Dose-Dependent Contribution of CD34-Positive Cell Transplantation to Concurrent Vasculogenesis and Cardiomyogenesis for Functional Regenerative Recovery After Myocardial Infarction

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Background—Multilineage developmental capacity of the $CD34^+$ cells, especially into cardiomyocytes and smooth muscle cells (SMCs), is still controversial. In the present study we performed a series of experiments to prove our hypothesis that vasculogenesis and cardiomyogenesis after myocardial infarction (MI) may be dose-dependently enhanced after $CD34^+$ cell transplantation.

Methods and Results—Peripheral blood $CD34^+$ cells were isolated from total mononuclear cells of patients with limb ischemia by apheresis after 5-day administration of granulocyte colony-stimulating factor. PBS and 1×10^3 (low), 1×10^5 (mid), or 5×10^5 (high) $CD34^+$ cells were intramyocardially transplanted after ligation of the left anterior descending coronary artery of nude rats. Functional assessments with the use of echocardiography and a microtip conductance catheter at day 28 revealed dose-dependent preservation of left ventricular function by $CD34^+$ cell transplantation. Necropsy examination disclosed dose-dependent augmentation of capillary density and dose-dependent inhibition of left ventricular fibrosis. Immunohistochemistry for human-specific brain natriuretic peptide demonstrated that human cardiomyocytes were dose-dependently observed in ischemic myocardium at day 28 (high, 2480±149; mid, 1860±141; low, 423±9; PBS, 0±0/mm^2; $P<0.05$ for high versus mid and mid versus low). Immunostaining for smooth muscle actin and human leukocyte antigen or *Ulex europaeus* lectin type I also revealed dose-dependent vasculogenesis by endothelial cell and SMC development after $CD34^+$ cell transplantation. Reverse transcriptase–polymerase chain reaction indicated that human-specific gene expression of cardiomyocyte (brain natriuretic peptide, cardiac troponin-I, myosin heavy chain, and Nkx 2.5), SMC (smooth muscle actin and sm22α), and endothelial cell (CD31 and KDR) markers were dose-dependently augmented in MI tissue.

Conclusions—Human $CD34^+$ cell transplantation may have significant and dose-dependent potential for vasculogenesis and cardiomyogenesis with functional recovery from MI. (***Circulation.*** **2006;113:1311-1325.**)

Key Words: angiogenesis ■ cell therapy ■ myocardial infarction ■ transplantation ■ vasculogenesis

The various cell types that make up the blood are of mesodermal origin and emanate from a common pool of hematopoietic stem cells (HSCs). During embryogenesis, hematopoietic and endothelial lineage cells[1,2] are derived from common progenitor cells (hemangioblasts).[3–6] In adults, $CD34^+$ cells had been considered to be HSCs and clinically applied to the field of hematology for HSC transplantation.[7,8] Recently, human peripheral blood $CD34^+$ cells were reported to be an endothelial progenitor cell (EPC)–enriched population as well as a HSC fraction.[9] Thereafter, therapeutic application of $CD34^+$ cells for vascular regeneration has been performed in many preclinical studies. In the case of immunodeficient rats with acute myocardial infarction (MI), transplanted human $CD34^+$ cells or ex vivo expanded EPCs incorporated into the site of myocardial neovascularization, differentiated into mature endothelial cells (ECs) (vasculo-

Editorial p 1275

Received February 8, 2005; revision received August 2, 2005; accepted September 13, 2005.

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The online-only Data Supplement can be found at http://circ.ahajournals.org/cgi/content/full/113/10/1311/DC1.

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Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/CIRCULATIONAHA.105.541268**

genesis), augmented capillary density, inhibited myocardial fibrosis and apoptosis, and preserved left ventricular (LV) function.[10-12] In our institution, a phase I/II clinical trial of CD34$^+$ cell transplantation has already been started in patients with chronic critical limb ischemia.

Recently, Orlic et al[13] and Jackson et al[14] reported translineage differentiation of adult mouse HSCs into cardiomyocytes when introduced into heart by intramyocardial injection or via the circulation. This is followed by demonstrating that mouse c-Kit–positive bone marrow cells differentiate into myocardium and blood vessels in vivo.[15] Further reports[16,17] presented development of human peripheral blood CD34$^+$ cells into cardiomyocytes, smooth muscle cells (SMCs), and ECs in a mouse model of acute MI. On the other hand, other investigators indicated that transplanted HSCs were unable to transdifferentiate into cardiomyocytes after MI.[18,19] Given these extensive and controversial investigations into the potency of so-called stem/progenitor cells derived from peripheral blood and bone marrow in experimental models, we conducted this study to investigate not only vasculogenesis but also cardiomyogenesis derived from human CD34$^+$ cells, the most practical human cells for clinical medicine, in a dose-dependent manner.

The present results of human CD34-positive cell transplantation into an immunodeficient rat MI model demonstrated that the collaborative multilineage differentiation potential of CD34$^+$ cells into not only ECs but cardiomyocytes and SMCs was enhanced by cell dose escalation and was conducive to heart regeneration in terms of functional and histological recovery through vasculogenesis and cardiomyogenesis.

Methods

Isolation of CD34$^+$ Cells From Patients With Critical Limb Ischemia

Peripheral blood total mononuclear cells were obtained from 2 male patients aged 21 and 40 years with Buerger disease by apheresis after 5-day subcutaneous administration of granulocyte colony-stimulating factor (G-CSF) (10 μg/kg per day). Bone marrow–derived CD34$^+$ cells were isolated from total mononuclear cells by the magnetic cell sorting system CliniMACS (Miltenyi Biotec Inc, Auburn, Calif).[20] These patients received intramuscular transplantation of 10^5 CD34$^+$ cells/kg according to the protocol of a phase I/II dose-escalation clinical trial. The CD34$^+$ cell fraction had a purity of >99%, as determined by fluorescence-activated cell sorting (FACS) analysis with the use of a CD34-specific monoclonal antibody (Becton Dickinson, San Jose, Calif) (Figure 1a and 1b). Remaining CD34$^+$ cells were used for the following experiments. Informed consent with regard to the cell therapy and experimental use of the remaining cells was obtained from the patients before the case registration. The clinical study protocol was approved by the institutional ethics committees of Kobe Institute of Biomedical Research and Innovation and Kobe City General Hospital.

Animals

Female athymic nude rats (F344/N Jcl rnu/rnu, CIEA Japan, Inc, Tokyo, Japan) aged 7 to 8 weeks and weighing 130 to 145 g were used in this study. The institutional animal care and use committees of RIKEN Center for Developmental Biology approved all animal procedures, including human cell transplantation.

Induction of MI and Cell Transplantation

Rats were anesthetized with ketamine and xylazine (60 and 10 mg/kg IP, respectively). MI was induced by ligating the left anterior descending coronary artery as described previously.[12] In brief, after the fourth to fifth intercostal space was opened, the heart was exteriorized, and the pericardium was incised. Thereafter, the heart was held with forceps, and MI was induced by ligating the left anterior descending coronary artery near its origin with a 6-0 Proline suture. Twenty minutes after MI, rats received intramyocardial transplantation of 1×10^5 (low group), 1×10^6 (mid group), or 5×10^6 (high group) CD34$^+$ cells resuspended with 120 μL of PBS or the same volume of PBS without cells (n=12 in each group when the first patient's cells were used; n=4 in each group for the second patient's cells). After the injection was completed, the thorax was closed.

Flow Cytometry Studies and Monoclonal Antibodies

Regular flow cytometric profiles were analyzed with a FACSCalibur analyzer and CELLQuest software (Becton Dickinson Immunocytometry Systems, Mountain View, Calif). The instrument was aligned and calibrated daily with the use of a 4-color mixture of CaliBRITE beads (BD Biosciences, San Jose, Calif) with FACSComp software (BD Biosciences). Dead cells were excluded from the plots on the basis of propidium iodide (PI) staining (Sigma Co, St Louis, Mo). CD34$^+$ cells were washed twice with Hanks' balanced salt solution (HBSS) containing 3.0% heat-activated fetal calf serum (FCS), incubated with 10 μL of FcR blocking regent to increase the specificity of monoclonal antibodies (Milteyi Biotic) for 20 minutes at 4°C, and incubated with the monoclonal antibodies for 30 minutes at 4°C. The stained cells were washed 3 times with PBS containing 3.0% FCS, resuspended in 0.5 mL of HBSS/3% FCS/PI, and analyzed by FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The following monoclonal antibodies were used to characterize the CD34$^+$ cell population: CD34-FITC (clone My10, BD), CD34-PE (clone 581, Pharmingen, San Diego, Calif), CD45-FITC (clone HI30, Pharmingen), CD31-FITC (clone WM59, Pharmingen), KDR (Sigma), AC133-APC (clone AC133, Pharmingen), VE-cadherin-APC (HyCult Biotechnology, Uden, The Netherlands), IgG1-FITC isotype controls (Pharmingen), IgG1-PE isotype controls (Pharmingen), IgG1-APC isotype controls (Pharmingen), IgG2a-APC isotype controls (Pharmingen), and PI (Sigma Chemical Co, St Louis, Mo).

Physiological Assessment of LV Function Using Echocardiography and Microtip Conductance Catheter

Transthoracic echocardiography (SONOS 5500, Philips Medical Systems) was performed to evaluate LV function immediately before and 5 and 28 days after MI. Under general anesthesia with ketamine and xylazine, LV end-diastolic and end-systolic dimensions (LVEDD and LVESD, respectively) and fractional shortening (FS) were measured at the midpapillary muscle level. Regional wall motion score (RWMS) was evaluated per published criteria.[21] All procedures and analyses were performed by an experienced researcher who was blinded to treatment.

Immediately after the final echocardiography on day 28, the rats underwent cardiac catheterization for more invasive and precise assessment of global LV function.[22] A 2.0F micromanometer-tipped conductance catheter (SPR 838, Millar Instruments Inc, Houston, Tex) was inserted via the right carotid artery into the LV cavity. LV pressure and its derivative (LV dP/dt) were continuously monitored with a multiple recording system (Pressure-Volume Conductance System ARIA and Pressure-Volume Analysis Using P-V Analysis Software [Millar Instruments Inc, Houston, Tex] and Power Laboratory DAQ System [ADInstrument, Australia]).

Heart rate, LV end-diastolic pressure (LVEDP), LV ejection fraction (EF), and the maximum and minimum LV dP/dt (+dP/dt and −dP/dt, respectively) were recorded continuously for 20 minutes. All data were acquired under stable hemodynamic conditions. All procedures and analyses were performed by an experienced researcher who was blinded to treatment.

Tissue Harvesting

All rats were killed 28 days after transplantation with an overdose of ketamine and xylazine. At necropsy, hearts were sliced in a broadleaf

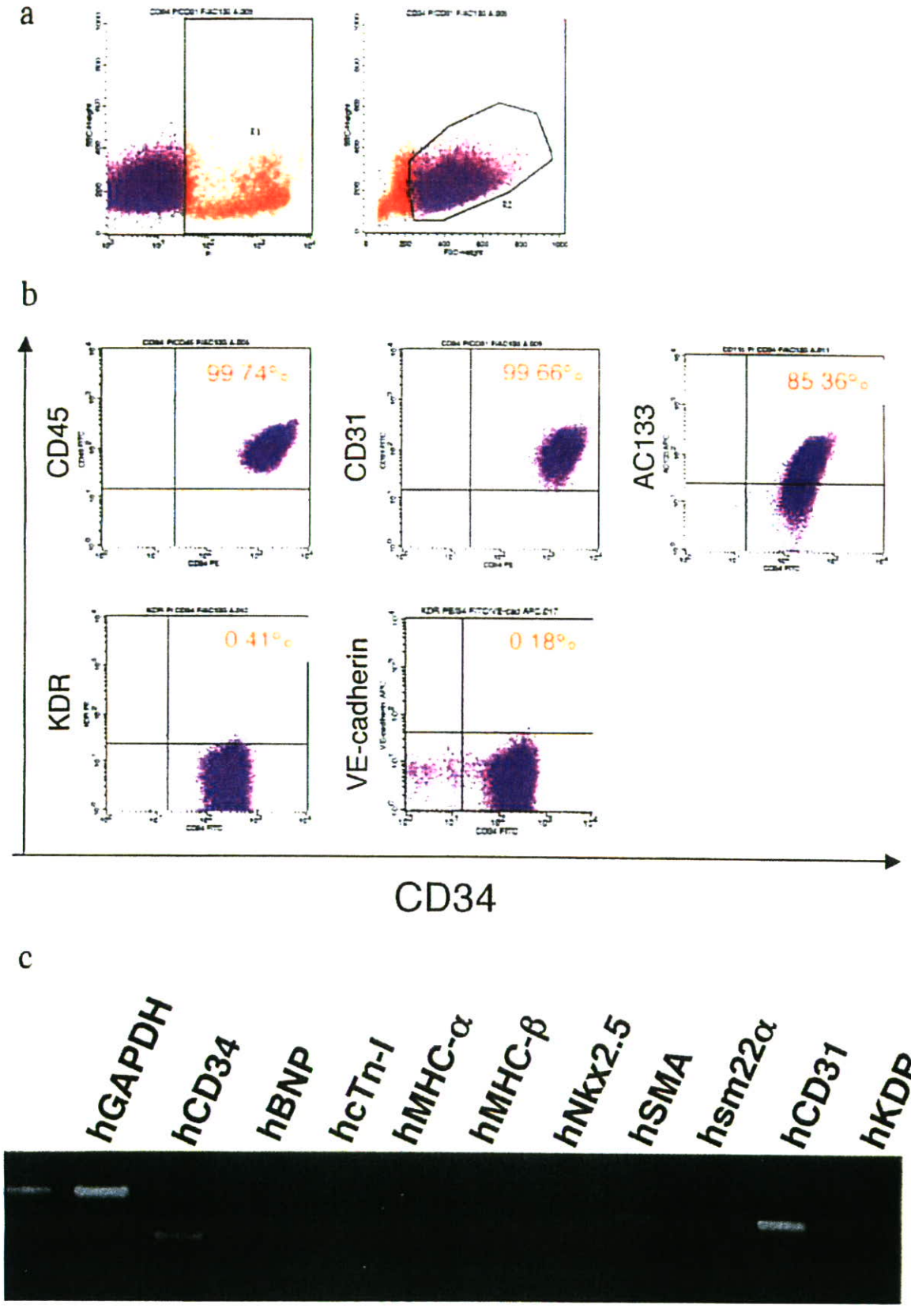

Figure 1. Representative FACS profile and RT-PCR of CD34+ cells isolated from a critically ischemic patient by magnetic cell sorting system. a, Dead cells were excluded by PI staining, and then mononuclear cell (MNC) population within live cells was assessed. b, The cells were stained with CD45, CD31, AC133, KDR, VE-cadherin, and CD34. Numbers are percentage of double-positive cells in each staining. c, RT-PCR analysis for human-specific genes of cardiomyocyte, SMC, and EC lineages in freshly isolated CD34+ cells.

fashion into 4 transverse sections from apex to base, embedded in OCT compound, snap-frozen in liquid nitrogen, and stored at −80°C for Masson trichrome staining, immunohistochemistry, and fluorescence in situ hybridization (FISH). Rat hearts in OCT blocks were sectioned, and 5-μm serial sections were collected on slides followed by fixation with 4.0% paraformaldehyde at 4°C for 5 minutes and stained immediately. Total RNA was isolated by selective dissection of peri-infarct area in LV myocardium for reverse transcriptase–polymerase chain reaction (RT-PCR).

Morphometric Evaluation of Capillary Density and Infarct Size

Histochemical staining with isolectin B4 (Vector Laboratories, Burlingame, Calif) was performed, and capillary density was morphometrically evaluated by histological examination of 5 randomly selected fields of tissue sections recovered from segments of LV myocardium subserved by the occluded left anterior descending coronary artery. Capillaries were recognized as tubular structures positive for isolectin B4. To elucidate the severity of myocardial fibrosis, Masson trichrome staining was performed on frozen sections from each tissue block, and the stained sections were used to measure the average ratio of fibrosis area to entire LV area (percent fibrosis area). All morphometric studies were performed by 2 examiners who were blinded to treatment.

Immunofluorescence Staining

To detect transplanted human cells in rat ischemic myocardium, immunohistochemistry was performed with following human-specific antibodies: human leukocyte antigen (HLA)–ABC (BD Pharmingen) and human nuclei antibody (HNA) (Chemicon International, Inc, Temecula, Calif) to detect various kinds of human cells, human-specific brain natriuretic peptide (hBNP),[23] which was kindly given by Dr Hiroshi Itoh of Kyoto University Kyoto, Japan, to detect human cardiomyocytes, and human-specific *Ulex europaeus* lectin type 1 (UEA-1) (Vector Laboratories, Inc)[24] for human ECs. Staining specificity of hBNP, HLA-ABC, HNA, and UEA-1 against human cells and lack of cross-reactivity to rat cells were confirmed by histochemical staining with the use of human and rat heart samples (data not shown). Double immunohistochemistry with HNA and cardiac troponin I (cTn-I) (Chemicon International, Inc) was performed to detect double-positive cells as human cardiomyocytes in rat myocardium. Double immunohistochemistry with HLA-ABC and smooth muscle actin (SMA) was performed to detect double-positive cells as human SMCs in rat myocardium. Similarly, double immunohistochemistry with hUEA-1 and SMA and for von Willebrand factor (vWF) (Chemicon International, Inc) and HNA was performed to detect UEA-1–positive cells and double-positive cells of vWF and HNA as human ECs in ischemic myocardium. The secondary antibodies for each immunostaining are as follows: FITC goat anti-mouse IgG (The Jackson Laboratory, Bar Harbor, Me) for hBNP staining, Alexa Flour 594–conjugated goat anti-mouse IgG_1 (Molecular Probes) for HLA-ABC staining, Alexa Flour 488–conjugated goat anti-mouse IgG_{2a} (Molecular Probes, Carlsbad, Calif) for cTn-I staining, Alexa Flour 488–conjugated goat anti-mouse IgG_{2a} (Molecular Probes) for SMA, Cy3-conjugated streptavidin (Jackson ImmunoResearch, West Grove, Pa) for hUEA-1, Alexa Flour 488– and 594–conjugated goat anti-mouse IgG1 for HNA, and Alexa Flour 594–conjugated goat anti-rabbit IgG for vWF. DAPI solution was applied for 5 minutes for nuclear staining. Number of human cardiomyocytes, total (both human and rat) cardiomyocytes in ischemic myocardium detected as hBNP-positive cells, number of human SMCs as double-positive cells for HLA and SMA, and number of human ECs as hUEA-1–positive cells 28 days after MI were morphometrically quantified with the use of 5 randomly selected fields (from peri-infarction area to fibrosis area) of tissue sections.

Fluorescence In Situ Hybridization

To identify whether cardiac repair occurred through cell fusion in rat ischemic myocardium, FISH was performed with human Y chromosome painting probe and rat genome probe (nick translation methods) in MI tissue. Tissue sections were fixed immediately with 4.0% paraformaldehyde at 4°C for 20 minutes and predenatured, dehydrated, and denatured according to the manufacturer's protocol. Sections were hybridized with a Cy-3–conjugated DNA probe for human Y chromosomes and a biotin-conjugated probe for rat genome overnight at 37°C. After posthybridization wash, TexRed-conjugated streptavidin was applied, and slides were counterstained with DAPI and examined.

RT-PCR Analysis of $CD34^+$ Cells and Ischemic Heart Tissue

Total RNA was obtained from freshly isolated peripheral blood $CD34^+$ cells of a patient and tissues of rat ischemic LV at day 28 with the use of TRIzol (Life Technologies, St Paul, Minn) according to the manufacturer's instructions. The first-strand cDNA was synthesized with the use of the RNA LA PCR Kit version 1.1 (Takara, Otsu, Japan), amplified by *Taq* DNA polymerase (Advantage-GC cDNA PCR Kit, Clontech and AmpliTaq Gold DNA polymerase, Applied Biosystems). PCR was performed with a PCR thermocycler (MJ Research PTC-225). The human GAPDH (hGAPDH), total (human and rat) GAPDH, human CD34 (hCD34), hBNP, human cardiac troponin-I (hcTn-I), human myosin heavy chain-β (hMHC-β), human KDR (hKDR), and human Nkx 2.5 (hNkx 2.5) were amplified by *Taq* DNA polymerase (AmpliTaq Gold DNA polymerase, Applied Biosystems) under the following conditions: 35 cycles of 30 seconds of initial denaturation at 94°C, annealing at 56°C for 1 minute, and 30 seconds of extension at 72°C according to the manufacturer's instructions. Human myosin heavy chain-α (hMHC-α), human SMA (hSMA), human sm22α (hsm22α), and human CD31 (hCD31) were amplified by *Taq* DNA polymerase (Advantage-GC cDNA PCR Kit, Clontech, Mountain View, Calif) under the following conditions: 37 cycles of 30 seconds of initial denaturation at 94°C, annealing at 68°C for 3 minutes, and 7 minutes of elongation at 64°C according to the manufacturer's instructions. Subsequently, PCR products were visualized in 1.5% ethidium bromide–stained agarose gels. Human heart RNA distributed from Clontech (premium RNA) was used as positive control. To quantify human-specific cardiomyogenic and vasculogenic gene expression in rat ischemic myocardium after human $CD34^+$ cell transplantation, we measured the band intensity of the RT-PCR image. Each gel was photographed onto positive/negative Polaroid film under ultraviolet illumination. The negative images were then captured with the use of an image scanner. After the images were recorded in a computer, the band intensities were processed with the NIH Image program (version 1.62) as described previously.[25] These band intensities were used to calculate the ratio of human-specific marker (hGAPDH, hBNP, hcTn-I, hMHC-α, hMHC-β, hNkx 2.5, hSMA, hsm22α, hKDR, and hCD31) expression to total GAPDH expression.

Primers

To avoid interspecies cross-reactivity of the primer pairs between human and rat genes, we designed the following human-specific primers using Oligo software (Takara). All primer pairs did not show cross-reactivity to rat genes (data not shown). Primer pairs were as follows: hBNP primer sequence (146 bp): sense GCA AAA TGG TCC TCT ACA CC; antisense CAG GAC TTC CTC TTA ATG CC; hcTn-I primer sequence (218 bp): sense AAT TGC AGC TGA AGA CTC TG; antisense GAC TTT TGC CTC TAT GTC GT; hMHC-β primer sequence (214 bp): sense GCT AAA GGT CAA GGC CTA CA; antisense GCA GAT CAA GAT CTG GCA AA; hNkx 2.5 primer sequence (205 bp): sense GAG AGT TTG TGG CGG CGA TT; antisense CGA CGG CGA GAT AGC AAA GG; hMHC-α primer sequence (413 bp): sense GTC ATT GCT GAA ACC GAG AAT G; antisense GCA AAG TAC TGG ATG ACA CGC T; hSMA primer sequence (485 bp): sense TCT GGA CGC ACA ACT GGC ATC GT; antisense TAC ATA GTG GTG CCC CCT GAT AG; hsm22-α primer sequence (468 bp): sense CGG CTG GTG GAG TGG ATC ATA; antisense CCC TCT GTT GCT GCC CAT CTG A; hCD31 primer sequence (469 bp): sense AGG TCA AGC AGC ATC GTG GTC AAC AT; antisense TTG TCT TTG AAT ACC GCA G; hCD34 primer sequence (380 bp): sense AAT GAG GCC ACA ACA

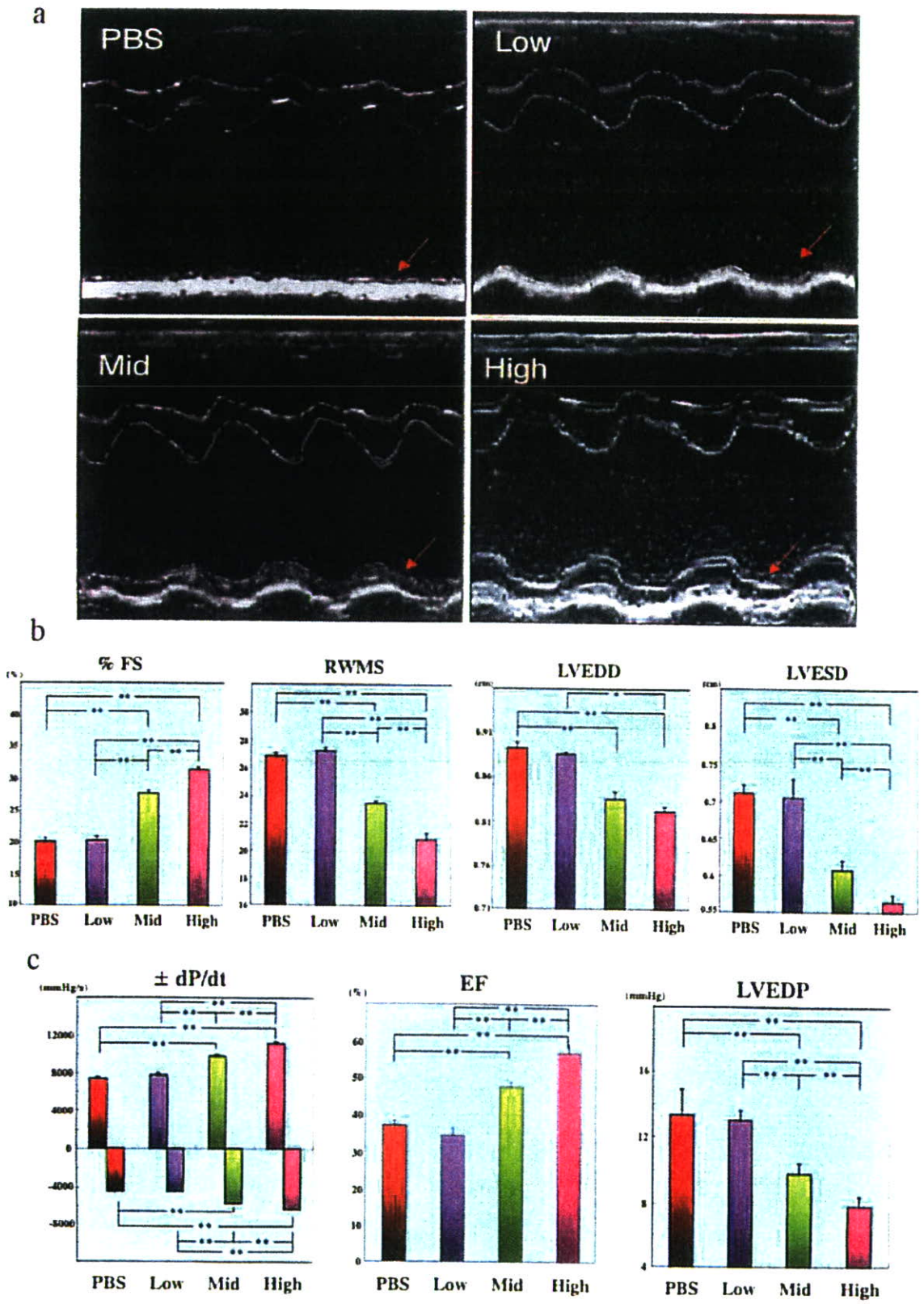

Figure 2. Functional evaluation of treated heart by echocardiography. a, Representative recording of M-mode echocardiography 28 days after cell or PBS administration. Lateral wall motion was dose-dependently preserved (arrow: endocardium in lateral wall). b, Echocardiographic parameters 28 days after cell transplantation. Global and regional LV function was dose-dependently preserved after CD34+ cell transplantation. *$P<0.05$, **$P<0.01$ (n=12 in all groups). c, Invasive hemodynamic parameters after CD34+ cell or PBS administration at day 28. The functional parameters were dose-dependently preserved after CD34+ cell transplantation. +dP/dt and −dP/dt indicate maximum and minimum derivative of LV pressure (positive values indicate +dP/dt, and negative values show −dP/dt). *$P<0.05$, **$P<0.01$ (n=12 in each group).

AAC ATC ACA; antisense CTG TCC TTC TTA AAC TCC GCA CAG C; hGAPDH primer sequence (596 bp): sense CTG ATG CCC CCA TGT TCG TC; antisense CAC CCT GTT GCT GTA GCC AAA TTC G; total GAPDH primer sequence (320 bp): sense GTG CCA GCC TCA TGT TCG TC; antisense CGC CAG TGT ACT CCA CGA CAT TTC G; hKDR primer sequence (468 bp): sense CAA ATG TGA AGC GGT CAA CAA AGT T; antisense ATG CTT TCC CCA ATA CTT GTC GTC T.

Statistical Analysis

The results were statistically analyzed with the use of a software package (Statview 5.0, Abacus Concepts Inc, Berkeley, Calif). All values were expressed as mean±SE. Paired *t* tests were performed for comparison of data before and after treatment. The comparisons among 4 groups were made with 1-way ANOVAs. Post hoc analysis was performed by Fisher protected least significant difference test. Differences of $P<0.05$ were considered statistically significant.

Results

Characterization of Freshly Isolated Peripheral Blood CD34$^+$ Cells

The CD34$^+$ cell fraction had a purity of >99%, as determined by FACS analysis with the use of anti-CD34, anti-CD45, anti-CD31, anti-AC133, anti-KDR, and anti-VE-cadherin monoclonal antibodies (Figure 1a and 1b). The RT-PCR analysis revealed the human-specific gene expression of CD34 and SMA but not of cardiomyocyte markers (hBNP, hcTn-I, hMHC-α, hMHC-β, and hNkx 2.5), hKDR, and hsm22α (Figure 1c).

Transplanted CD34$^+$ Cells Dose-Dependently Preserve LV Function After MI

There were no significant differences in preoperative echocardiographic parameters, LVEDD, LVESD, FS, and RWMS among high, mid, low, and PBS groups. Echocardiography on day 5 revealed that the functional parameters were also similar in all groups (data not shown). Echocardiography performed 4 weeks after cell transplantation demonstrated that LVEDD was significantly smaller in the high group than in the low and PBS groups ($P<0.05$ versus low and $P<0.01$ versus PBS). LVEDD was also significantly smaller in the mid group than in the PBS group ($P<0.01$). However, LVEDD was similar in the high and mid groups and in the low and PBS groups (Figure 2a and 2b). LVESD 4 weeks after MI was significantly smaller in the high group than in the mid group and in the mid group than in the low group (high group, 0.564±0.01; mid group, 0.607±0.013; low group, 0.705±0.025; PBS group, 0.711±0.011 cm; $P<0.01$ for high versus mid and mid versus low) (Figure 2b). FS was significantly greater in the high group than in the mid group and in the mid group than in the low group (high group, 31.4±0.43%; mid group, 27.7±0.45%; low group, 20.2±0.58%; PBS group, 20.0±0.54%; $P<0.01$ for high versus mid and mid versus low) (Figure 2a and 2b). RWMS was significantly better preserved in the high group than in the mid group and in the mid group than in the low group (high group, 20.8±0.46; mid group, 23.4±0.15; low group, 27.2±0.2; PBS group, 26.8±0.24; $P<0.01$ for high versus mid and mid versus low). LVEDD, LVESD, FS, and RWMS 4 weeks after transplantation in the low group were not significantly different from those in the PBS group (Figure 2a and 2b).

Invasive Hemodynamic Parameters After CD34$^+$ Cell or PBS Administration at Day 28

Group	n	Heart Rate, bpm	Systolic Left Ventricular Pressure, mm Hg	Diastolic Left Ventricular Pressure, mm Hg
PBS	12	296±1.9	110±1.9	5.5±1.4
Low	12	302±2.0	112±3.0	3.5±0.5
Mid	12	300±2.4	117±1.8	3.9±0.5
High	12	300±2.1	120±3.1	3.3±0.4

Invasive hemodynamic study performed 4 weeks after transplantation revealed that heart rate and diastolic blood pressure were similar in each group. Systolic blood pressure was significantly greater in the high and mid groups than in the PBS group ($P<0.05$) (Table). The +dP/dt, absolute value of −dP/dt, and EF were significantly greater in the high group than in the mid group and in the mid group than in the low group (+dP/dt: high group, 11 131±106; mid group, 9772±111; low group, 7734±160; PBS group, 7322±233 mm Hg/s; $P<0.01$ for high versus mid and mid versus low) (Figure 2c) (−dP/dt: high group, −6403±209; mid group, −5753±170; low group, −4413±230; PBS group, −4415±212 mm Hg/s; $P<0.01$ for high versus mid and mid versus low) (Figure 2c) (EF: high group, 56.8±2.3%; mid group, 47.5±1.4%; low group, 34.2±2.1%; PBS group, 36.9±1.4%; $P<0.01$ for high versus mid and mid versus low) (Figure 2c). In addition, LVEDP 4 weeks after ischemia was significantly smaller in the high group than in the mid group and in the mid group than in the low group (high group, 7.8±0.6; mid group, 9.8±0.6; low group, 13.0±0.7; PBS group, 13.3±1.6 mm Hg; $P<0.01$ for high versus mid and mid versus low) (Figure 2c). The +dP/dt, −dP/dt, EF, and LVEDP 4 weeks after transplantation in the low group were not significantly different from those in the PBS group.

Thus, transplantation of high and mid doses of CD34$^+$ cells, but not the low dose, significantly preserved global and regional LV function after MI. The functional effect of CD34$^+$ cells was dose-dependently observed.

Morphometric Evaluation of Capillary Density and Infarct Size

Myocardial neovascularization assessed by capillary density on day 28 was dose-dependently enhanced in rats receiving CD34$^+$ cell transplantation (high group, 714.3±25.0; mid group, 535.8±31.0; low group, 320.9±36.0; PBS group, 291.3±19.0/mm^2; $P<0.01$ for high versus mid and mid versus low). Capillary density in the low group was similar to that in the PBS group (Figure 3a and 3b). LV remodeling evaluated by the percent fibrosis area was dose-dependently inhibited in rats receiving CD34$^+$ cell transplantation (high group, 16.0±2.6%; mid group, 22.4±1.9%; low group, 30.7±3.9%; PBS, 31.5±0.7%; $P<0.01$ for high versus mid and mid versus low groups). Percent fibrosis area was similar in the low and PBS groups (Figure 3c and 3d).

Thus, transplantation of high and mid CD34$^+$ cells, but not the low dose, significantly preserved LV structural integrity

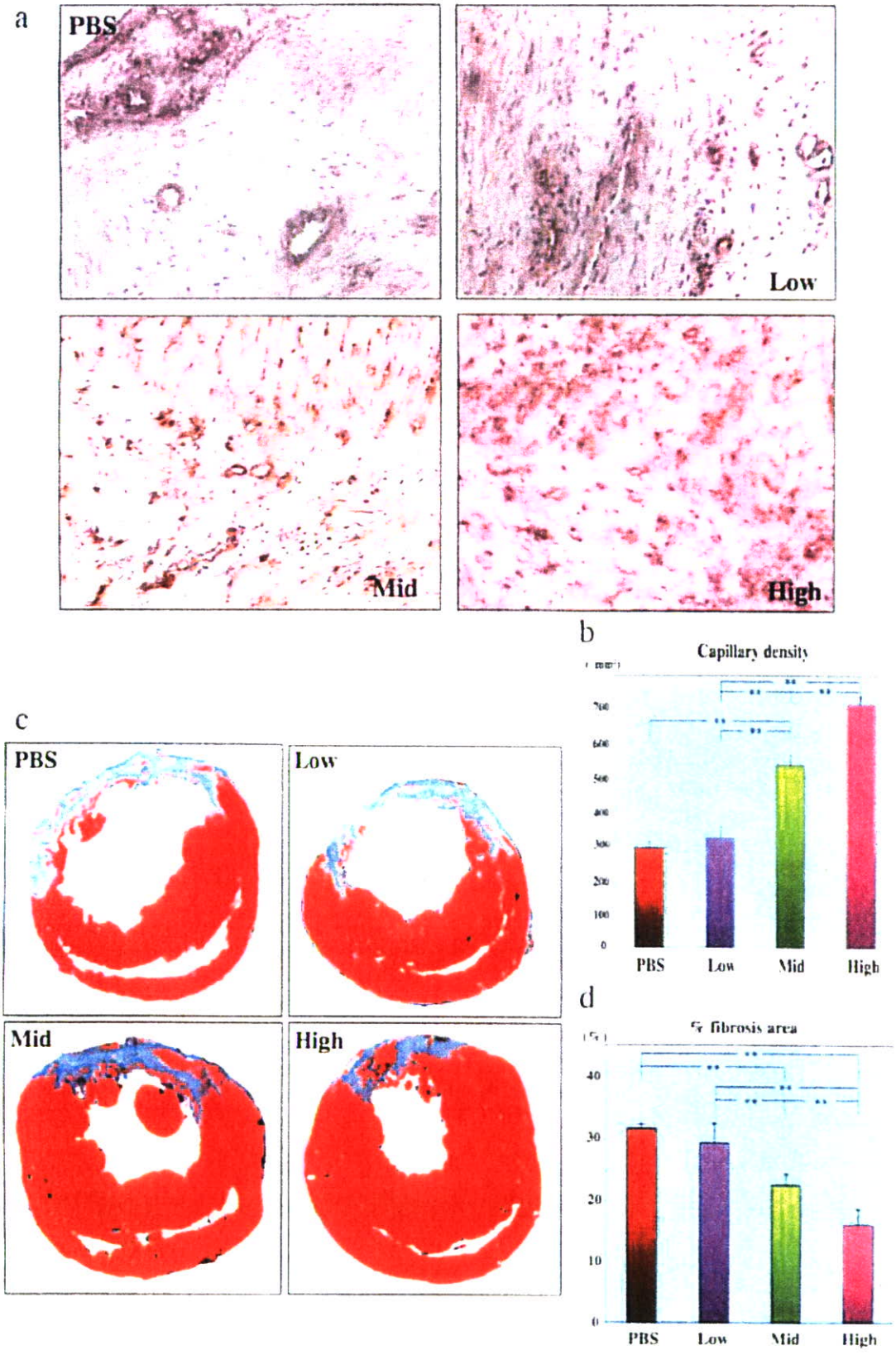

Figure 3. Histological evaluation of myocardial neovascularization and LV remodeling after MI. a, Representative immunostaining for isolectin B4 in each group at day 28 (magnification ×200). b, Capillary density in rats receiving CD34⁺ cells or PBS at day 28. Ischemic neovascularization was dose-dependently enhanced after CD34⁺ cell transplantation. $^{**}P<0.01$ (n=8 in all groups). c, Representative Masson trichrome staining at day 28 in each group. d, Ratio of fibrosis area/LV area (percent fibrosis area) at day 28 in each group. LV remodeling after MI was dose-dependently inhibited after CD34⁺ cell transplantation. $^{**}P<0.01$ (n=8 in each group).

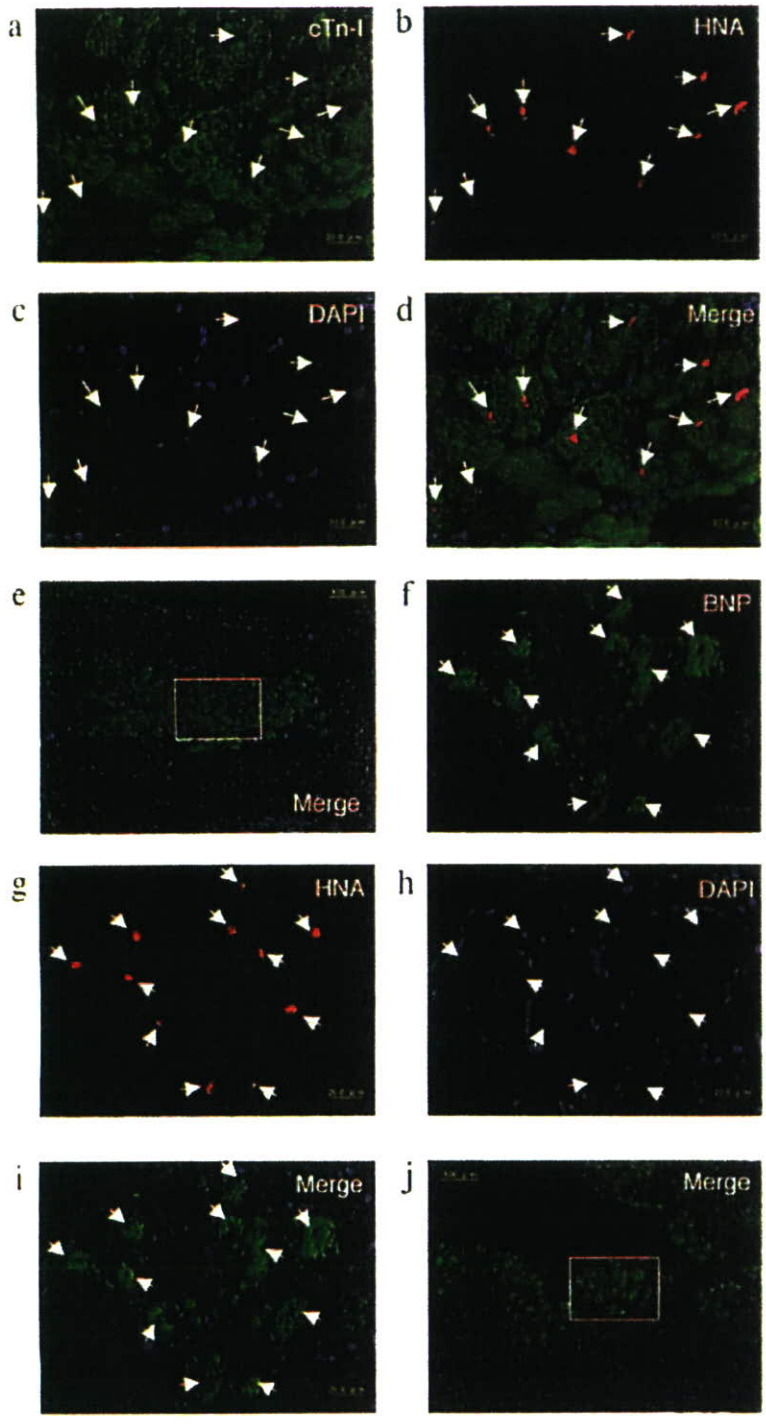

Figure 4. Histological evaluation of human cardiomyocyte development in rat ischemic myocardium. a to e, Representative double immunofluorescence staining for cTn-I and HNA in high-dose group at day 28. Human cardiomyocytes were identified as double-positive cells for cTn-I (green) and HNA (red). a, cTn-I, magnification ×400; b, HNA, ×400; c, DAPI, ×400; d, merge, ×400; e, merge, ×100. White arrows show nuclei of human cardiomyocytes. f to j, Representative double-immunofluorescence staining for hBNP and HNA in high-dose group at day 28. Human cardiomyocytes were identified as double-positive cells for hBNP (green) and HNA (red). f, hBNP, magnification ×400; g, HNA, ×400; h, DAPI, ×400; i, merge, ×400; j, merge, ×100. White arrows show nuclei of human cardiomyocytes. k, Representative double-immunofluorescence staining for cTn-I and HNA at day 28 in each group. Human cardiomyocytes were identified as double-positive cells for cTn-I (green) and HNA (red) (magnification ×400). White arrows show nuclei of human cardiomyocytes. The double-positive cells for cTn-I and HNA derived from transplanted cells were dose-dependently observed in ischemic myocardium (magnification ×400). l, Representative fluorescence immunohistochemical images for hBNP at day 28 in each group. Green fluorescence shows hBNP-positive cells, and blue indicates DAPI for nuclear staining. The hBNP-positive cardiomyocytes derived from transplanted cells were dose-dependently observed in ischemic myocardium (magnification ×200). m, Human cardiomyocytes (CMC) (hBNP-positive cardiomyocytes: black bar) and total (both human and rat) cardiomyocytes (white bar) on day 28 were dose-dependently observed in the ischemic myocardium. $^{*}P<0.05$, $^{**}P<0.01$ (n=8 in all groups).

after MI. The histological efficacy of CD34^{+} cells was dose-dependently observed.

Transplanted hCD34^{+} Cells Dose-Dependently Differentiate Into Cardiomyocytes

Differentiated human cardiomyocytes derived from the transplanted CD34^{+} cells were mainly identified in the rat peri-infarct myocardium by double staining both for cTn-I and HNA (Figure 4a to 4e) and for hBNP and HNA (Figure 4f to 4j). These findings suggest that transplanted CD34^{+} cells have potency of differentiation into cardiomyocytes. Double immunohistochemistry with hBNP and HNA also revealed specificity of the hBNP antibody for human cells in rat myocardium. Dose-dependent distribution of human cardiomyocytes in rat myocardium was observed both in samples stained with cTn-I and HNA (Figure 4k) and in samples stained with hBNP (Figure 4l). In fact, the numbers of hBNP-positive cardiomyocytes were dose-dependently observed in ischemic myocardium at day 28 (high group, 2480±149; mid group, 1860±141; low group, 423±9; PBS

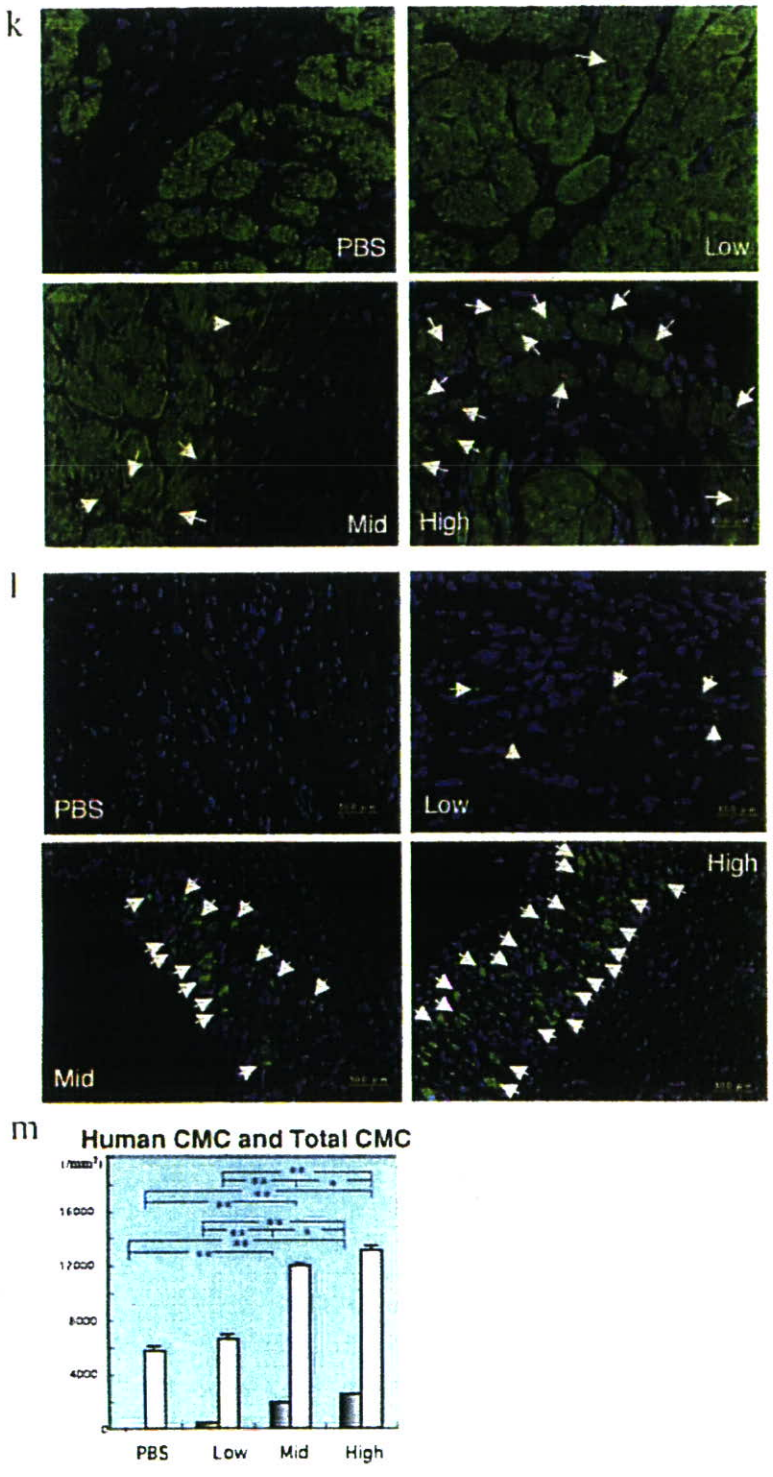

Figure 4. Continued

group, 0±0/mm²; $P<0.05$ for high versus mid and mid versus low groups). Total (both human and rat) cardiomyocytes were also dose-dependently observed in ischemic myocardium at day 28 (high group, 13 102±298; mid group, 11 936±238; low group, 6564±369; PBS group, 5707±300/mm²; $P<0.05$ for high versus mid and mid versus low groups) (Figure 4l and 4m). Similar dose-dependent cardiomyogenesis was observed when CD34⁺ cells from another patient were used (Figure I in the online-only Data Supplement). These findings strongly suggest that transplanted CD34⁺ cells may have dose-dependent potency of differentiation into cardiomyocytes.

Transplanted hCD34⁺ Cells Dose-Dependently Differentiate Into SMCs

Human SMCs derived from the transplanted CD34⁺ cells were mainly identified in the vasculatures within the peri-infarct area

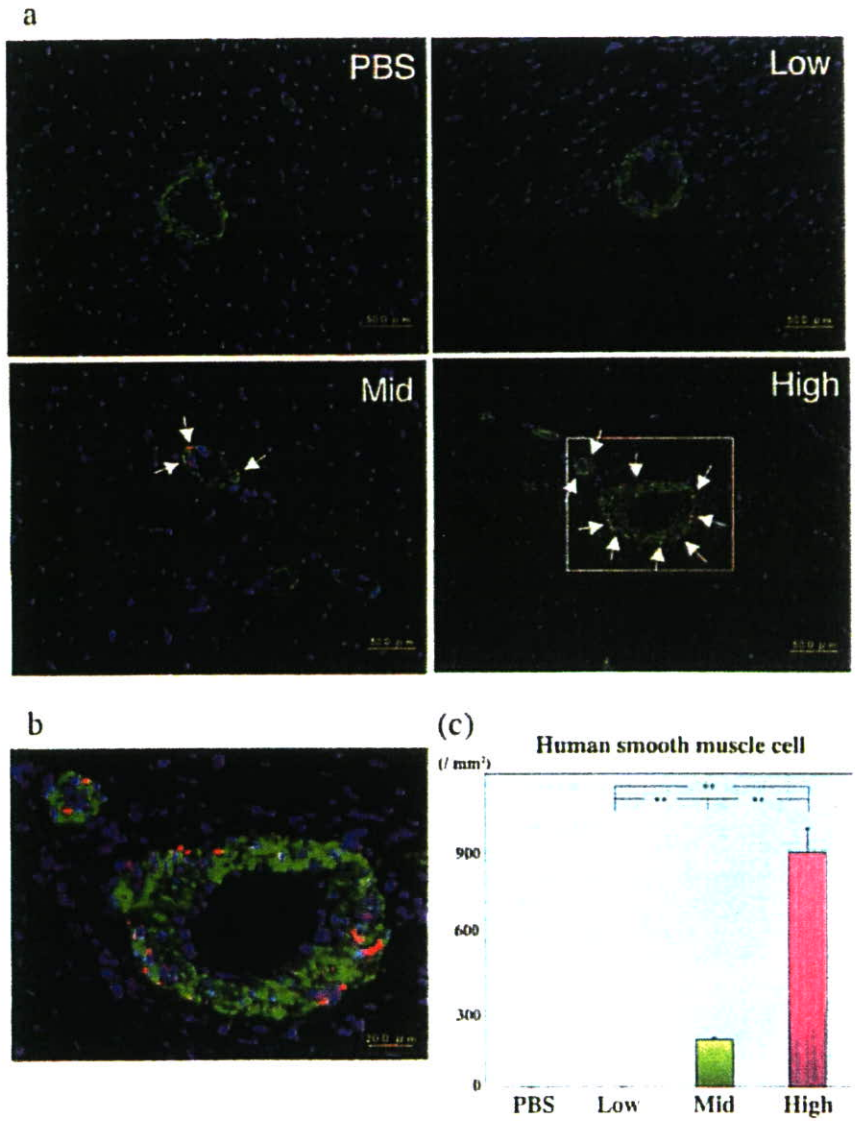

Figure 5. Histological evaluation of human SMC development. a, Representative double-immunofluorescence staining for SMA and HLA-ABC at day 28 in each group (magnification ×200). Human vascular SMCs were identified as double-positive cells for SMA (green) and HLA-ABC (red). b, Representative double-immunofluorescence staining for SMA and HLA-ABC at day 28 in high group (magnification ×400). c, Human SMCs on day 28 were dose-dependently observed in rat ischemic myocardium. $^{**}P<0.01$ (n=8 in each group).

by double staining for SMA and HLA-ABC. Identified human SMCs were dose-dependently observed after $CD34^+$ cell transplantation (high group, 895±95; mid group, 180±11; low group, 0±0; PBS group, 0±0/mm²; $P<0.01$ for high versus mid and mid versus low groups). In contrast, differentiated human SMCs were not identified in PBS and low groups (Figure 5a to 5c). Similar dose-dependent SMC commitment was observed when $CD34^+$ cells from another patient were used (Figure II in the online-only Data Supplement). These findings suggest that transplanted $CD34^+$ cells may have dose-dependent potency of differentiation into SMCs.

Transplanted $hCD34^+$ Cells Dose-Dependently Differentiate Into ECs

We confirmed the specificity of UEA-1 staining for human ECs using double immunohistochemistry with UEA-1 and HNA (Figure 6a to 6e). Differentiated human ECs derived from the transplanted $CD34^+$ cells were observed in the vasculatures within the peri-infarct area by UEA-1 staining. Identified UEA-1–positive cells were greater in higher-dose groups than lower-dose groups (high group, 3373±363; mid group, 980±211; low group, 226±35; PBS group, 0±0/mm²; $P<0.05$ for high versus mid and mid versus low groups). In contrast, differentiated human ECs were not identified in the PBS group (Figure 6f, 6g). Similar dose-dependent endothelial commitment was observed when $CD34^+$ cells from another patient were used (Figure III in the online-only Data Supplement).

Thus, locally transplanted $CD34^+$ cells were incorporated not only into ECs but also into mature SMCs, resulting in contribution to vasculogenesis in ischemic myocardium.

FISH Analysis of Transplanted $CD34^+$ Cell–Derived Cardiomyocytes

To determine whether cardiac repair occurred through cell fusion in MI tissue, we performed FISH with human Y chromosomes and rat genome probe. The specificity of the probes was tested in tissues of normal rat heart and rat heart immediately after human cell transplantation. We confirmed that these 2 probes did not cross-react with cells of the other species (data not shown). The FISH analysis revealed the

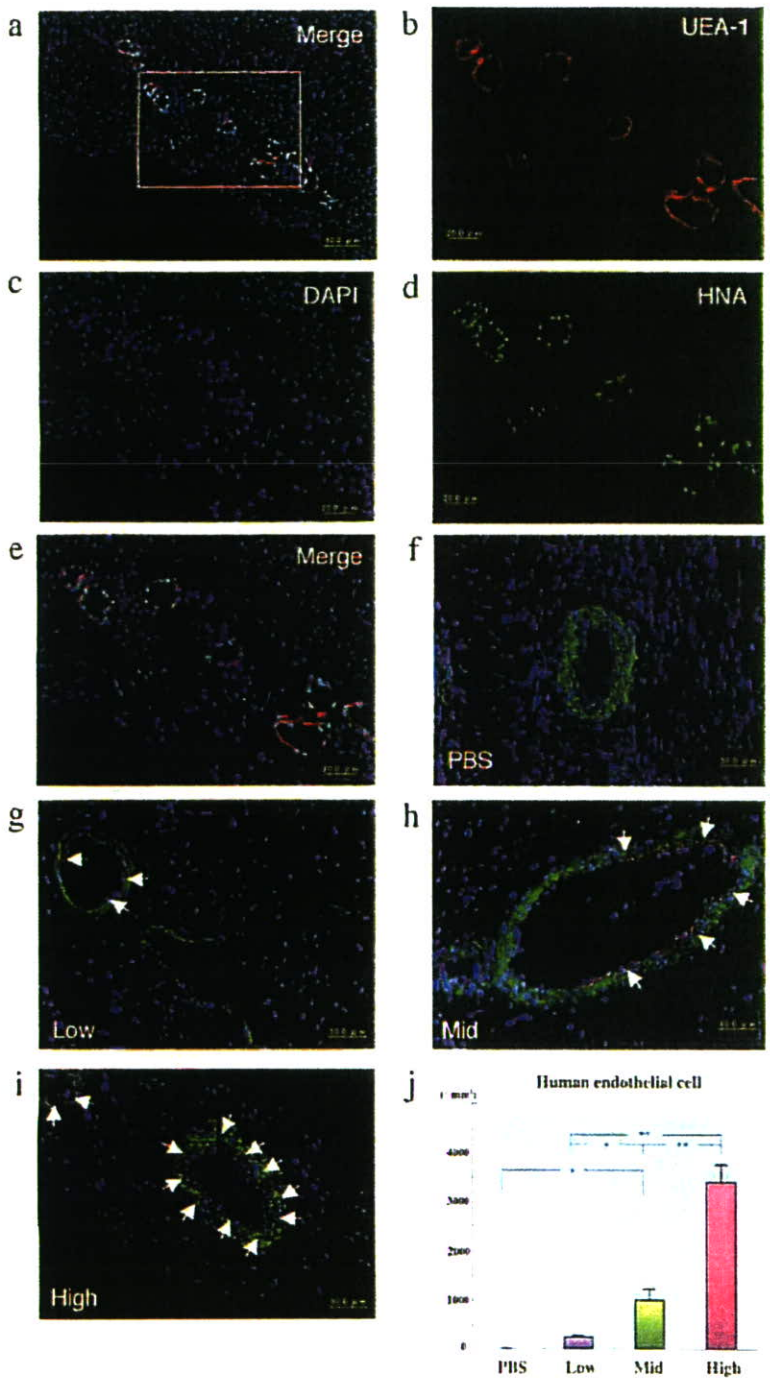

Figure 6. Histological evaluation of human EC development. a to e, Representative double-immunofluorescence staining for UEA-1 (red) and HNA (green) in high-dose group at day 28. Human ECs were identified as double-positive cells for UEA-1 and HNA. a, Merge, magnification ×100; b, UEA-l, ×400; c, DAPI, ×400; d, HNA, ×400; e, merge, ×400. f to j, Representative double-immunofluorescence staining for HNA (green) and UEA-1 (red) at day 28 in each group (magnification ×200). Human ECs were identified as UEA-1–positive cells (arrow). In the PBS group (f), differentiated human ECs were not identified. In the low group (g), differentiated human ECs were rarely identified. In the mid group (h), human ECs were more frequently identified than in the low and PBS groups. In the high group (i), human ECs were further more frequently identified than in other groups. j, Human ECs on day 28 were dose-dependently observed in the ischemic myocardium. $^{*}P<0.05$, $^{**}P<0.01$ (n=8 in all groups).

existence of cardiomyocytes in which human Y chromosome was paired with rat genome (cell fusion) as well as those without genome (no fusion) (Figure 7a to 7d). These findings indicate that both cell fusion and multilineage differentiation without fusion may be involved in transformation of transplanted CD34+ cells into cardiomyocytes.

Dose-Dependent Gene Expression of Human-Specific Cardiac, Smooth Muscle, and Endothelial Lineage Cell Markers in Rat Ischemic Myocardium After CD34+ Cell Transplantation

To further ensure the immunohistochemical results with regard to cardiomyogenesis and vasculogenesis by the molecular approach, we performed RT-PCR with rat ischemic myocardium by using human-specific primer BNP, cTn-I, MHC-α, MHC-β, and Nkx 2.5 as human cardiomyocyte lineage markers, human-specific primer sm22α and SMA as human SMC markers, and human-specific primer CD31 and KDR as human EC markers.

The RT-PCR analysis revealed dose-dependent expression of human-specific cardiomyogenic, arteriogenic, and vasculogenic genes in rat ischemic myocardium after human CD34+ cell transplantation with the use of the NIH Image program (version 1.62) (Figure 8a and 8b). Notably, gene expression of all markers except hSMA was not detected in freshly isolated

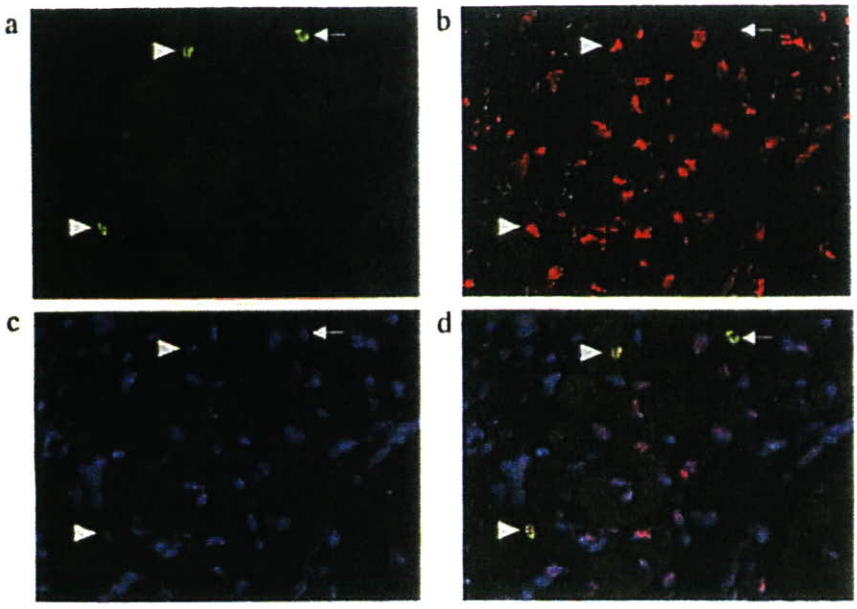

Figure 7. Representative FISH analysis at day 28 with the use of human Y chromosome probe and rat genome probe to assess cardiomyogenesis mechanism. Human cardiomyocytes due to cell fusion (arrow heads) were identified as double-positive cells for human Y chromosomes (yellow) and rat genome (red). Human cardiomyocytes developed without cell fusion (arrow) were identified as only positive cells for human Y chromosome. a, FISH image for human Y chromosome probe; b, FISH image for rat genome probe; c, DAPI staining; d, merged image.

$CD34^+$ cells (Figure 1c) but was dose-dependently augmented in ischemic myocardium after cell transplantation.

Discussion

Abrupt occlusion of coronary arteries causes MI, which leads to massive cardiomyocyte loss and consequently deterioration of cardiac function because cardiomyocytes have severely limited capacity to be divided and thus replace the damaged tissue. Progressive heart failure is a major cause of death or frequent hospitalization in patients after MI. Although MI is classified as vascular (coronary artery) disease, therapeutic strategies should be focused on regenerating not only blood vessels but also cardiac muscle to improve the poor prognosis of the disease.

Compelling evidence suggests that transplantation of bone marrow–derived $CD34^+$ cells or cultured EPC-enriched population contributes to preservation of LV function after MI through enhancing ischemic neovascularization.[10–12] The mechanism of this therapeutic effect was previously considered to be incorporation, differentiation, and proliferation of EPCs for new blood vessel formation.[9,11,26] Recently, Badorff et al[27] reported in vitro transdifferentiation of EPCs into functional cardiomyocytes. Yeh et al[10] also demonstrated in vivo differentiation of $CD34^+$ cells into cardiomyocytes and SMCs in a mouse model of acute MI. Regeneration of SMCs as well as ECs may result in mature vasculogenesis, which is more potent for blood flow recovery in ischemic myocardium compared with capillary formation by EC-only regeneration. These findings lead to a novel concept that $CD34^+$ cell transplantation may contribute to cardiomyogenesis and vasculogenesis, which may be an ideal strategy to treat MI. On the other hand, Balsam et al[15] and Murry et al[19] reported that mouse bone marrow HSCs isolated as Lin^- c-Kit^+ cells or c-Kit^+ $Thy1.1^+$ Lin-Sca-1^+ cells do not transdifferentiate into cardiomyocytes in infarcted myocardium. Several points should be considered carefully with regard to this discrepancy, such as the difference in species (human versus murine), cell populations ($CD34^+$ cells versus Lin c-Kit^+ cells or c-Kit^+ $Thy1.1^+$ Lin-Sca-1^+), cell doses to transplant, or cell delivery methodologies. Given the controversy, the question is whether improvement of myocardial function after EPC transplantation was due to myocardial preservation through the signal from enhanced vasculogenesis as well as due to regenerative cardiomyogenesis by transplanted cells. To solve this issue, we tried to confirm the lineage potency and the tissue plasticity of $CD34^+$ cells by transplanting the cells into immunodeficient rats with MI in a dose-ranging fashion. From a practical point of view, a significant contribution of cardiomyogenesis and vasculogenesis to LV functional recovery after MI may not be expected if such translineage differentiation is a rare event after $CD34^+$ cell transplantation. To detect the translineage differentiation of human $CD34^+$ cells into rat myocardium, we performed not only immunohistochemistry but also RT-PCR for human-specific markers of cardiomyocytes, SMCs, and ECs. These sensitive assessments revealed dose-dependent augmentation of cardiomyogenesis and vasculogenesis of human $CD34^+$ cells in rat infarcted myocardium. The translineage potential was accompanied with dose-dependent enhancement of capillary density, inhibition of LV fibrosis, and preservation of LV function. These findings suggest that transplantation of a higher dose of $CD34^+$ cells may be more potent for therapeutic application to the damaged myocardium than a lower dose. Another interesting finding in this study is that these favorable effects of $CD34^+$ cells were not significantly observed in the low-dose group (1×10^5 cells/kg). To our knowledge, information about a noneffective dose of $CD34^+$ cells has never been provided. Taken together, the present results strongly suggest the therapeutic importance of the cell dosage in the actual clinical application.

It is unclear what mechanism of $CD34^+$ cells is involved in multilineage commitment and significant incorporation for functional organogenesis. One mechanism for multilineage commitment is the translineage differentiation of

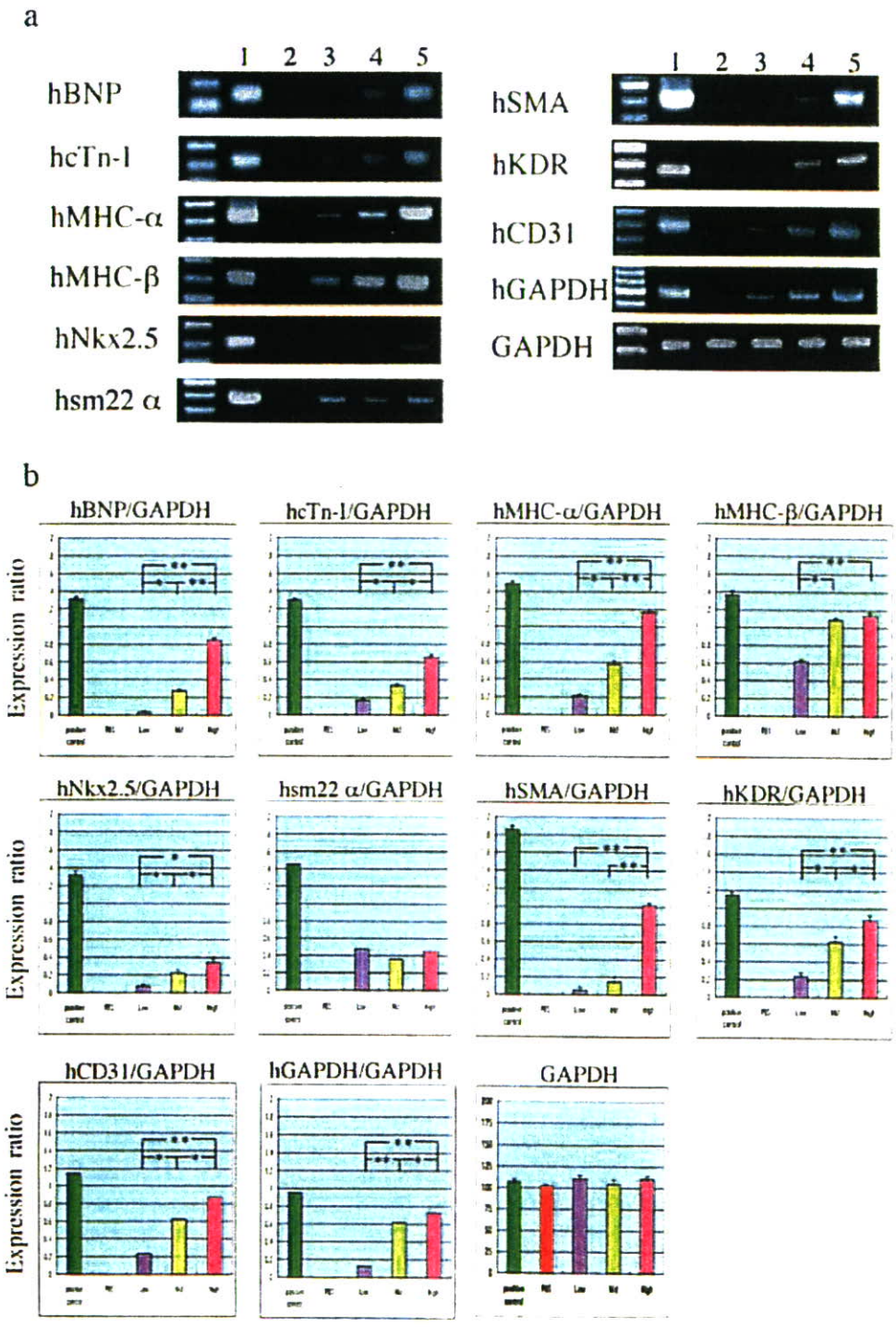

Figure 8. a, RT-PCR analysis to evaluate expression of human-specific genes of cardiomyocyte (hBNP, hcTn-I, hMHC-α, hMHC-β, and hNkx 2.5), SMC (hsm22α and hSMA), and EC (hKDR and hCD31) lineages in rat ischemic myocardium at day 28. Lane 1, human heart (positive control); lane 2, PBS group; lane 3, low group; lane 4, mid group; lane 5, high group. b, The ratio of gene expression of each human-specific marker to total (rat and human) GAPDH in MI tissue was dose-dependently augmented after CD34+ cell transplantation. Gene expression of total GAPDH mRNA levels was similar in all groups. *$P<0.05$, **$P<0.01$ (n=4 in each group).

already committed EPCs or HSCs among CD34+ cells through transdifferentiation or cell fusion. Recently, Zhang et al[7] demonstrated that 70% of newly formed cardiomyocytes derived from CD34+ cells were developed through a cell fusion mechanism between human and mouse cells, whereas CD34+-derived ECs are mainly not developed by cell fusion. The FISH analyses in this study revealed that the mechanism of cardiomyogenic plasticity of CD34+ cells involves both cell fusion and the multilineage differentiation without fusion, although the functional contribution of both mechanisms to myocardiogenesis remains uncertain.

The other possible mechanism of multilineage differentiation is due to the original multipotency of the CD34+ cell population. Recently, peripheral blood CD34+ cells were proved to contain a cell fraction expressing not only hematopoietic and endothelial but also cardiac, muscle, liver, and neural lineage markers after mobilization following G-CSF administration or myocardial ischemia,[28,29] whereas this issue is not determined if multilineage cells are derived from pluripotent stem cell or various lineage progenitor cell mixtures in CD34+ cells. When the magnificent incorporation of CD34+ cell–derived cardiomyocytes and SMCs is taken into account, the mechanism is likely due to programmed lineage commitment in the myocardial ischemia environment, although we have not defined each responsible cell fraction for lineage diversification in CD34+ cells.

The cooperative signal from vasculogenesis to cardiomyogenesis must also be considered further in the regenerative process through multilineage commitments by CD34+ cells. Cardiomyogenesis and vasculogenesis are closely regulated in terms of microenvironmental interaction during the developmental stage. Recently, Shen et al[30] proved the significance of vascular signals for postnatal neural stem cell biology, as formerly indicated in the case of liver and pancreas development in embryo.[31,32] Microenvironmental interaction between myocardial and vascular lineage cells involves not only paracrine regulatory factors but also direct cellular communications in developing CD34+ cells. We speculate that an enhanced vasculogenesis signal may exert cellular commitment and development of CD34+ cells into myocardial cells as a cooperative organogenesis mechanism.

In conclusion, after transplantation of bone marrow–derived CD34+ cells, the collaborative multilineage differentiation potential of CD34+ cells not only into ECs but also into cardiomyocytes and SMCs was enhanced by cell dose escalation and was conducive to heart regeneration in terms of functional and histological recovery through vasculogenesis and cardiomyogenesis.

Acknowledgments

This work was supported by Health and Labor Sciences research grants (H14-trans-001, H17-trans-002) from the Japanese Ministry of Health, Labor, and Welfare. We thank Yumiko Masukawa and Tomoko Itoh for secretarial assistance. Human-specific antibody against brain natriuretic peptide (hBNP) was a generous gift from Dr Hiroshi Itoh of Kyoto University, Kyoto, Japan.

Disclosures

None.

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CD34-Positive Cells Exhibit Increased Potency and Safety for Therapeutic Neovascularization After Myocardial Infarction Compared With Total Mononuclear Cells

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Background—We compared the therapeutic potential of purified mobilized human $CD34^+$ cells with that of mobilized total mononuclear cells (tMNCs) for the preservation/recovery of myocardial tissue integrity and function after myocardial infarction (MI).

Methods and Results—$CD34^+$ cells were purified from peripheral blood tMNCs of healthy volunteers by magnetic cell sorting after a 5-day administration of granulocyte colony-stimulating factor. Phosphate-buffered saline (PBS), 5×10^5 $CD34^+$ cells/kg, 5×10^5 tMNCs/kg (low-dose MNCs [loMNCs]), or a higher dose of tMNCs (hiMNCs) containing 5×10^5 $CD34^+$ cells/kg was transplanted intramyocardially 10 minutes after the induction of MI in athymic nude rats. Hematoxylin and eosin staining revealed that moderate to severe hemorrhagic MI on day 3 was more frequent in the hiMNC group than in the PBS and $CD34^+$ cell groups. Immunostaining for human-specific CD45 revealed abundant distribution of hematopoietic/inflammatory cells derived from transplanted cells in the ischemic myocardium of the hiMNC group. Capillary density on day 28 was significantly greater in the $CD34^+$ cell group (721.1±19.9 per 1 mm^2) than in the PBS, loMNC, and hiMNC groups (384.7±11.0, 372.5±14.1, and 497.5±24.0 per 1 mm^2) ($P<0.01$). Percent fibrosis area on day 28 was less in the $CD34^+$ cell group (15.6±0.9%) than in the PBS, loMNC, and hiMNC groups (26.3±1.2%, 27.5±1.8%, and 22.2±1.8%) ($P<0.05$). Echocardiographic fractional shortening on day 28 was significantly higher in the $CD34^+$ cell group (30.3±0.9%) than in the PBS, loMNC, and hiMNC groups (22.7±1.5%, 23.4±1.1%, and 24.9±1.7%; $P<0.05$). Echocardiographic regional wall motion score was better preserved in the $CD34^+$ cell group (21.8±0.5) than in the PBS, loMNC, and hiMNC groups (25.4±0.4, 24.9±0.4, and 24.1±0.6; $P<0.05$).

Conclusions—$CD34^+$ cells exhibit superior efficacy for preserving myocardial integrity and function after MI than unselected circulating MNCs. (***Circulation.* 2006;114:2163-2169.**)

Key Words: angiogenesis ■ endothelium ■ ischemia ■ progenitor cells ■ stem cells

Since endothelial progenitor cells (EPCs) were identified as circulating CD34 antigen–positive mononuclear cells,[1] the therapeutic potential of purified EPCs or total (unpurified) mononuclear cells (tMNCs) containing both EPC and non-EPC fractions has been evaluated in many preclinical and clinical studies. Transplantation of purified EPCs augments ischemic neovascularization in mice with hind-limb ischemia,[2,3] rats with acute myocardial ischemia,[4,5] and swine with chronic myocardial ischemia.[6] Recent pilot clinical trials also have suggested the therapeutic potential of EPC transplantation in patients with coronary artery disease.[7,8] On the other hand, tMNC transplantation has been further reported. Trials using tMNCs have demonstrated their therapeutic efficiency

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to enhance ischemic neovascularization in animal studies[9,10] and human clinical trials.[11,12] Although tMNCs consist mainly (>99%) of non-EPCs and contribute to limited vasculogenic volume by EPCs, transplantation of the non-EPC fraction stimulates secretion of angiogenic cytokines in ischemic tissue.[10] However, the fate of the non-EPC fraction after transplantation into ischemic sites is not well known. The non-EPC fraction of hematopoietic cells might cause excess inflammation in the ischemic

Received March 3, 2006; de novo received June 8, 2006; revision received August 23, 2006; accepted September 8, 2006.

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Circulation is available at http://www.circulationaha.org

DOI: 10.1161/CIRCULATIONAHA.106.644518

tissue. The possibility for non-EPCs to differentiate into undesired lineage cells such as osteoblasts, chondroblasts, fibroblasts, adipocytes, or ectopic myocytes also remains to be clarified.

To the best of our knowledge, no report has compared the therapeutic potential and safety of EPC transplantation with those of tMNC administration. Accordingly, we performed transplantation of human EPCs compared with tMNCs in a model of rat myocardial infarction (MI) and investigated the effects of these 2 potential cellular therapies for ischemic neovascularization, inhibition of left ventricular (LV) remodeling, and preservation of LV function after acute MI.

Methods

These experiments were performed as a part of a pre-IND (investigational new drug) study, which supported a clinical trial of autologous $CD34^+$ cell transplantation in patients with coronary artery disease. Results of this study were submitted to the US Food and Drug Administration with the clinical protocol, which was approved in November 2003.

Cell Collection and Isolation

All procedures were approved by our institutional ethics committees. Peripheral blood tMNCs were obtained from 3 healthy volunteers who underwent leukoapheresis after subcutaneous administration of granulocyte colony-stimulating factor ($5\ \mu g \cdot kg^{-1} \cdot d^{-1}$) for 5 days. We performed fluorescent-activated cell sorter analysis to examine the frequency of $CD34^+$ cells in the tMNCs. $CD34^+$ cells were isolated from tMNCs with the Isolex 300i $CD34^+$ cell isolation system (Baxter, Deerfield, Ill) as an EPC-enriched fraction. Fluorescent-activated cell sorter analysis revealed that the frequency of $CD34^+$ cells in the tMNCs was $1.7\pm0.9\%$ and the purity of isolated $CD34^+$ cells was $90.1\pm8.1\%$.

Induction of Myocardial Ischemia and Cell Transplantation

All procedures were performed in accordance with the policies of our Institutional Animal Care and Use committees. Female athymic nude rats (Hsd:RH-rnu rats, Harlan Sprague Dawley, Indianapolis, Ind) 6 to 8 weeks of age were anesthetized with ketamine hydrochloride (75 mg/kg IP) and xylazine (10 mg/kg IP). Myocardial ischemia was induced by permanently ligating the left anterior descending (LAD) coronary artery under controlled ventilation.[4] Ten minutes after the LAD was ligated, 100 μL phosphate-buffered saline (PBS), 5×10^5 $CD34^+$ cells/kg, 5×10^5/kg of tMNCs (low-dose MNCs [loMNCs]), or a higher dose of tMNCs (hiMNCs) calculated to contain 5×10^5 $CD34^+$ cells/kg were injected intramyocardially into 5 sites in the ischemic LAD territory with a 27G needle (20 μL to each site) (n=9 to 11 in each group). All cells were suspended with 100 μL PBS. Cell number of the hiMNC group was determined from the results of the fluorescent-activated cell sorter analysis for CD34 described above. The ischemic zone was macroscopically identified by the pale color of the anterior and lateral walls immediately after LAD ligation.[5] Induction of myocardial ischemia and cell transplantation was performed by experienced researchers who were blinded to treatment assignment.

Histological Assessment of Transplanted Animals

Rats were anesthetized with ketamine hydrochloride and xylazine 3 days (n=6 to 8) and 28 days (n=8 to 11) after cell transplantation. Peripheral blood was obtained from the abdominal aorta of each rat for hematological examinations such as blood cell count, hemoglobin, and hematocrit and blood chemical examinations, including blood urea nitrogen, creatinine, alanine transaminase, aspartate transaminase, creatine kinase, lactic dehydrogenase, troponin I, and blood sugar. Immediately after blood collection, rats were killed with an overdose of ketamine hydrochloride. At necropsy, organs, comprising brain, lung, heart, liver, spleen, kidney, and ovary, from each animal were collected, weighed, and fixed with 4% paraformaldehyde. Hearts were also sliced in a bread-loaf fashion into 8 transverse sections from apex to base. In 3 additional rats in each group, heart samples collected on day 3 were similarly sliced, embedded in optimal cutting temperature compound, snap-frozen in liquid nitrogen, and stored at −80°C. Frozen heart samples were similarly obtained on day 28 in 5 additional rats in each group.

Paraffin-embedded tissues of all organs were stained with hematoxylin and eosin to histologically examine adverse events after cell transplantation. Severity of hemorrhagic infarction in ischemic myocardium on day 3 also was evaluated semiquantitatively using the hematoxylin and eosin–stained samples as follows: 0=none, 1=mild, 2=moderate, and 3=severe. Masson-trichrome staining was performed using the paraffin-embedded heart sections obtained 28 days after transplantation to measure the average ratio of fibrosis area to the entire LV area. Histochemical staining for the murine-specific endothelial cell marker isolectin B4 (Vector Laboratories, Burlingame, Calif)[4] was performed using the heart samples obtained 28 days after treatment. Capillary density was evaluated morphometrically by histological examination of 5 randomly selected fields of tissue sections recovered from segments of LV myocardium subserved by the occluded LAD. Capillaries were recognized as tubular structures positive for isolectin B4.

Frozen heart samples obtained on day 3 were used for immunohistochemistry with human-specific antibody against CD45 (BD Biosciences, San Jose, Calif) to identify hematopoietic/inflammatory cells derived from transplanted human cells in rat ischemic myocardium. Frozen samples on day 28 were used for immunohistochemistry with antibodies against human nuclear antigen (HNA), cardiac troponin I, and von Willebrand factor (vWF) (all from Chemicon International, Temecula, Calif) to detect cardiomyocytes and endothelial cells derived from transplanted human cells.

All morphometric studies were performed by 2 examiners who were blinded to treatment assignment.

Physiological Assessment of LV Function

Transthoracic echocardiography (SONOS 5500, Phillips Technologies, Bothell, Wash) was performed 28 days after transplantation. Fractional shortening was measured at the middle papillary muscle level. Regional wall motion score was examined per published criteria.[13] All procedures and analyses were performed by an experienced researcher who was blinded to treatment.

Statistical Analysis

Results were statistically analyzed with the use of the Statview 5.0 software package (Abacus Concepts Inc, Berkeley, Calif). Severity scores were examined across groups through the use of the Kruskal-Wallis test, followed by the Wilcoxon rank-sum test with the simple Bonferroni method (a value of $P<0.05/6$ was considered statistically significant). Intergroup comparison of incidence of moderate to severe hemorrhagic infarction on day 5 was assessed by χ^2 test (a value of $P<0.05$ was considered significant). Echocardiographic and histological values on day 28 were expressed as mean±SE. Scheffé's test was performed for the multiple comparisons after analysis of variance between groups. In Scheffé's test, a value of $P<0.05$ was considered statistically significant.

The authors had full access to the data and take responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results

Exacerbation of Hemorrhagic MI Is Evident 3 Days After Transplantation of hiMNCs but Not loMNCs and Purified $CD34^+$ Cells

The weight of all organs was similar in all groups on days 3 and 28. Hematoxylin and eosin staining for all organs

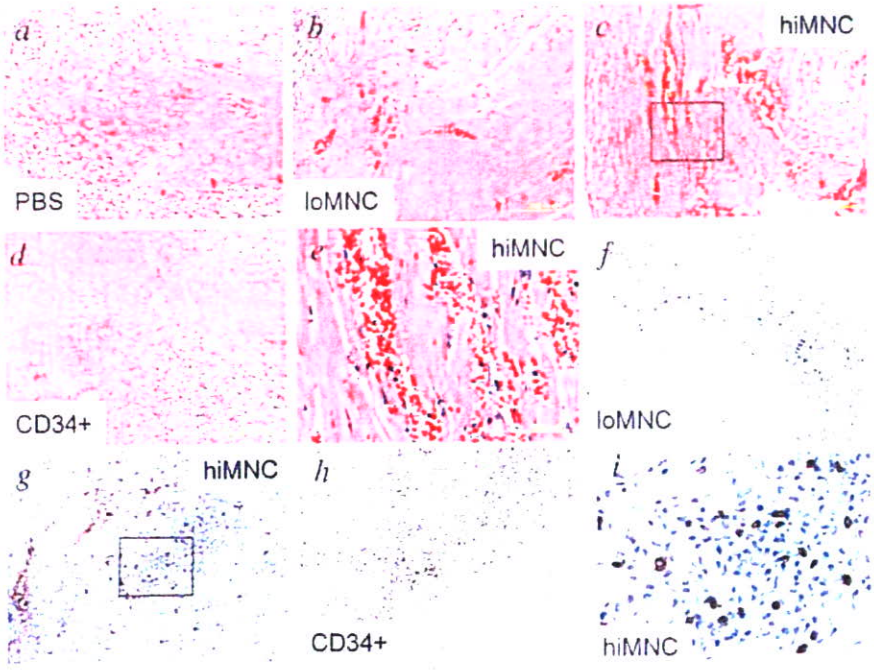

Figure 1. a–e, Representative light-microscope findings in infarcted myocardium 3 days after cell transplantation (hematoxylin and eosin staining; a–d, ×10; e, ×40). a, Rat receiving PBS; b, rat receiving 5×10^5 tMNC/kg (loMNC); c, e, rat receiving hiMNCs containing 5×10^5 CD34⁺ cells/kg; d, rat receiving 5×10^5 CD34⁺ cells/kg (CD34⁻). f–i, Representative findings of immunohistochemistry for human-specific CD45 (f–h, ×10; i, ×40). Abundant distribution of human CD45⁺ cells was observed in the hiMNC group (g and i) but not in the loMNC (f) and CD34⁺ cell (h) groups.

except the heart disclosed no abnormal findings on days 3 and 28. Results of hematologic and blood chemical tests were similar in all groups on days 3 and 28 (data not shown).

Hematoxylin and eosin staining of myocardial tissue samples on day 3 revealed that the frequency of moderate to severe hemorrhagic MI was significantly greater in rats receiving hiMNCs compared with the PBS and CD34⁺ cell groups (hiMNC, 87.5%, n=8; PBS, 33.3%, n=6; CD34⁺, 12.5%, n=8; $P=0.04$ versus PBS and $P=0.003$ versus CD34⁺ cell group). Frequency of severe hemorrhagic MI also was greater in the hiMNC group than in the CD34⁺ cell group (hiMNC, 50.0%; CD34⁺ cell, 0.0%; $P=0.02$). The severity score of hemorrhagic MI had a tendency to be greater in the hiMNC group (50.0% severe, 37.5% moderate, 12.5% mild, and 0.0% none) than in the PBS group (16.7% severe, 16.7% moderate, 50.0% mild, and 16.7% none) and CD34⁺ cell group (0.0% severe, 12.5% moderate, 75.0% mild, and 12.5% none); however, these differences were not statistically significant ($P=0.04$ versus PBS, $P=0.01$ versus CD34⁺) because a value of $P<0.05/6$ was considered significant by Bonferroni's method. The severity of hemorrhagic infarction was similar in the PBS, loMNC (28.6% severe, 28.6% moderate, 28.6% mild, and 14.3% none; n=7), and CD34⁺ cell groups (Figure 1a through 1e).

Immunohistochemistry for human-specific CD45 revealed more abundant distribution of human CD45⁺ cells within the ischemic myocardium of the hiMNC group compared with the CD34⁺ cell and loMNC groups. The human CD45⁺ cells were mainly round without a tubular structure, a finding that strongly suggests differentiation of transplanted human cells into hematopoietic/inflammatory cells in the rat ischemic myocardium. Human-specific CD45⁺ cells were not observed in the PBS group (Figure 1f through 1i).

These results suggest that transplantation of unselected human MNCs may worsen hemorrhagic MI, perhaps via distribution of hematopoietic/inflammatory cells into the acutely ischemic myocardium. This unfavorable phenomenon was not observed after transplantation of loMNCs and CD34⁺ cells.

Transplanted CD34⁺ Cells Differentiate More Abundantly Into Cardiomyocytes and Endothelial Cells in the Infarcted Myocardium on Day 28 Compared With Unpurified tMNCs

Double immunostainings for HNA and cardiac troponin I to detect transplanted human cell–derived cardiomyocytes and for HNA and vWF to identify human cell–derived endothelial cells were performed using samples of the infarcted myocardium at day 28. These stainings revealed that double-positive cells for HNA and cardiac troponin I were identified only in rats receiving CD34⁺ cells but not in the hiMNC, loMNC, and PBS groups (Figure 2a through 2h). Similarly, double-positive cells for HNA and vWF were abundant in the CD34⁺ cell group and rare in the hiMNC group. The double-positive cells were not observed in the loMNC and PBS groups (Figure 2i through 2p).

These results suggest that purified CD34⁺ cell transplantation may have more potential for cardiac myoangiogenesis compared with total MNC transfer.

Transplantation of CD34⁺ Cells Further Augments Ischemic Neovascularization and Inhibits LV Remodeling on Day 28 Compared With That of Unpurified tMNCs

Capillary density 28 days after treatment was significantly greater in the CD34⁺ cell group (721.1±19.9 per 1 mm²) than in the PBS, loMNC, and hiMNC groups (384.7±11.0, 372.5±14.1, and 497.5±24.0 per 1 mm², respectively)

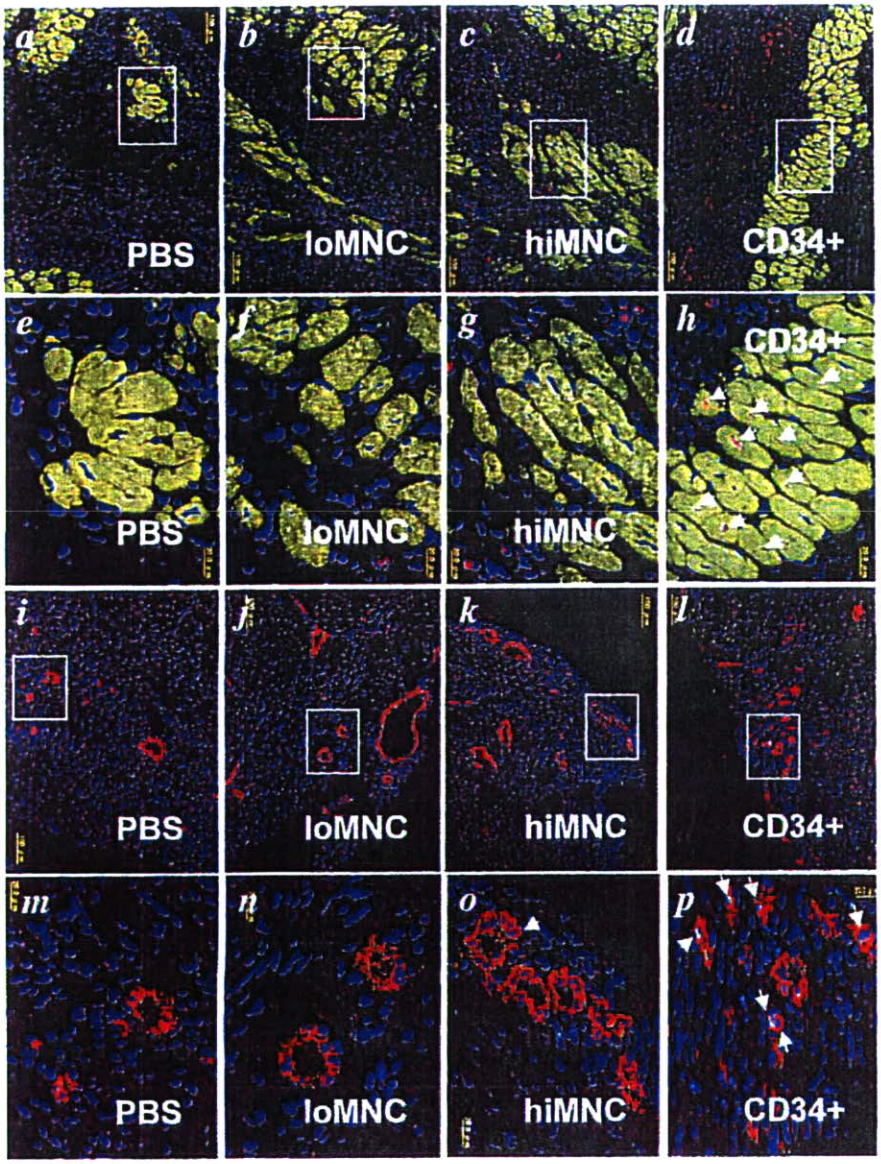

Figure 2. a–h, Representative double immunohistochemistry for HNA (red) and cardiac troponin I (green) in PBS (a, e), loMNC (b, f), hiMNC (c, g), and $CD34^-$ cell (d, h) groups. Blue fluorescence demonstrates 4′6-diamidino-2-phenylindole (DAPI) for nuclear staining. a–d, ×10; e–h, ×40; arrows indicate nuclei of cardiomyocytes expressing HNA (purple, double positive for HNA and DAPI), which indicates differentiation of transplanted human cells into cardiomyocytes. The cardiomyogenic differentiation was identified only in the $CD34^-$ cell group. i–p, Representative double immunostaining for HNA (green) and vWF (red) in the PBS (i, m), loMNC (j, n), hiMNC (k, o), and $CD34^-$ cell (l, p) groups. Blue fluorescence demonstrates DAPI for nuclear staining. i–l, ×10; m–p, ×40; arrows show nuclei of endothelial cells expressing HNA (pale green, double positive for HNA and DAPI), which indicates differentiation of transplanted human cells into endothelial cells. The vasculogenic differentiation was abundantly detected in the $CD34^-$ cell group and rarely in the hiMNC group but not in the loMNC and PBS groups.

($P<0.0001$ versus PBS, loMNC, and hiMNC groups). Capillary density on day 28 also was significantly greater in the hiMNC group than in the PBS and loMNC groups ($P=0.003$ versus PBS group, $P=0.001$ versus loMNC group). Capillary density on day 28 in the loMNC group was not significantly different from that in the PBS group (Figure 3a through 3e).

The ratio of percent fibrosis area to entire LV area was significantly lower in the $CD34^-$ cell group ($15.6\pm0.9\%$) than in the PBS, loMNC, and hiMNC groups ($26.3\pm1.2\%$, $27.5\pm1.8\%$, and $22.2\pm1.8\%$, respectively) ($P=0.0003$ versus PBS group, $P<0.0001$ versus loMNC group, $P=0.02$ versus hiMNC group). This ratio was similar between the PBS, loMNC, and hiMNC groups (Figure 3f through 3j).

Thus, transplantation of hiMNCs significantly augmented ischemic neovascularization; however, transplantation of $CD34^-$ cells enhanced new blood vessel formation to a greater degree than when the same dose of $CD34^-$ cells was administered within an unselected MNC population. Furthermore, only transplantation of $CD34^-$ cells significantly inhibited LV remodeling after MI.

Transplantation of $CD34^+$ Cells Preserves LV Function After Myocardial Ischemia

By day 28 after treatment, fractional shortening was significantly higher in the $CD34^-$ cell group ($30.3\pm0.9\%$) than in the PBS, loMNC, and hiMNC groups ($22.7\pm1.5\%$, $23.4\pm1.1\%$, and $24.9\pm1.7\%$, respectively) ($P=0.007$ versus PBS, $P=0.02$ versus loMNC, $P=0.049$ versus hiMNC group). Fractional shortening on day 28 was similar in the PBS, loMNC, and hiMNC groups (Figure 4a and 4b). Regional wall motion score was better preserved in the $CD34^-$ cell group (21.8 ± 0.5) than in the PBS, loMNC, and hiMNC groups (25.4 ± 0.4, 24.9 ± 0.4, and 24.1 ± 0.6, respectively) ($P=0.0004$ versus PBS, $P=0.002$ versus loMNC, $P=0.02$ versus hiMNC group). Regional wall motion score was similar in the PBS, loMNC, and hiMNC groups (Figure 4a and 4c).

Thus, echocardiographic examination performed in the chronic phase after MI suggests that transplantation of

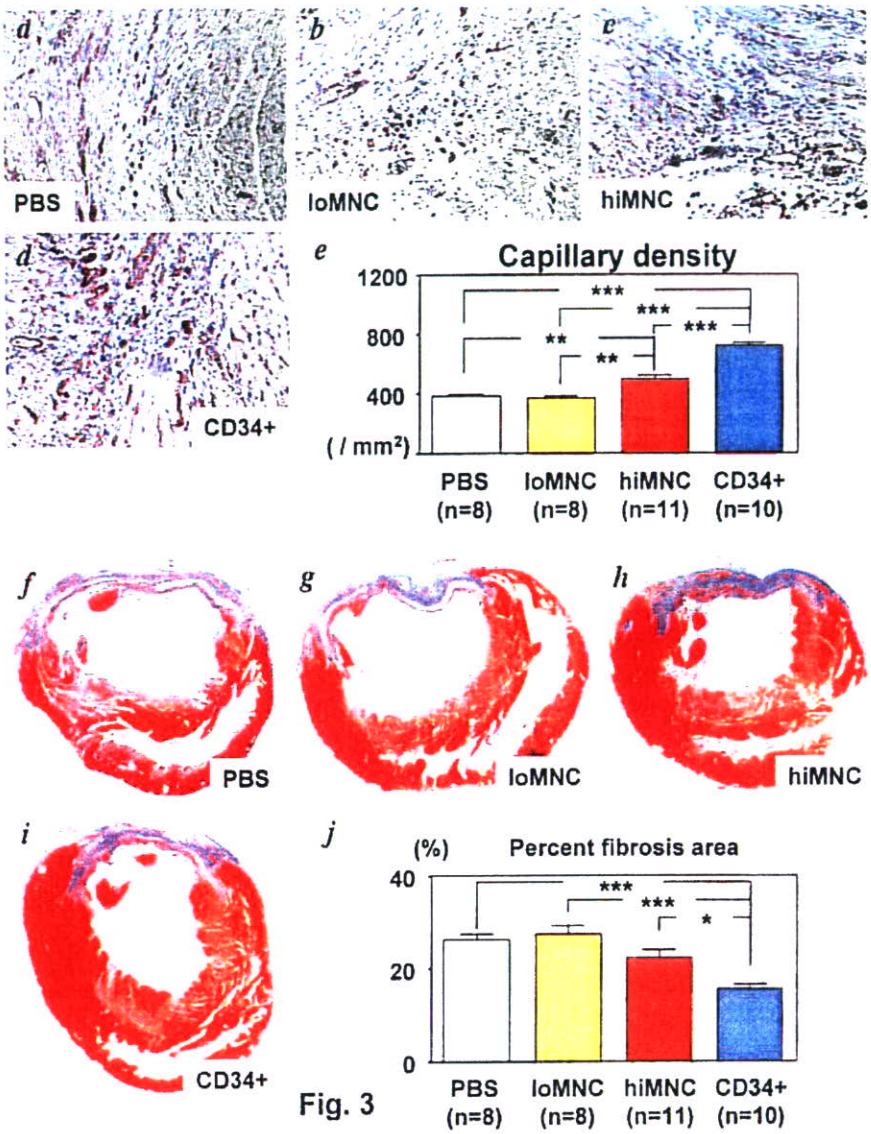

Fig. 3

Figure 3. a–d, Representative findings of histochemical staining for isolectin B4 in the ischemic myocardium 4 weeks after treatment (×20). a, Rat receiving PBS; b, rat receiving loMNCs; c, rat receiving hiMNCs; d, rat receiving 5×10^5 CD34$^+$ cells/kg (CD34$^+$). e, Capillary density in ischemic myocardium 4 weeks after each treatment. f–i, Representative findings of elastic tissue trichrome staining in heart samples 4 weeks after MI. These samples were obtained from rats receiving PBS (f), loMNCs (g), hiMNCs (h), and CD34$^+$ cells (i). j, Ratio of fibrosis area to entire LV area (percent fibrosis area) 4 weeks after each treatment. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

CD34$^+$ cells may have a favorable impact on the preservation of global and regional LV function. Transplantation of higher doses of unselected tMNCs also had a tendency to preserve LV contractility after MI, but this change was not significant.

Discussion

In the present study, dosages of CD34$^+$ cells and tMNCs were determined on the basis of our previous animal study in anticipation of a future clinical trial. In the previous study[6] evaluating intramyocardial transplantation of CD34$^+$ cells into rats with MI, the effective cell dose for ischemic neovascularization and preservation of LV function was 10^5 cells per rat, which is equivalent to 5 to 7×10^5 cells/kg. Previous clinical reports in the hematology field indicated that the estimated number of autologous CD34$^+$ cells obtained by single leukoapheresis after a 5-day administration of granulocyte colony-stimulating factor is 5 to 10×10^5 cells/kg.[14,15] Therefore, we anticipated that transfer of 5×10^5 CD34$^+$ cells/kg would be both an effective and a clinically realistic dose. To precisely assess the difference of safety and therapeutic potential between purified CD34$^+$ cells and tMNCs, we also included 2 treatment groups of tMNCs: the same total dose of tMNCs (loMNC) as the CD34$^+$ cells (5×10^5 cells/kg) and high-dose tMNCs (hiMNC) containing an equivalent dose of CD34$^+$ cells (5×10^5 CD34$^+$ cells/kg).

Histological findings in the acute phase of MI (on day 3) revealed that the incidence of moderate to severe hemorrhagic infarction, which is one of the prognostic signs of irreversible myocardial and microvascular damage after MI,[16,17] was significantly greater after hiMNC transplantation than PBS or CD34$^+$ cell injection. This unfavorable phenomenon was not observed in the loMNC group. These findings suggest that intramyocardial transplantation of tMNCs into acutely ischemic myocardium may be safe up to 5×10^5 cells/kg but may worsen hemorrhagic infarction at higher doses (10^7 cells/kg). The present findings also indicate that the hemorrhagic issue is not present in the case of CD34$^+$ cell transplantation at a dose of up to 5×10^5 cells/kg. The exact mechanism of hemorrhagic infarction in the hiMNC group is unknown; however, immunohisto-