in a 22% decrease in pulmonary vascular resistance (12.6±1.5 to 9.8±1.3 Wood units, P<0.05) (Figure 3). Inhaled AM did not significantly alter systemic vascular resistance. The ratio of pulmonary vascular resistance to systemic vascular resistance was decreased significantly at the end of inhalation (0.63±0.08 to 0.55±0.07, P<0.05). These hemodynamic effects of AM lasted for >45 minutes.

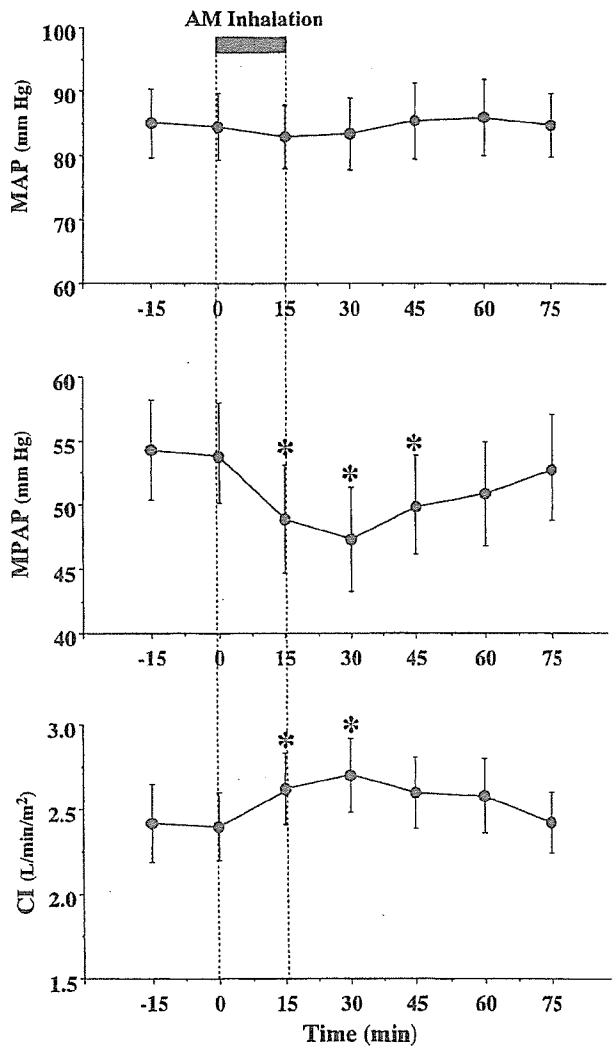

Figure 2. Changes in mean arterial pressure (MAP), mean pulmonary arterial pressure (MPAP), and cardiac index (CI) by inhalation of aerosolized AM in patients with idiopathic pulmonary arterial hypertension. Data are mean±SEM. *P<0.05 vs value at time 0.

No significant change in heart rate, pulmonary capillary wedge pressure, or right atrial pressure was observed. There was no significant change in arterial oxygen saturation (94±3% to 93±3%).

Effects of AM Inhalation on Exercise Capacity and Ventilatory Efficiency

As the limiting symptom at the end of exercise, 6 patients reported muscle weakness and 4 reported dyspnea. There was no difference in these symptoms when exercise testing was performed with or without inhalation of AM. Inhalation of AM altered neither heart rate nor blood pressure either at rest or at peak exercise (Table 2). Inhalation of AM significantly increased peak workload (86±5 to 93±6 W, P<0.05) (Table 2). AM also significantly increased peak $\dot{V}O_2$ (14.6±0.6 to 15.7±0.6 mL · kg^{-1} · min^{-1}, P<0.05) (Figure 4). Inhalation of AM significantly increased $\Delta\dot{V}O_2/\Delta W$ ratio (6.3±0.4 to

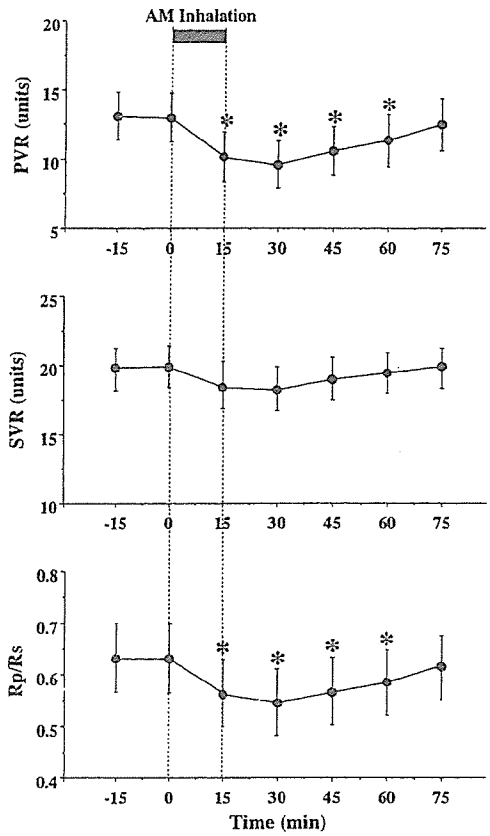

Figure 3. Changes in pulmonary vascular resistance (PVR), systemic vascular resistance (SVR), and ratio of pulmonary vascular resistance to systemic vascular resistance (Rp/Rs) by inhalation of aerosolized AM in patients with idiopathic pulmonary arterial hypertension. Data are mean±SEM. *$P<0.05$ vs value at time 0.

7.0±0.5 mL · min^{-1} · W^{-1}, $P<0.05$). AM did not significantly alter the $\dot{V}_E$-$\dot{V}_{CO_2}$ slope (Table 2). No significant changes in arterial oxygen saturation were observed either at rest or at peak exercise. In 1 patient with NYHA class IV who did not undergo cardiopulmonary exercise testing, the distance walked in 6 minutes increased from 150 to 180 m by inhalation of AM.

Discussion

In the present study, we demonstrated that inhalation of AM improved hemodynamics with pulmonary selectivity and exercise capacity in patients with idiopathic pulmonary arterial hypertension.

AM is one of the most potent endogenous vasodilators in the pulmonary vascular bed.[25–27] The vasodilatory effect is mediated by cAMP-dependent and nitric oxide–dependent mechanisms.[28,29] Endogenous AM production is enhanced in a variety of cardiovascular diseases through a compensatory mechanism.[14,30] Nonetheless, additional supplementation of AM has beneficial effects in these diseases.[18,19] These results suggest that endogenous AM level is not sufficient to improve deteriorated conditions despite the increased AM production. Interestingly, Champion et al[31] have shown that intratracheal gene transfer of calcitonin gene–related peptide, a member of the same peptide family as AM, to bronchial

TABLE 2. Changes in Exercise Parameters by Inhalation of AM or Placebo

Variables	Placebo	AM	P
Peak workload, W	86±5	93±6	<0.05
HR, bpm			
Rest	75±5	75±3	NS
Peak	144±6	148±6	NS
MAP, mm Hg			
Rest	85 3	87 ' 5	NS
Peak	108±5	110±6	NS
Peak Borg score (D/L)	17/18	18/18	NS
Peak $\dot{V}_{O_2}$, mL · kg^{-1} · min^{-1}	14.6±0.6	15.7±0.6	<0.05
$\Delta\dot{V}_{O_2}/\Delta W$ ratio, mL · min^{-1} · W^{-1}	6.3±0.4	7.0±0.5	<0.05
$\dot{V}_E$-$\dot{V}_{CO_2}$ slope	37±2	36±2	NS
Sa_{O_2}, %			
Rest	97±1	97±1	NS
Peak	95±1	95±1	NS

HR indicates heart rate; MAP, mean arterial pressure; Peak Borg score (D/L), Borg score at peak exercise (dyspnea/leg fatigue); Peak $\dot{V}_{O_2}$, peak oxygen consumption; $\Delta\dot{V}_{O_2}/\Delta W$ ratio, $\dot{V}_{O_2}$ increase per unit workload; $\dot{V}_E$-$\dot{V}_{CO_2}$ slope, slope of regression line of relation between $\dot{V}_E$ and $\dot{V}_{CO_2}$; and Sa_{O_2}, arterial oxygen saturation. Data are mean±SEM.

epithelial cells attenuates chronic hypoxia-induced pulmonary hypertension in the mouse. These results raise the possibility that intratracheal delivery of a vasodilator peptide may be sufficient to alter pulmonary vascular function. In fact, in the present study, inhalation of AM significantly decreased pulmonary vascular resistance, whereas it did not alter systemic arterial pressure or systemic vascular resistance. The ratio of pulmonary vascular resistance to systemic vascular resistance was reduced significantly by AM inhalation. These results suggest that inhaled AM improves hemodynamics with pulmonary selectivity. This is consistent with earlier findings that inhaled prostacyclin or its analogue iloprost acts transepithelially with pulmonary selectivity and improves pulmonary hypertension.[20,21] Inhalation of AM slightly but significantly increased cardiac index in patients with idiopathic pulmonary arterial hypertension. Considering the strong vasodilator activity of AM in the pulmonary vasculature, the significant decrease in cardiac afterload may be responsible for increased cardiac index with

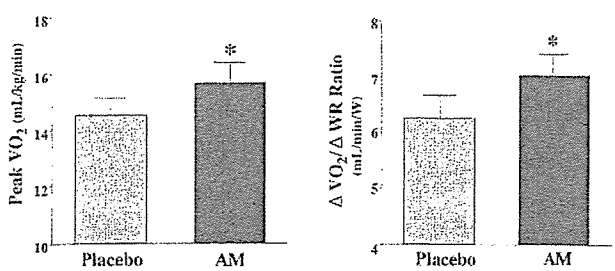

Figure 4. Changes in peak oxygen consumption (peak $\dot{V}_{O_2}$) and ratio of change in oxygen uptake to that in work rate ($\Delta\dot{V}_{O_2}/\Delta W$ ratio) by inhalation of aerosolized AM or placebo in patients with idiopathic pulmonary arterial hypertension. Data are mean±SEM. *$P<0.05$ vs placebo.

AM. Interestingly, the hemodynamic effects of inhaled AM lasted for >45 minutes. A previous study demonstrated that intravenous injection of AM produces a long-lasting vasodilator response because of its long half-life (≈15 minutes).[32] The half-life of plasma AM after inhalation was longer (20 minutes). Thus, inhalation of AM may cause relatively long-lasting pulmonary vasodilator activity in patients with idiopathic pulmonary arterial hypertension. In the present study, plasma cAMP level increased after AM inhalation, suggesting that the hemodynamic effects of AM may be mediated by activation of cAMP.

Earlier studies have shown that peak $\dot{V}O_2$ during exercise is markedly lower in patients with idiopathic pulmonary arterial hypertension than in healthy subjects.[33,34] Peak $\dot{V}O_2$ is determined primarily by the maximal cardiac output during exercise and the potential for O_2 extraction by the exercising muscle.[35] Thus, the decreased peak $\dot{V}O_2$ may reflect insufficient oxygen delivery to the body during exercise, at least in part because of an inadequate increase in cardiac output under conditions of severe pulmonary hypertension. In the present study, inhalation of AM significantly increased peak $\dot{V}O_2$ in patients with pulmonary hypertension. AM also increased the $\Delta\dot{V}O_2/\Delta W$ ratio, which indicates oxygen transport per unit workload to the exercising legs. These results suggest that inhalation of AM improves exercise capacity in patients with idiopathic pulmonary arterial hypertension. It is possible that an increase in cardiac output during exercise may contribute to increases in peak $\dot{V}O_2$ and the $\Delta\dot{V}O_2/\Delta W$ ratio.

The major limitation of this pilot trial relates to the lack of a randomized, placebo-controlled group in acute hemodynamic studies, which was as result not only of invasive assessment of hemodynamics but also of the limited number of patients available. Nevertheless, cardiopulmonary exercise testing was performed in a double-blind, randomized, crossover design. Thus, it is unlikely that the hemodynamic effects of inhaled AM are attributable to the placebo effect.

Inhalation therapy may be more simple, noninvasive, and comfortable than continuous intravenous infusion therapy. An experimental study demonstrated that repeated inhalation of AM (for 30 minutes, 4 times a day) inhibited monocrotaline-induced pulmonary hypertension and markedly improved survival in rats.[36] Recently, pulmonary delivery of a dry-powder insulin has been shown to improve glycemic control without adverse pulmonary effects.[37] Although further studies are necessary to maximize the efficiency and reproducibility of pulmonary AM delivery, combining AM inhalation therapy with other modalities that have a different mode of action may have beneficial effects in patients with idiopathic pulmonary arterial hypertension.

Conclusions

These preliminary results suggest that inhalation of AM may have beneficial effects on pulmonary hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension.

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