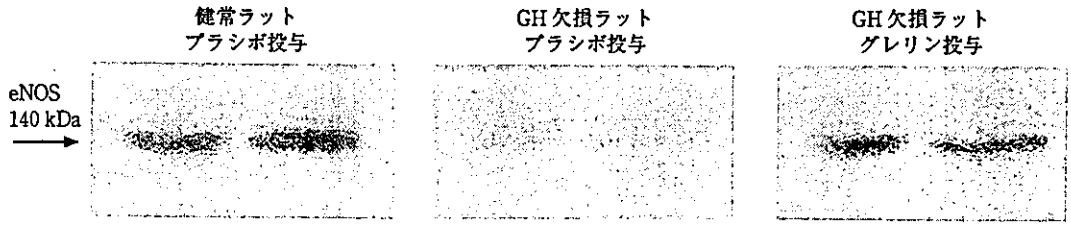

図2 グレリンを皮下投与されたラットにおける内皮型一酸化窒素合成酵素(eNOS)蛋白の発現[15]

機能の調節に重要な役割を果たし，血管の恒常性の維持や動脈硬化の進展予防に関与する可能性が示唆された．

おわりに

これまでの研究から，グレリンは悪液質を有する慢性心不全において，代償的に増加し，異化に傾いたエネルギーバランスの不調和の改善に関与していると考えられる．またその強力なGH分泌作用またはグレリン独自の作用により，心機能および内皮機能の維持にも関与していると考えられ，グレリンは循環器系における恒常性の維持に重要な役割を担っていることが示唆された．

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特　論

アドレノメデュリンと細胞治療

Combined therapy with adrenomedullin and cell transplantation

岩瀬　俊　　永谷憲歳

Key words：adrenomedullin, cell therapy

はじめに

近年，成人個体の骨髄および末梢血中における血管内皮前駆細胞(EPC)の存在が報告されてから，循環器領域における細胞移植治療に関する研究が活発に行われている[1]．実際，臨床においても自己の骨髄単核球(MNC)を用いた血管新生療法が主に閉塞性動脈疾患症例に対して行われている[2]．しかし，現在の方法では細胞採取時の侵襲が大きいことや，時間経過とともに治療効果が減弱することなどの問題点が存在する．したがって，細胞治療の移植効果を上げる機能強化法の開発が必要である．

アドレノメデュリン(adrenomedullin: AM)は，北村，寒川らによってヒト褐色細胞腫より発見された強力な血管拡張ペプチドである[3]．AMは近年その血管拡張作用に加え，血管新生および胎児期の血管形成においても重要な役割を果たしていることが明らかとなっている[4]．AMはこれらの多面的作用を有することより，現在の血管再生を主眼とした細胞移植治療においてその効果を相加的または相乗的に増強する可能性がある．

本稿では，①AMの血管新生作用およびEPC，MNCに対する相互作用，②疾患動物モデル(原発性肺高血圧および末梢動脈閉塞症)に対するAMと細胞移植治療併用の治療効果に関して，今まで著者らが行ってきた検討を文献的考察も含めて報告する．

1. AMの血管新生作用ならびにEPC，MNCに対する相互作用

AMはPI3K-Akt経路を活性化することで血管内皮細胞の生存，遊走，増殖に関与することが知られている．更に近年，AMがPI3K-Akt経路を介して血管新生作用を有することが相次いで報告されている[5,6]．著者らもウサギ下肢虚血モデルにAM遺伝子を導入すると，虚血下肢において著明な血管新生が誘導されることならびに，AMの血管新生作用は代表的な血管新生因子であるVEGFを介さない独自の作用であることを報告してきた[7]．

EPCは生体内で虚血や血管内皮障害が起きたときに，その障害部位へ遊走，付着して血管内皮細胞に分化して血管を形成する能力を有するが[1]，移植に必要な細胞数を確保するのに多量の血液が必要である．著者らはAMがEPCの機能増強作用を有するか否かについて*in vitro*での検討を行った．正常電ゼラチンを用いてAM遺伝子をEPCに導入すると(図1)，EPCのアポトーシスが抑制され，増殖が促進された．更にAM遺伝子導入EPCはEPC単独の10倍以

Takashi Iwase, Noritoshi Nagaya: Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute　国立循環器病センター研究所再生医療部

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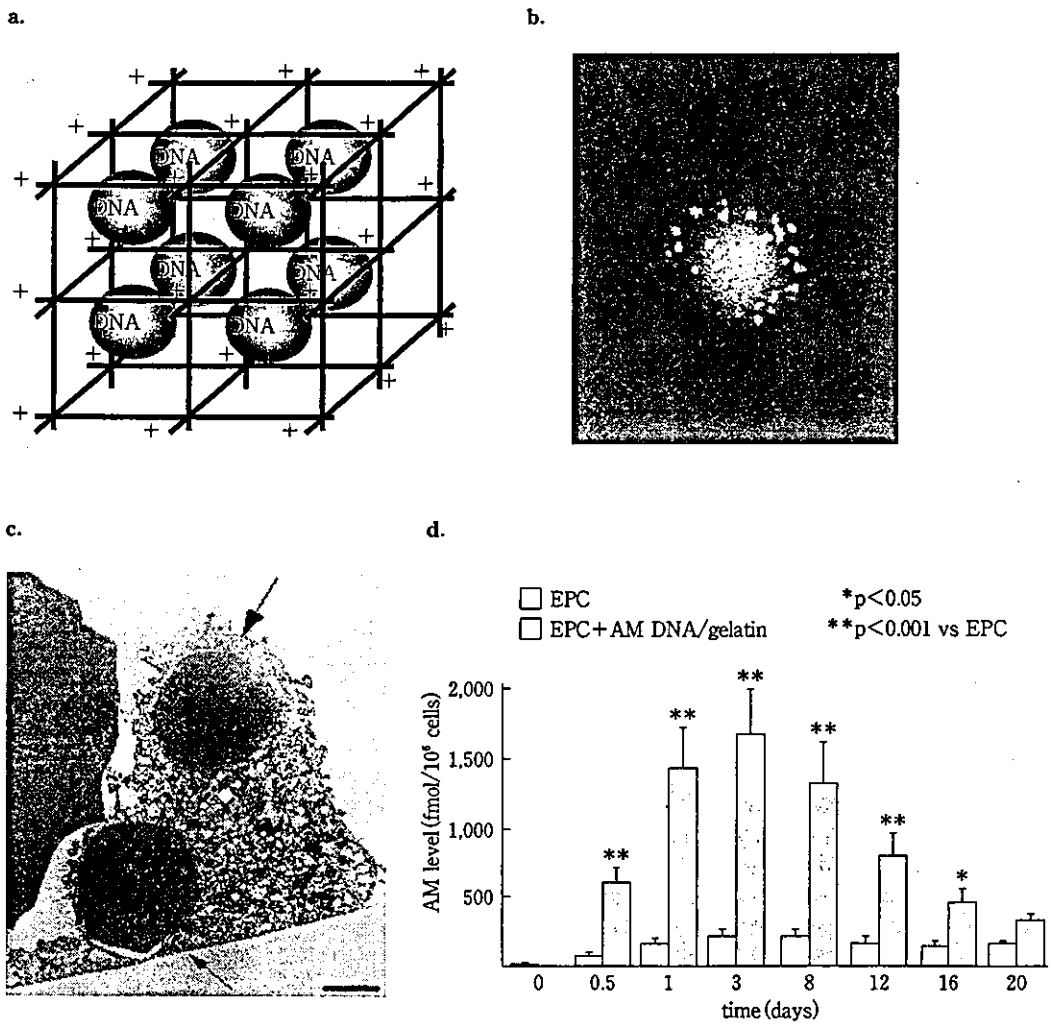

図1　ゼラチンを用いたEPCに対するAM遺伝子の導入[8]

DNA-ゼラチン複合体のシェーマ(a)とその実像(b).
電顕像でEPCがDNA-ゼラチン複合体を貪食するのが確認された(c).
ゼラチンによる遺伝子導入後のEPC培養液中のAM濃度(d).

上のAMを分泌し，2週間以上発現が持続した[8]．以上よりAM遺伝子をEPCに組み込むことで，①EPCの機能強化に結びつくのみならず，②ベクターとして局所まで運ばれることで(cell-based gene therapy)，AMの多彩な作用が効率良く局所で発揮されることが期待できると考えられた．

同様にMNCとAMの相互作用についても検討した．無血清培地にて24時間培養すると多数のMNCがアポトーシスを来すが，AMは用量依存性にMNCのアポトーシスを抑制した(図2-a)．一方，PI3K阻害薬であるwortmanninを加えるとAMの効果が抑制されることから，AMのMNCに対する抗アポトーシス作用はPI3K-Akt経路を介したものであると考えられた．更にAMはMNCの血管内皮細胞に対する接着を促進し，MNCからEPCへの分化を促進した．これらの結果よりAMは独自の血管新生作用のみならず，EPCおよびMNCの移植効果増強作用も有する可能性が示された．

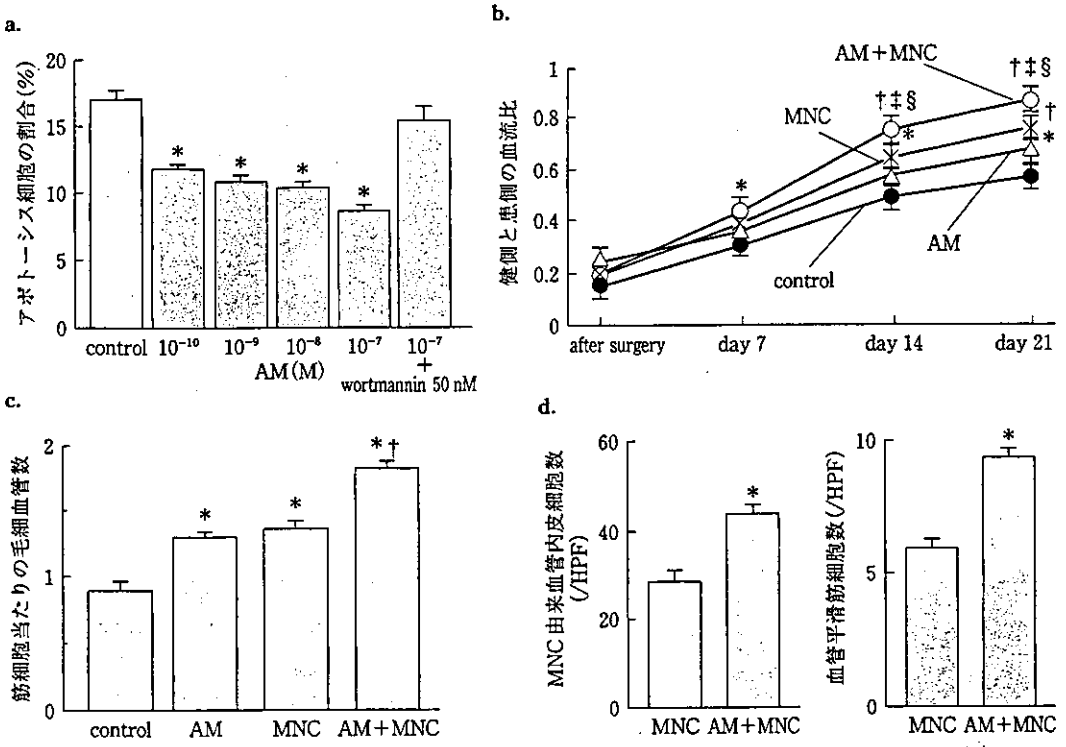

図2 AMとMNCの相互作用

AMのMNCに対する抗アポトーシス作用(a)，AM投与，MNC移植およびAMとMNC併用投与の下肢虚血ラットに対する治療効果(b-d)．

a: *$p<0.01$ vs control.

b: レーザードプラによる下肢血流の評価

control: 下肢虚血ラットにvehicle投与，AM: 下肢虚血ラットにAM投与，MNC: 下肢虚血ラットにMNC移植，AM+MNC: 下肢虚血ラットにAMとMNC併用投与．

*$p<0.05$, †$p<0.01$ vs control, ‡$p<0.01$ vs AM, §$p<0.05$ vs MNC.

c: 毛細血管数の評価

*$p<0.01$ vs control, †$p<0.01$ vs AMおよびMNC.

d: AMの移植したMNCに対する分化促進作用

*$p<0.01$ vs MNC.

2. AM遺伝子とEPCを用いた肺高血圧治療

原発性肺高血圧症は原因不明の肺動脈性肺高血圧症であり，発病してから死亡するまで平均3年と非常に予後不良な疾患である．本疾患に対しては現在もなお決定的な治療法がなく，新たな治療法の開発が望まれている状況である．

近年，原発性肺高血圧症の発症原因として肺血管内皮の機能障害が報告されている[9]．一つは肺血管内皮機能の障害による血管作動物質のバランス破綻(収縮因子>拡張因子)である．また病的な肺血管内皮の異常増殖も機序の一つとして想定されている[10]．以上より，肺血管内皮機能障害の病態に着目した治療として，①肺血管内皮細胞で産生される拡張因子の補充と収縮因子の抑制，②正常な肺血管内皮細胞の再生促進という治療法が考えられる．

そこでAM遺伝子とEPCの移植併用による肺高血圧治療効果を，ラット肺高血圧モデルを用いて検討した[8]．ヒト臍帯血から単核球を分離し，VEGF下で培養することでEPCを得た．

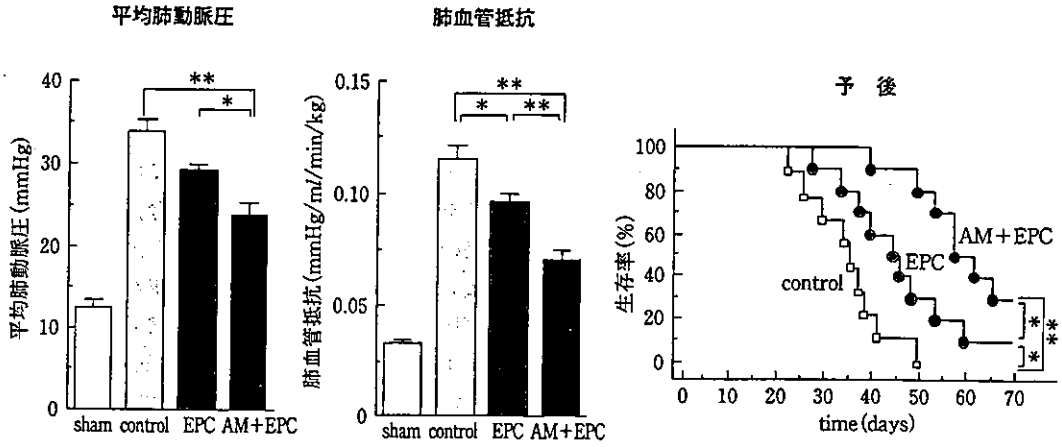

図3 AM遺伝子導入EPCの移植による平均肺動脈圧，肺血管抵抗，肺高血圧ラットの予後改善効果[8]

sham: 正常ラット，control: モノクロタリン肺高血圧ラットへvehicle投与，EPC: 肺高血圧ラットへEPC投与，AM+EPC: 肺高血圧ラットへAM遺伝子導入EPC投与．
$^{*}p<0.05$, $^{**}p<0.001$.

正常のラットにEPCを経静脈投与しても肺組織への付着は認められなかったが，モノクロタリンで肺血管内皮細胞や間質に障害を与えた後に投与すると，EPCは肺細動脈と間質に付着し成熟した血管内皮細胞となった．またEPCの移植は肺組織の血管数を増加させた．しかし血行動態的には平均肺動脈圧の有意な変化はなく，肺血管抵抗のわずかな改善が認められたのみであった(図3)．したがってEPCの移植のみでは肺高血圧治療効果には限界があると思われた．一方，AM遺伝子を導入したEPCを静脈内投与したところ[8]，コントロール群およびEPC移植単独群と比べて平均肺動脈圧を有意に低下させ，生存率を著明に改善させた．

AMは，特異的受容体が体血管よりむしろ肺血管に多数存在すること[11]，血管平滑筋細胞および血管内皮細胞に働き血管拡張を来すことから[12]，AM遺伝子導入EPC移植は正常な血管内皮の再生を促すのみならずAMによる肺血管拡張作用および血管新生作用があいまって肺高血圧の軽減に結びついたと思われる．既存の治療に抵抗性の原発性肺高血圧症患者が少なからず存在することを考えると，このAM遺伝子とEPCを用いた細胞移植治療は重症肺高血圧症に対する新たな治療法となる可能性がある．

3. 末梢動脈閉塞症に対するMNCを用いた細胞移植治療

続いて末梢動脈閉塞症(閉塞性動脈硬化症，Buerger病)に対するAMとMNC移植の併用療法の有効性を，下肢虚血動物モデルを用いて検討した．

ラットの左総腸骨動脈を結紮・切除して下肢虚血を作成し，AM単独投与，MNC移植単独およびAMとMNCの併用投与を行い，血管新生の程度について各群で比較検討した．3週間後の比較ではAM単独投与およびMNC移植単独においてコントロールと比較して有意な血流増加を認めた．更にAMとMNCの併用投与においては投与1週間後より顕著な血流増加を認め，3週間後の評価でも単独治療群よりも有意な血流増加を呈した(図2-b)．毛細血管の増加もAMとMNCの併用投与で最も著明であった(図2-c)．MNC移植単独およびAMとMNC併用投与はともに移植したMNCから血管内皮細胞への分化を認めたが，MNC単独と比較してAM併用投与では，より多くのMNC由来血管内皮細胞が確認された．更にAMとMNC併用

投与において血管の成熟に必要とされる血管平滑筋および壁細胞がMNC由来血管内皮細胞の周囲に多数確認された(図2-d). 以上のことよりAM単独投与, MNC単独投与でも血管新生作用があるが, 併用投与により血管新生効果が更に増強されることが明らかになった. またAM投与は移植したMNCの血管内皮細胞への分化を促進するのみならず, 成熟した血管の再生を促したものと考えられた. また心筋虚血モデルにおける検討においても同様の結果が得られた.

*in vitro*での検討およびこれらの動物実験の結果から, AMとMNCの併用投与は, AM独自の血管新生, 血管拡張作用に加え, 移植したMNCのアポトーシス抑制効果, 接着および内皮化を促進することで, 移植したMNCの組織への生着を保ち, 血管新生作用の増強および効果的な血管再生に結びついたと考えられた.

おわりに

今回, AMの遺伝子導入もしくはペプチド投与による細胞治療との併用療法の有効性について報告した. AMの遺伝子導入に用いた正帯電ゼラチンに関する詳細な記述は今回割愛するが, 従来のウイルスベクターを用いた遺伝子導入と比較して安全で倫理面の問題も少なく近い将来の臨床応用が期待される. また著者らは既に急性心不全症例や原発性肺高血圧症症例に対してAMのペプチドとしての投与を行い, 安全性を確認している[13,14]. 今回報告したMNCとAM併用療法に関しては, 末梢動脈閉塞症症例を対象とした臨床試験による有効性の確認が予定されており, 既に国立循環器病センターの倫理委員会で承認されている. AMは今回紹介した作用以外にも心筋細胞のアポトーシス抑制など幅広い生理作用を有しており, 様々な循環器疾患において細胞治療の効果を増強することが期待される.

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

Noritoshi Nagaya,[1,2] Takafumi Fujii,[3] Takashi Iwase,[1] Hajime Ohgushi,[4] Takefumi Itoh,[1] Masaaki Uematsu,[5] Masakazu Yamagishi,[2] Hidezo Mori,[3] Kenji Kangawa,[6] and Soichiro Kitamura[7]

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. $33 \pm 2\%$, $P < 0.05$). MSC transplantation decreased left ventricular end-diastolic pressure and increased left ventricular maximum dP/dt (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.

Address for reprint requests and other correspondence: N. Nagaya, Dept. of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan (E-mail: nnagaya@ri.ncvc.go.jp).

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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

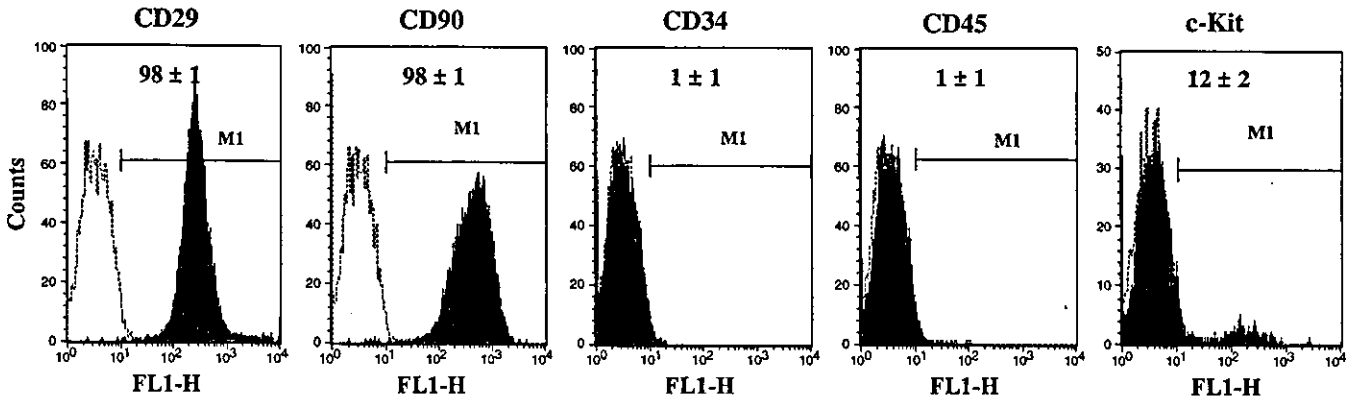

Fig. 1. Flow cytometric analysis of adherent, spindle-shaped mesenchymal stem cell (MSC) population expanded to 4–5 passages. Most of the cells expressed CD29 and CD90 but were negative for CD34 and CD45. Some cells were positive for c-Kit. MI, myocardial infarction.

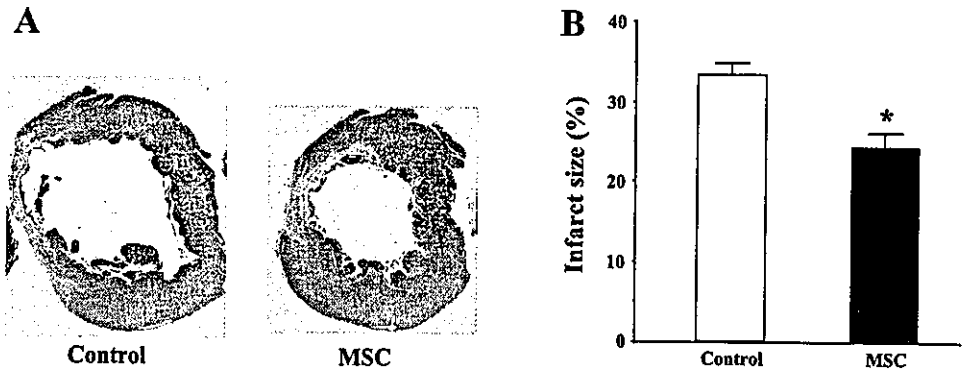

Fig. 2. Effect of MSC transplantation on myocardial infarct size 4 wk after myocardial infarction. *A*: representative Masson's trichrome-stained myocardial sections from control and MSC groups. *B*: quantitative analysis demonstrating that MSC transplantation significantly decreased infarct size. Values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

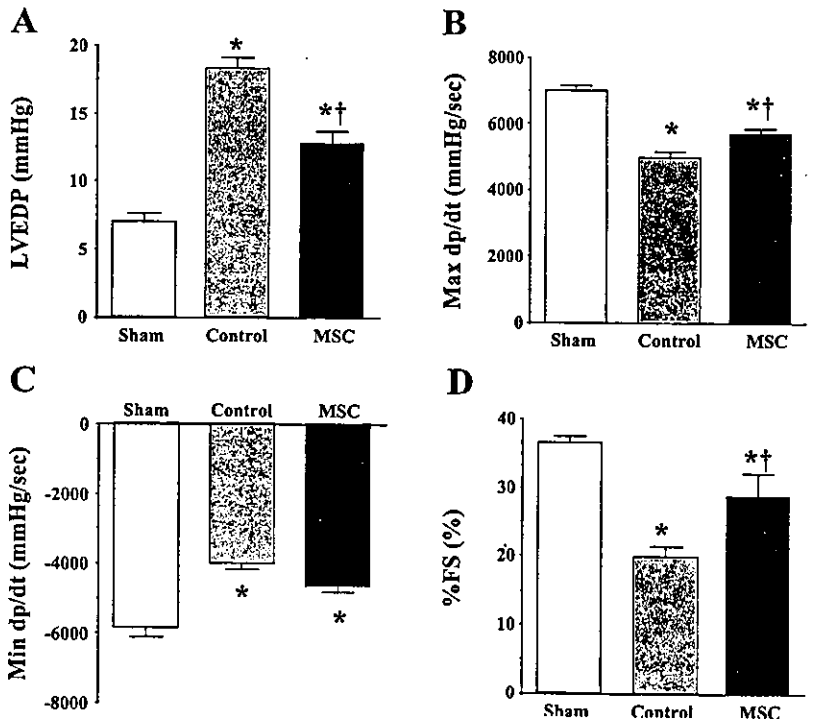

Fig. 3. Effects of MSC transplantation on hemodynamic parameters. LVEDP, LV end-diastolic pressure (*A*); max dP/d*t*, LV maximum dP/d*t* (*B*); Min dP/d*t*, LV minimum dP/d*t* (*C*); %FS, LV fractional shortening (*D*). Values are means ± SE. $*P < 0.05$ vs. sham. $†P < 0.05$ vs. control.

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

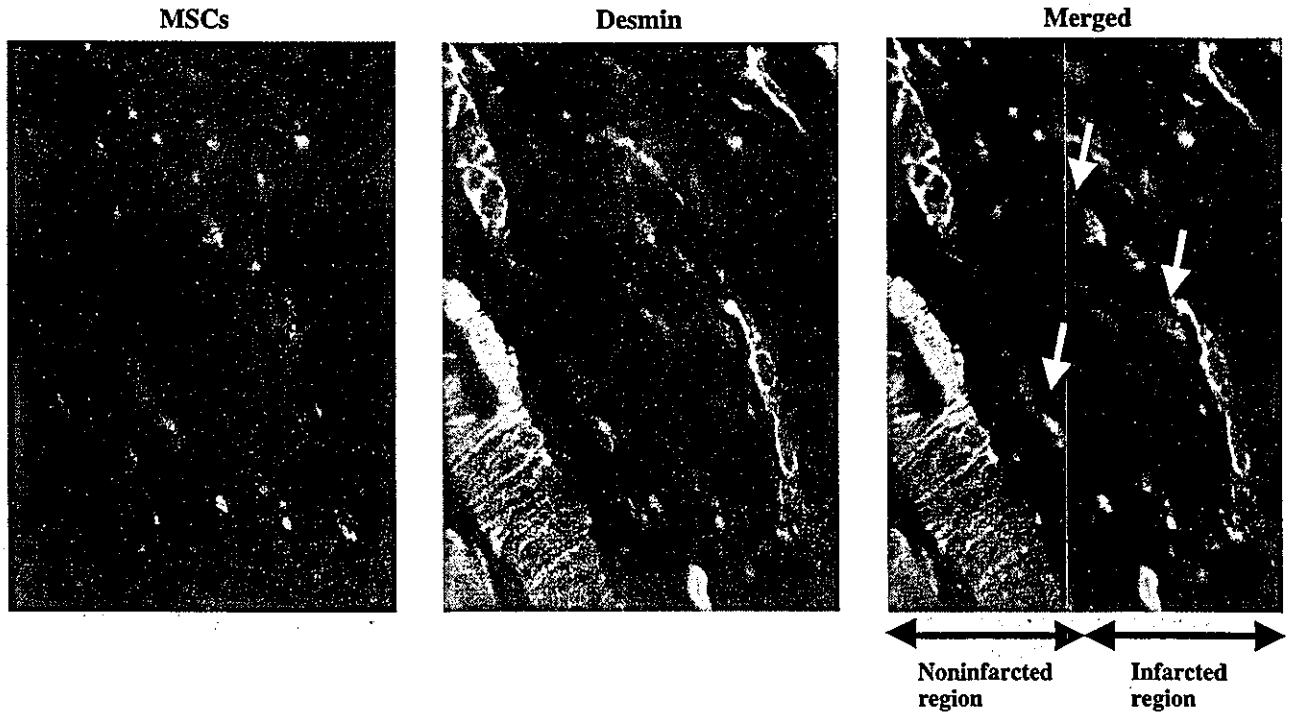

Fig. 4. Distribution of intravenously administered MSCs in myocardium after acute myocardial infarction. Red fluorescence-labeled MSCs were incorporated into ischemic boundary zone of the heart. These cells were positive for desmin (arrows), a cardiac marker. Magnification ×400.

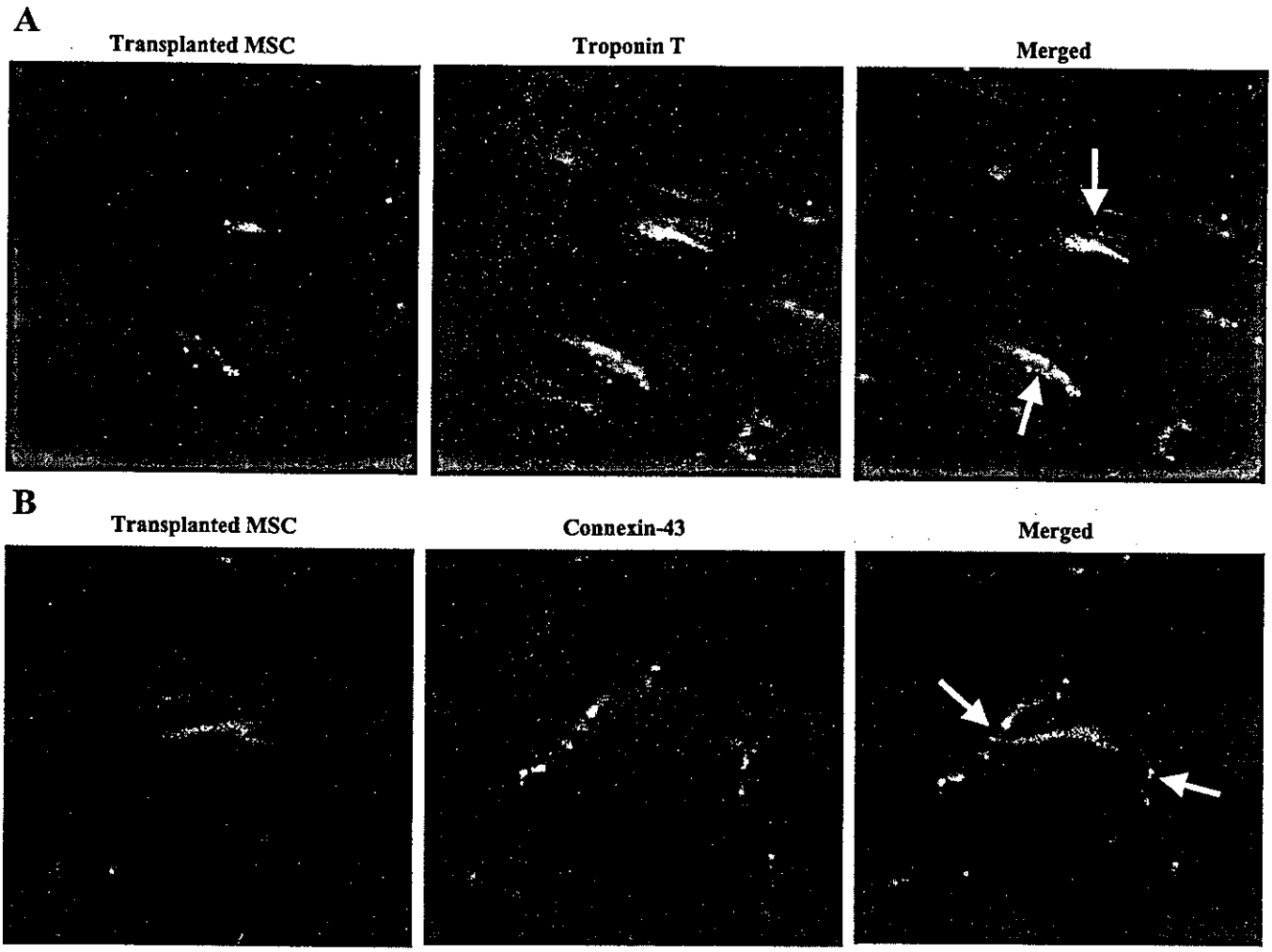

Fig. 5. Differentiation of transplanted MSCs in ischemic myocardium. Engrafted MSCs were positive (arrows) for cardiac troponin T (*A*) and connexin43 (*B*). Magnification ×400.

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)

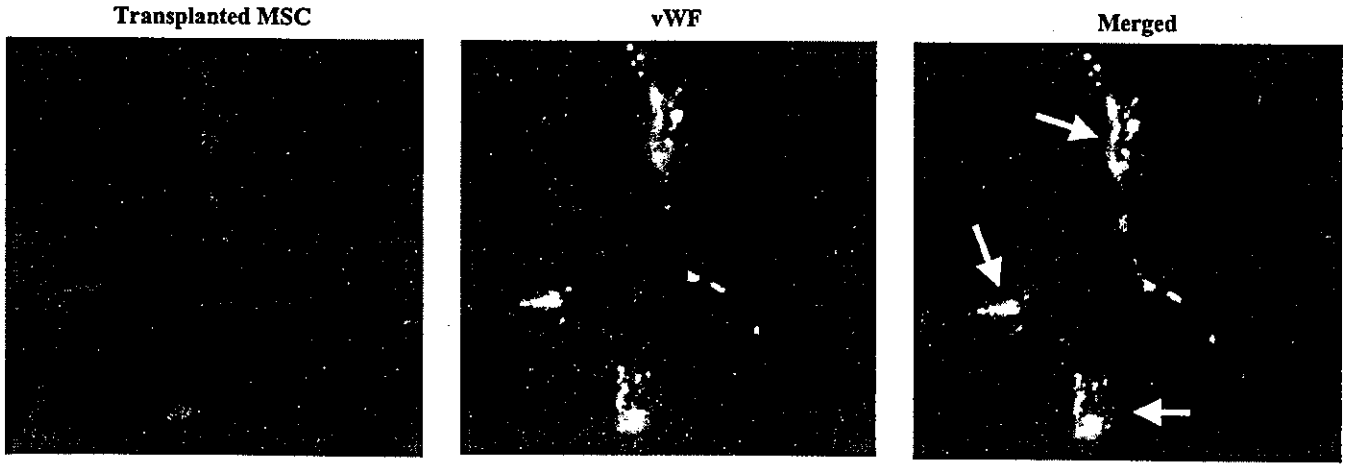

Fig. 6. Transplanted MSCs were positive for von Willebrand factor (vWF) and formed vascular structures. Magnification ×400.

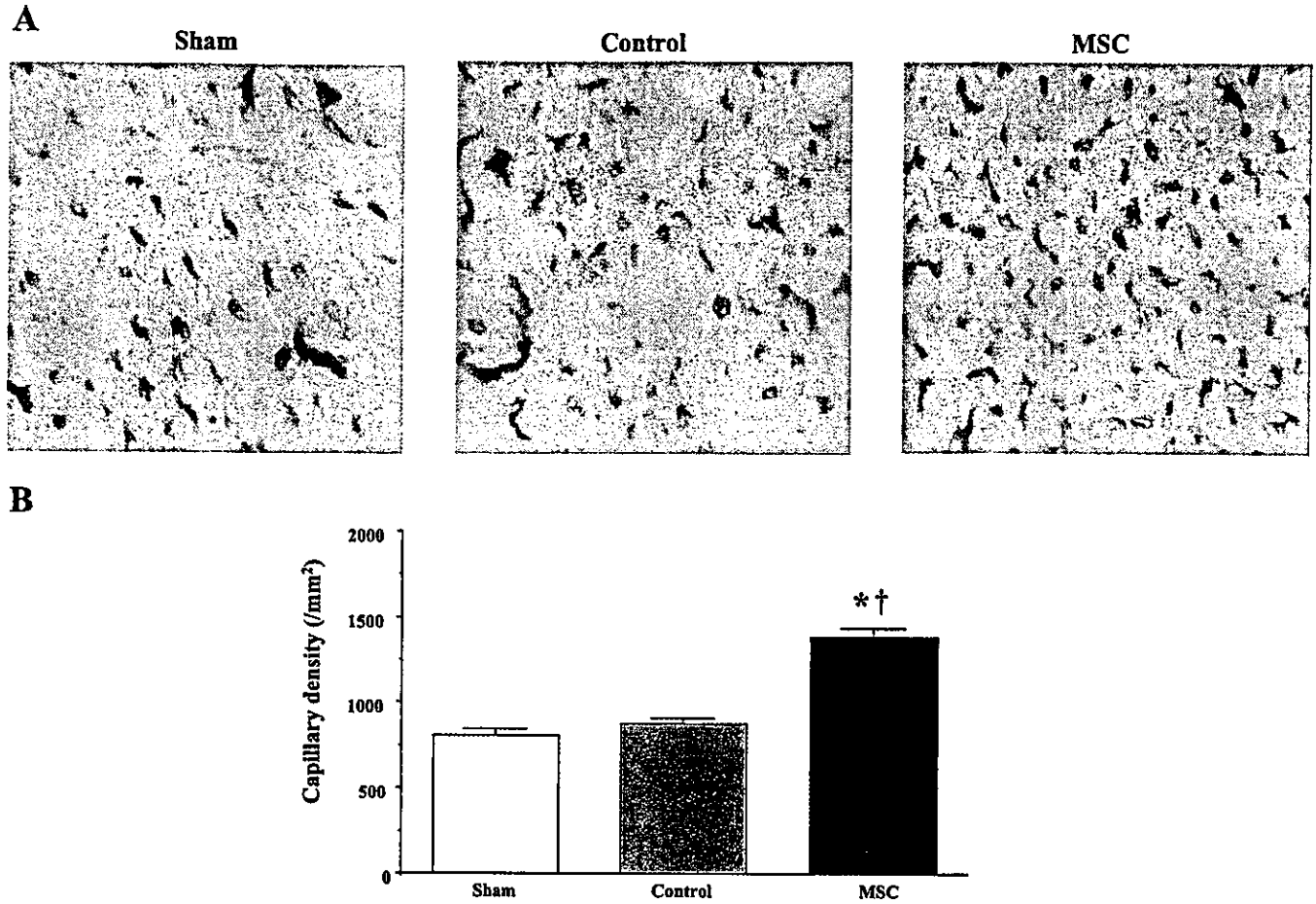

Fig. 7. *A*: representative samples of alkaline phosphatase staining in peri-infarct area. Magnification ×200. *B*: quantitative analysis of capillary density in peri-infarct area. Values are means ± SE. $*P < 0.05$ vs. sham. $\dagger P < 0.05$ vs. control.

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/d*t*, and a decrease in minimum LV dP/d*t*. 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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Adrenomedullin enhances therapeutic potency of bone marrow transplantation for myocardial infarction in rats

Takafumi Fujii,[1] Noritoshi Nagaya,[2,3] Takashi Iwase,[2] Shinsuke Murakami,[2] Yoshinori Miyahara,[1] Kazuhiro Nishigami,[3] Hatsue Ishibashi-Ueda,[5] Mikiyasu Shirai,[1] Takefumi Itoh,[2] Kozo Ishino,[6] Shunji Sano,[6] Kenji Kangawa,[4] and Hidezo Mori[1]

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.

Address for reprint requests and other correspondence: N. Nagaya, Dept. of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan (E-mail: nnagaya@ri.ncvc.go.jp).

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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.

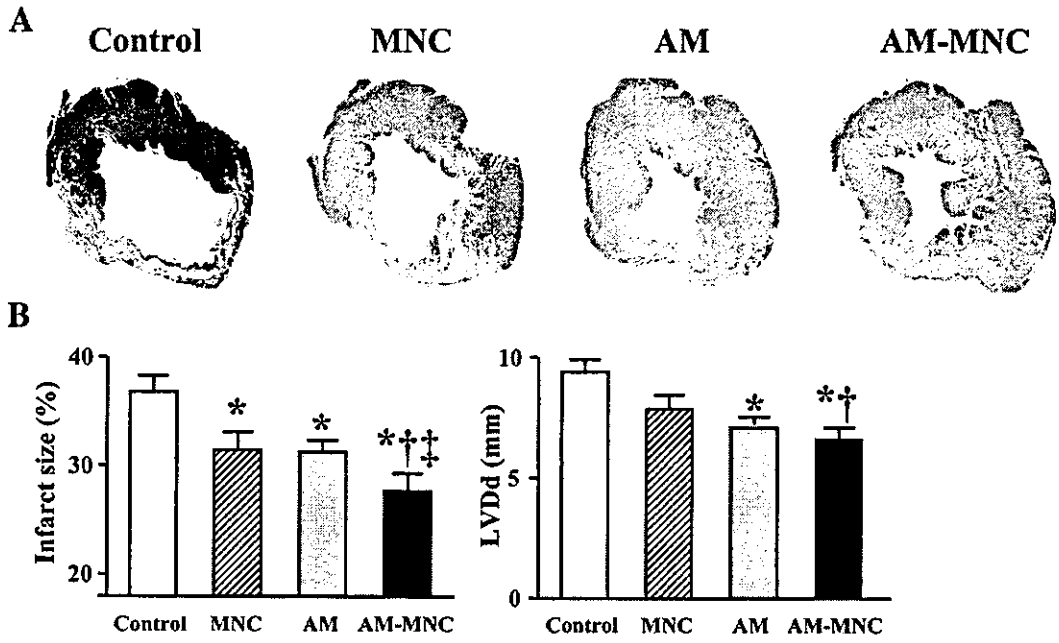

Fig. 1. *A*: representative examples of Masson trichrome-staining of transverse sections of left ventricular (LV) myocardium 4 wk after coronary ligation. *B*: quantitative analysis of infarct size and LV chamber size. Infarcted area and LV end-diastolic diameter (LVDd) of the adrenomedullin-mononuclear cell (AM-MNC) group were significantly smaller than those of the other groups. Values are means ± SE. *$P < 0.05$ vs. control; †$P < 0.05$ vs. MNC; ‡$P < 0.05$ vs. AM.

RESULTS

Infarct size and ventricular weight. Moderate-to-large infarcts were observed in the control group after coronary ligation (Fig. 1). However, infarct size was smaller in the MNC, AM, and AM-MNC groups than in the control group. In particular, it was very small in the AM-MNC group. Quantitative analysis also demonstrated that cardiac infarct size in the AM-MNC group was smallest among the four groups. Right ventricular weight was significantly lower in the AM and AM-MNC groups than that in the control group (Table 1). LV weight did not significantly differ among the four groups.

Echocardiographic findings. LV diastolic dimension was smallest in the AM-MNC group, followed by the AM, MNC, and control groups (Fig. 1). LV fractional shortening in the AM-MNC group was also higher than that in the control, MNC, and AM groups (Table 2). Diastolic thickness of the anterior wall was significantly attenuated in the MNC, AM, and AM-MNC groups compared with the control group.

Hemodynamics. Cardiac output in the AM-MNC group was significantly higher than that in the control, MNC, and AM groups (Fig. 2). LV end-diastolic pressure in the MNC, AM, and AM-MNC groups was significantly lower than that in the control group. LV maximum change in pressure over time (dP/dt) in the MNC and AM-MNC group were significantly higher than that in the control group. Similarly, LV minimum dP/dt was significantly decreased only in the AM-MNC group.

Capillary density. Alkaline phosphatase staining of ischemic myocardium showed marked augmentation of neovascularization in the MNC, AM, and AM-MNC groups compared with the control group (Fig. 3*A*). Quantitative analysis demonstrated that capillary density was significantly higher in the AM-MNC group than in the MNC and AM groups (Fig. 3*B*). Cartilage, bone, or fat was not observed in the transplanted area. No tumor-like cells were seen.

Antiapoptotic effect of AM on MNC. Red fluorescence-labeled MNC were detected in each recipient heart 72 h after transplantation (Fig. 4). TUNEL-positive cells were frequently observed in the MNC group. In contrast, these apoptotic cells

Table 1. *Physiological profiles of four experimental groups*

	Control	MNC	AM	AM-MNC
Number	13	14	14	15
Body weight, g	274±3	285±5	287±3	305±4*
Heart rate, bpm	410±24	404±30	398±33	387±36
MAP, mmHg	101±11	104±13	103±9	116±14*
LV wt/body wt, g/kg	2.4±0.2	2.5±0.2	2.6±0.1	2.5±0.2
RV wt/body wt, g/kg	1.1±0.1	0.9±0.1	0.8±0.1*	0.7±0.1*

Values are means ± SE; number is number of rats in each group. Control group, myocardial infarction rats given vehicle; MNC group, those given mononuclear cells; AM, those given adrenomedullin; AM-MNC, those given AM and MNC; MAP, mean arterial pressure; LV, left ventricle; RV, right ventricle. *$P < 0.05$ vs. control.

Table 2. *Echocardiographic findings*

	Control	MNC	AM	AM-MNC
LVDd, mm	9.9±0.2	8.3±0.3	7.3±0.2*	6.9±0.3*†
LVDs, mm	8.4±0.3	6.6±0.4	5.8±0.2*	5.1±0.2*
%FS, %	14±1	22±1*	21±1*	26±1*†‡
AWT diastole, mm	1.0±0.2	1.3±0.3*	1.3±0.3*	1.4±0.4*
PWT diastole, mm	1.5±0.5	2.2±0.4	2.1±0.4	2.2±0.4

Values are means ± SE. LVDd, LV diastolic dimension; LVDs, LV systolic dimension; %FS, LV fractional shortening; AWT, anterior wall thickness; PWT, posterior wall thickness. *$P < 0.05$ vs. control; †$P < 0.05$ vs. MNC; ‡$P < 0.05$ vs. AM.

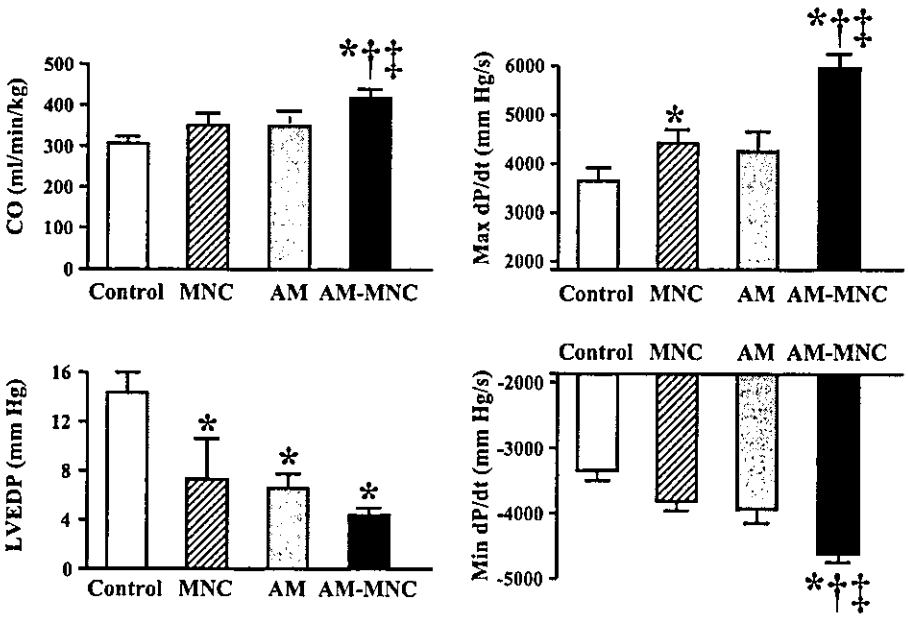

Fig. 2. Effects of AM infusion and MNC transplantation on hemodynamic parameters. CO, cardiac output; LVEDP, LV end-diastolic pressure; Max dP/d*t*, LV maximum change in pressure over time; Min dP/d*t*, LV minimum dP/d*t*. Values are means ± SE. *$P < 0.05$ vs. control; †$P < 0.05$ vs. MNC; ‡$P < 0.05$ vs. AM.

were hardly detected in the AM-MNC group. Semiquantitative analysis demonstrated that the number of TUNEL-positive MNC was significantly lower in the AM-MNC group than in the MNC group. Similarly, the number of caspase-3-positive MNC was significantly lower in the AM-MNC group than in the MNC group. These results suggest that infusion of AM inhibits apoptosis of transplanted MNC.

In vitro, serum starvation induced MNC apoptosis. When incubated in the presence of AM (1×10^{-7} M), the percentage of TUNEL-positive cells decreased significantly (19 ± 1 to 9 ± 1%, $P < 0.05$). However, pretreatment with wortmannin, a PI3-kinase inhibitor, diminished the antiapoptotic effect of AM (17 ± 1%).

Differentiation of MNC into endothelial lineage. Four weeks after transplantation, fluorescence-labeled transplanted cells

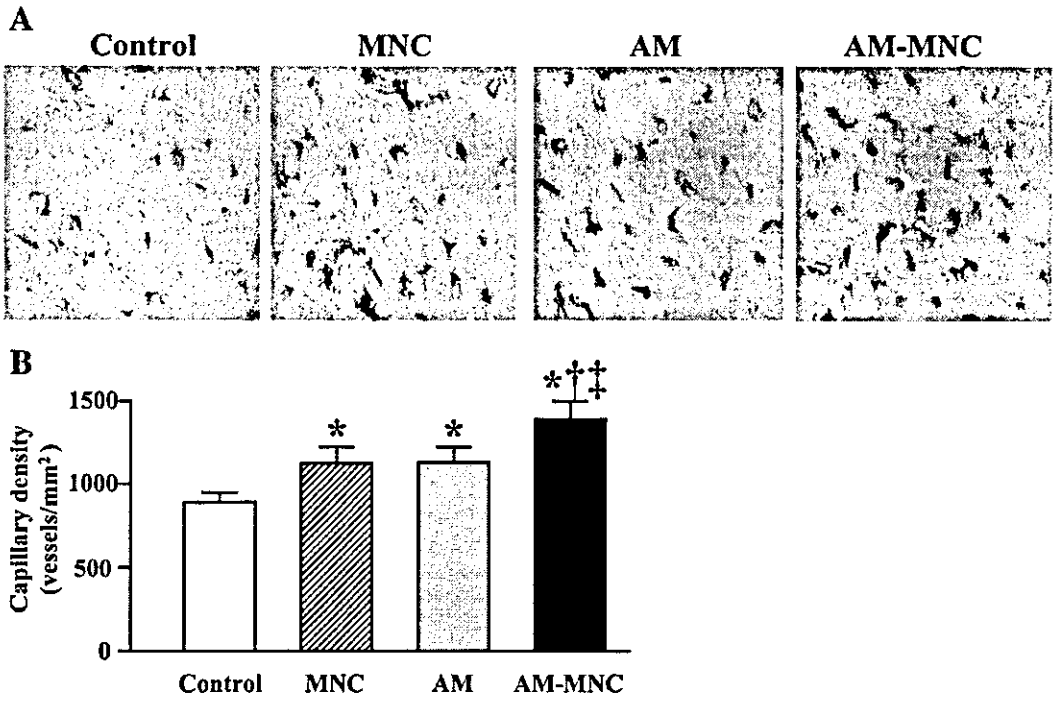

Fig. 3. *A*: representative examples of alkaline phosphatase staining in peri-infarct area. A combination of AM infusion and MNC transplantation markedly induced myocardial neovascularization. Magnification, ×200. *B*: quantitative analysis of capillary density in peri-infarct area. Capillary density in the AM-MNC group was significantly higher than that in the MNC and AM groups. Values are means ± SE. *$P < 0.05$ vs. control; †$P < 0.05$ vs. MNC; ‡$P < 0.05$ vs. AM.

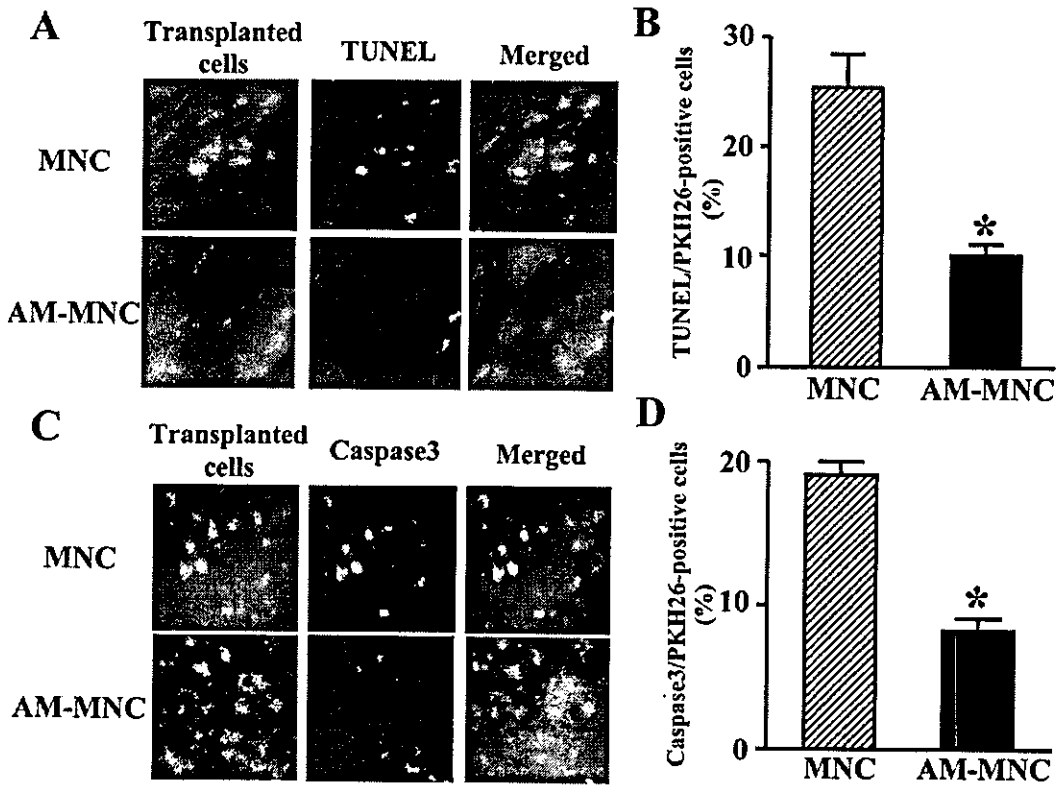

Fig. 4. Detection of transplanted cell apoptosis. *A*: representative photographs of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Red fluorescence (PKH26) marks transplanted MNC; green fluorescence indicates TUNEL-positive cells. TUNEL-positive cells were frequently observed in the MNC group, whereas they were hardly detected in the AM-MNC group. Magnification, ×400. *B*: semiquantitative analysis of TUNEL-positive cells in the PKH26-positive (transplanted) cells. *C*: representative photographs of caspase-3 staining. Red fluorescence (PKH26) marks transplanted MNC; green fluorescence indicates caspase-3-positive cells. *D*: semiquantitative analysis of caspase-3-positive cells in the PKH26-positive cells. Values are means ± SE. $*P < 0.05$ vs. control.

were more frequently observed in the AM-MNC group than in the MNC group (6.4 ± 0.4 to 3.1 ± 0.2%, $P < 0.05$). Moreover, some of the transplanted cells were positive for UEA-1 lectin in the AM-MNC group (Fig. 5*A*), suggesting differentiation of MNC into vascular endothelial cells. Semiquantitative analysis demonstrated that the number of DAPI/PKH26 double-positive cells (viable transplanted cells) was significantly higher in the AM-MNC group than in the MNC group (Fig. 5*B*). Moreover, the ratio of lectin-positive cells to DAPI/PKH26 double-positive cells was significantly higher in the AM-MNC group than in the MNC group. The ratio of DAPI/PKH26 double-positive cells to lectin-positive cells was small, but significantly higher in the AM-MNC group than in the MNC group (23.9 ± 0.9 to 17.2 ± 0.6%, $P < 0.01$). Transplanted MNC were negative for troponin T or α-smooth muscle actin-positive cells. Some of the transplanted MNC were positive for ED1, a marker of macrophage (data not shown).

DISCUSSION

In the present study, we demonstrated that *1*) infusion of AM enhanced the angiogenic potency of MNC in a rat model of acute myocardial infarction, resulting in decreased infarct size and improved cardiac function. We also demonstrated that *2*) AM induced angiogenesis and inhibited apoptosis of the transplanted MNC. Thus a combination of AM and MNC may have beneficial effects in rats with myocardial infarction, partly through the angiogenic potency of AM itself and through its antiapoptotic effect on MNC.

Bone marrow-derived MNC include a variety of stem and progenitor cells (1, 15, 19), some of which can differentiate into endothelial cells and secrete numerous cytokines and chemokines (6, 9, 10). Earlier studies (6, 9, 10, 23, 25) have shown that autologous bone marrow transplantation induces angiogenesis and improves LV function in animals and humans. However, some patients are refractory to this cell therapy. Thus an approach to augment the angiogenic potency of MNC transplantation is required.

The present study showed that MNC transplantation or AM infusion alone reduced infarct size. A combination of AM infusion and MNC transplantation resulted in further decreases in infarct size and LV chamber size. MNC transplantation or AM administration modestly improved LV function. On the other hand, a combination of MNC and AM significantly improved cardiac performance compared with MNC or AM alone, as indicated by increases in cardiac output, fractional shortening, and LV maximum dP/d*t*. Earlier studies (6, 9, 10) have reported that MNC transplantation induces therapeutic angiogenesis and preserves LV function through inhibition of cardiomyocyte apoptosis in animal models of myocardial infarction. We have shown that AM infusion during the acute phase of ischemia-reperfusion inhibits apoptosis of cardiomyocytes and produces hemodynamic improvement in an animal

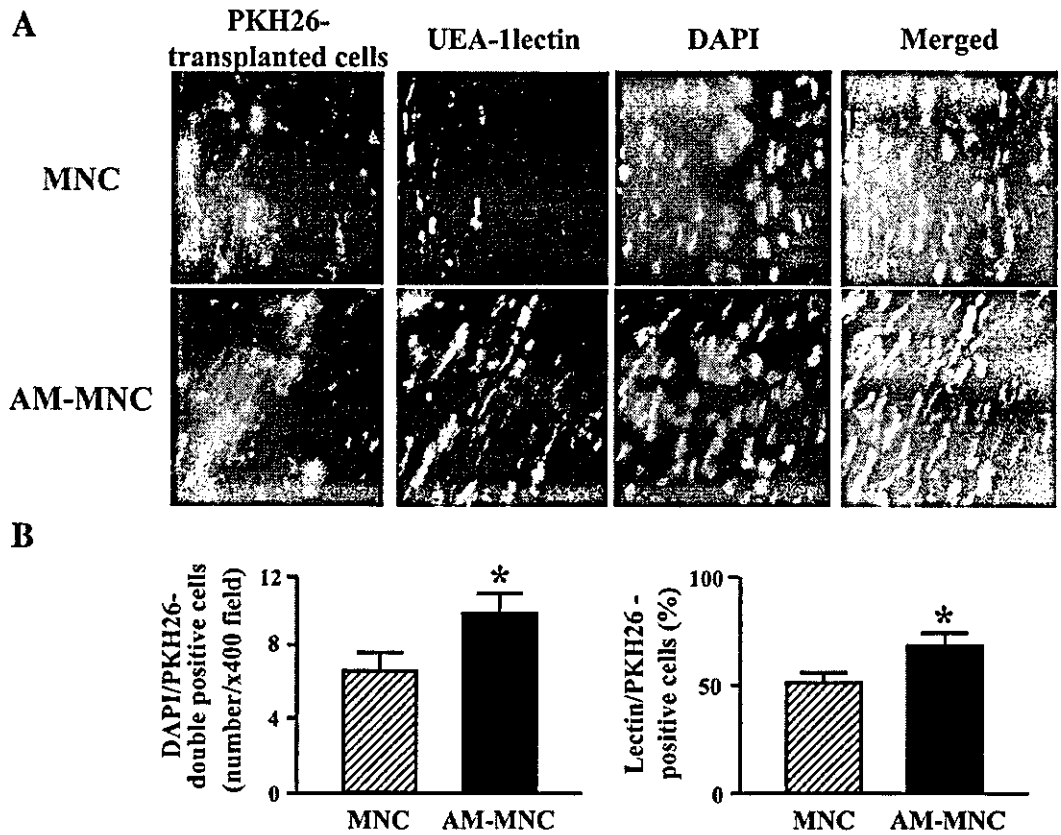

Fig. 5. *A*: representative examples of MNC differentiation into endothelial lineage. Red fluorescence (PKH26) marks transplanted cells; green fluorescence indicates ulex europaeus (UEA)-1 lectin, a marker for vascular endothelial cells. Most of the transplanted cells differentiated into endothelial cells in the AM-MNC group. Magnification, ×400. *B*: quantitative analysis of living transplanted cells and endothelial differentiation. The number of living cells after transplantation was significantly higher in the AM-MNC group than in the MNC group. The ratio of lectin-positive cells to living transplanted cells was significantly higher in the AM-MNC group than in the MNC group. Values are means ± SE. $*P < 0.05$ vs. control. DAPI, 4′,6′-diamidino-2-phenylindole.

study (18). These findings suggest that the reduction of infarct size induced by this combination therapy may be attributable to additive cardioprotective effects of MNC and AM.

The present study showed that AM infusion significantly increased capillary density in ischemic myocardium. Furthermore, AM infusion plus MNC transplantation demonstrated a further increase in capillary density compared with AM or MNC alone. Contribution of transplanted MNC to neovascularization (the ratio of DAPI/PKH26 double-positive cells to lectin-positive cells) was significantly greater in the AM-MNC group than in the MNC group. A recent study (14) has reported that AM promotes proliferation and migration of human umbilical vein endothelial cells and enhances angiogenesis in a murine gel plug assay through the PI3-kinase/Akt pathway. We have also shown that intramuscular administration of AM DNA induces therapeutic angiogenesis in a rabbit model of chronic hindlimb ischemia via activation of Akt (24). These findings suggest that the beneficial effects of combination therapy using AM and MNC may be attributable, in part, to the angiogenic properties of AM itself. Thus it is possible that AM infusion and MNC transplantation induce additive effects on myocardial damage after myocardial infarction. However, it still remains unknown whether AM infusion plus MNC transplantation induces synergetic effects.

An earlier study has demonstrated that ischemia and mechanical stress induce apoptosis of transplanted cells in the early stage after MNC transplantation (9). These results raise the possibility that the angiogenic potency of MNC transplantation is attenuated by MNC apoptosis. Kim et al. (7) have demonstrated that AM inhibits apoptosis of endothelial cells through the PI3-kinase/Akt pathway in vitro. Activation of the PI3-kinase/Akt pathway has been shown to inhibit apoptosis of endothelial progenitor cells and enhance neovascularization (11). In the present study, AM infusion significantly inhibited MNC apoptosis in ischemic tissue. In vitro, we showed that the antiapoptotic effect of AM on MNC was mediated by activation of the PI3-kinase/Akt pathway. Thus AM may enhance the therapeutic potency of MNC transplantation through a direct action of AM on MNC survival. Moreover, immunohistological examination demonstrated that infusion of AM increased the number of lectin-positive (endothelial) cells in transplanted MNC. These findings raise the possibility that AM may enhance differentiation of MNC into the endothelial lineage. Thus AM may directly act on transplanted MNC, which may result in synergetic effects on the ischemic myocardium.

This study includes some study limitations. Although the labeling efficacy of PKH26 has been shown to persist for >8 wk without cell toxicity (3, 4), the used vital marker PKH26