Corrosion casts allow the visualization of small arteries less than 100 μm in diameter,[11] although it is impossible to examine in live animals or ex vivo beating hearts, and the complete distension of the vessels depends on several factors such as elastic properties of the vessel wall, viscosity of the infused material, and pressure of infusion. Conventional angiography, which has been widely performed in clinical practice, can be also undergone in live animals repeatedly. However, previous reports indicated that conventional angiography systems, which could not visualize arteries less than 200 μm in diameter,[15,16] have insufficient resolution to visualize full extent of collateral formation. The resolutional limitation may lead to underestimation of angiogenic potential of the SCTx, because improvement of collateral-dependent flow typically results from the proliferation of vessels less than 180 μm in diameter.[17–19] The indispensable angiographic assessment in small animals has never been established.

Synchrotron radiation (SR) has been investigated as a novel approach for animal studies because intravenous coronary angiography, a relatively less invasive technique compared with selective coronary angiography, was begun at the end of 1970's. Research groups have improved imaging systems in SR facilities for future clinical application.[20] Aside from the intravenous coronary angiography, Mori et al[15,16] recently developed a new angiography system called SR microangiography (SRM), which was an intraarterial microangiography system. In this system, monochromatic SR is used as an x-ray source, which energy was adjusted to 33.2 keV just above the iodine K-edge energy to produce the highest contrast image of the iodine contrast material, and a high-fidelity video system is also used as a detector, which has the potential to visualize small vessels (diameter <50 to 100 μm). Thereafter, many researchers have used the SRM to visualize penetrating transmural coronary arteries in the canine hearts,[21] collateral microvessels following therapeutic angiogenesis in rat model of hind limb ischemia,[22] vasodilatation of arterial circle of cerebrum and its branches of the dogs,[23] and tumor-derived angiogenic vessels of the rabbits[24] at the Photon Factory in Tsukuba, Japan. However, the previous SRM system was unable to visualize coronary arteries, their branches, and collateral vessels in beating hearts of small animals because of the still inappropriate image quality. Currently, new SRM system with spatial resolution in the μm range has been developed in the SPring-8 (Japan Synchrotron Radiation Research Institute) in Sayo, Japan. Recently, Kidoguchi et al[25] have applied the new SRM system to visualize branches of rat middle cerebral arteries and successfully depict the vessels as small as 30 μm in diameter at 9.5 μm of detector pixel size. In this study, we used the new generation SRM to visualize rat coronary vessels as small as 20 μm in diameter at 4.5 μm of pixel size and evaluated coronary vascular function in response to vasodilator under fast beating condition. Here, we report usefulness of the third generation SRM to visualize collateral vessels and quantify the effect of therapeutic neovascularization by bone marrow (BM)-derived CD34+ cell transplantation in rats with MI.

Methods

Isolation of CD34+ Cells From Patients With Critical Limb Ischemia

Peripheral blood total mononuclear cells (tMNCs) were obtained from 3 male patients 71, 63, and 60 years of age with atherosclerotic peripheral artery disease by apheresis after 5-day subcutaneous administration of G-colony stimulating factor (CSF) (10 μg/kg/d). CD34+ cells or CD34− cells were isolated from the tMNCs by a magnetic cell sorting system, CliniMACS (Miltenyi Biotec).[26] The CD34+ cell fraction had a purity of >99%, as determined by fluorescence-activated cell sorting (FACS) analysis using a monoclonal antibody specific for human CD34 (Becton Dickinson). CD34+ cells in this study were $CD31^{bright}$, $AC133^{bright}$, and $CD45^{dim}$ but negative for KDR and VE-cadherin. In contrast, CD34− cells were positive for CD45 and CD31, but negative for AC133, KDR, and VE-cadherin. The FACS results suggest that freshly-isolated CD34+ cells are immature population responsible for hematopoietic stem cells, endothelial progenitor cells, and hemangioblasts, whereas the CD34− cells are not considered to be either immature or mature endothelial lineage cells (supplemental Figure I, available online at http://atvb.ahajournals.org).

These patients received intramuscular transplantation of 10^5 CD34+ cells/kg according to the protocol of a phase I/II dose-escalation clinical trial. Remaining CD34+ or CD34− cells were used for following experiments. Informed consent regarding the cell therapy and experimental use of the remaining cells was obtained from each patient 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; CLEA Japan, Tokyo, Japan) aged 7 to 8 weeks and weighing 145–160 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. All of our experiments on imaging of the rat hearts with MI also conformed to the SPring-8 Guide for Care and Use of Laboratory Animals in SRM examination.

Induction of Myocardial Infarction and Cell Transplantation

Rats were anesthetized with ketamine and xylazine (60 mg/kg and 10 mg/kg, respectively, IP). MI was induced by ligating left anterior descending coronary artery (LAD) as described previously.[7–9] Twenty minutes after MI, rats received intramyocardial transplantation of 1×10^5 CD34− cells or 1×10^5 CD34+ cells resuspended with 100 μL of PBS or the same volume of PBS without cells (n=9 in each group). To evaluate incorporation and development of the transplanted cells in MI tissue, CD34+ cells or CD34− cells labeled with fluorescent carbocyamine 1, 1′-dioctadecyl1–1 to 3,3,3,3′-tetramethylindocarbocyanine perchlorate (DiI) dye (Molecular Probes, Carlsbad, CA) were intramyocardially transferred into athmic nude rats (n=3) after MI.[9]

Imaging System

SRM experiments were performed at the 2nd optical hatch of the BL28B2 beamline in the SPring-8. Monochromatic synchrotron radiation with an energy level of 33.2 keV was obtained from the beamline. An X-ray imaging system needs to have high shutter speed to make sharp and blur-free images of fast-moving hearts, and for this purpose we developed a shutter system using a rotating disk with radial slots rotating around an axis parallel to the X-ray beam. The shortest shutter open time was 0.1 ms. X-rays transmitted through the object are detected by the X-ray direct-conversion type detector incorporating the X-ray SATICON pick-up tube. For high-resolution, real-time imaging (7.0 μm or 4.5 μm pixel size, 30 frames/second), the monochromatized x-ray obtained from the third generation SR source and the new rotating disk shatter were used.

Differences in Characteristics of Synchrotron Radiation System Between the Photon Factory and the SPring-8

	Photon Factory	SPring-8
Input field of view, mm	50×50 or 20×20	7.0×7.0 or 4.5×4.5
Pixel size, μm	48×48 or 19×19	7.×7.0 or 4.5×4.5
Spatial resolution, μm	30	6
Minimum detectable vessel diameter, μm	50–100	20
Shortest shutter open time, msec	17	2

Sequential images were obtained with an input field of view of 7.0 mm × 7.0 mm or 4.5 mm × 4.5 mm. Image signals were converted into digital format and stored in a frame memory with a 1024×1024 pixels format and 10-bit resolution. Improved points in the new generation SR imaging system in the Spring-8 compared with the previous version in the Photon Factory in Tsukuba, Japan is shown in the Table.

Coronary Microangiography

Transplanted immunodeficient rats were anesthetized with pentobarbital and anticoagulant heparin intraperitoneally. After thoracotomy, the heart and aortic arch were rapidly excised and immersed in perfusion solution. The pericardium was quickly removed under immersion and aorta was prepared for cannulation. The heart was mounted on an aortic cannula, and then pulmonary artery was cut near its origin. Throughout the experiment, aortic retrograde perfusion at a constant flow rate (4.0 mL/min) with oxygenated perfusion solution drawn from a temperature-regulated reservoir (37°C) was started according to the Langendorf technique, as described in detail previously.[27] The perfusion solution was of the following composition (in mmol/L): NaCl, 118.5; $NaHCO_3$, 25.0; KCl, 3.2; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 1.4; glucose, 11.0. The solution was filtered before use and gassed continuously with 90% O_2/10% CO_2 (pH 7.4 at 37°C). Perfusion fluid was directed into coronary arteries to perfuse the entire ventricular mass of the heart. Contractile function and regular heart rhythm returned within a few seconds, and maximum function was established in several minutes. After stabilization of heart rate and perfusion pressure in the ex vivo beating hearts under the Langendorf perfusion, SRM at baseline was performed in each animal. The microangiographic images were taken at base, mitral papillary muscle, and apical levels. Microangiography was performed with an automated injector (Nemoto Kyorindo) which was programmed to reproducibly deliver 0.4 mL/sec of nonionic contrast media containing 37% iodine (Iopamiron 370; Nihon Schering) for 4 sec. After the baseline angiography were taken, sodium nitroprusside (SNP) (Roche), an endothelium-nondependent vasodilator, was added to oxigenized Krebs-Henseleit solution while keeping the perfusate concentrations and the flow rate. The concentration of SNP used in this study was 1×10^{-6} mol/L, which corresponds to values validated as the most suitable concentration of SNP to assess the vasodilating effect in a previous study.[28] Microangiography was similarly performed to visualize dilated coronary vessels. Each imaging started 1 to 2 seconds before contrast media infusion, so that background pictures without contrast media could be taken for later computed analysis.

Tissue Harvest

After SRM, hearts were sliced in a broad-leaf fashion into 4 transverse sections from apex to base, embedded in OCT compound, snap frozen in liquid nitrogen (LN_2), and stored at −80°C for immunohistochemistry. 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).

Angiographic Assessment of Collateral Vessel Formation

Collateral flow filling to the LAD territory pre and post SNP was graded angiographically in a blinded manner by use of the Rentrop scoring system.[7] To quantify development of collateral vessels, angiographic microvessel density (AMVD) in the occluded LAD area both pre and post SNP was measured by following computed analysis. Hearts were divided into 4 parts from ligation point to apex, then we measured vessel densities in each part. Region of interest was determined in LAD perfusing area but without visible major branches of the LAD. The images immediate before (background) and during contrast media infusion were captured by an image scanner. After the image capture, vessel density in each part was obtained by subtracting the background density from the angiographic density processed with the NIH image program (v. 1.62) as described previously.[29] Average value of the vessel densities in 4 portions was calculated as the AMVD for each imaging procedure. The ratio of AMVD post SNP to pre SNP (AMVD ratio) was also calculated. These data analyses were performed by 2 blinded observers.

Morphometric Evaluation of Capillary Density

Histochemical staining with isolectin B4 (Vector Laboratories) was performed, and capillaries were recognized as tubular structures positive for isolectin B4. Histological capillary density was evaluated by morphometric examination of 5 randomly selected fields of tissue sections recovered from segments of LV myocardium subserved by the occluded LAD.[7–9] All morphometric studies were performed by 2 examiners who were blinded to treatment.

Statistical Analysis

The results were statistically analyzed with the use of a software package (Statview 5.0, Abacus Concepts Inc). All values were expressed as mean±SE. Paired *t* tests were performed for comparison of data between day 5 and day 28, and between pre and post SNP infusion. The comparisons among 3 groups were made with 1-way ANOVAs. Post hoc analysis was performed by Fisher protected least significant difference test. Correlation between histological and microangiographic vessel densities was analyzed by linear regression test. Differences of $P<0.05$ were considered statistically significant.

Results

Rentrop Score Pre and Post SNP Infusion

SRM was performed to evaluate collateral vessel development by elucidating Rentrop score, a semiquantitative grading of collateral flow filling into the occluded coronary artery,[10] 5 and 28 days after cell transplantation. SRM on day 5 demonstrated that the LAD was totally occluded at the ligation point and collateral flow filling into the distal LAD was not well visualized in all groups (Figure 1a). Angiographic Rentrop score at day 5 was not significantly different in each group (Figure 1c). In contrast, SRM on day 28 revealed better visualization of collateral vessels into the distal LAD area in CD34+ cell group compared with both CD34− cell and PBS groups. Collateral vessels were generated from left circumflex artery or proximal site of LAD. Diameter of the collateral vessels was generally 20 to 120 μm, which is apparently invisible size in conventional angiography (Figure 2a). Rentrop score at day 28 was significantly greater in CD34+ cell group than either CD34− cell or PBS group (CD34+, 1.6±0.2; CD34−, 0.6±0.2; PBS, 0.4±0.2, $P<0.01$ for CD34+ versus CD34− and PBS) (Figure 2c).

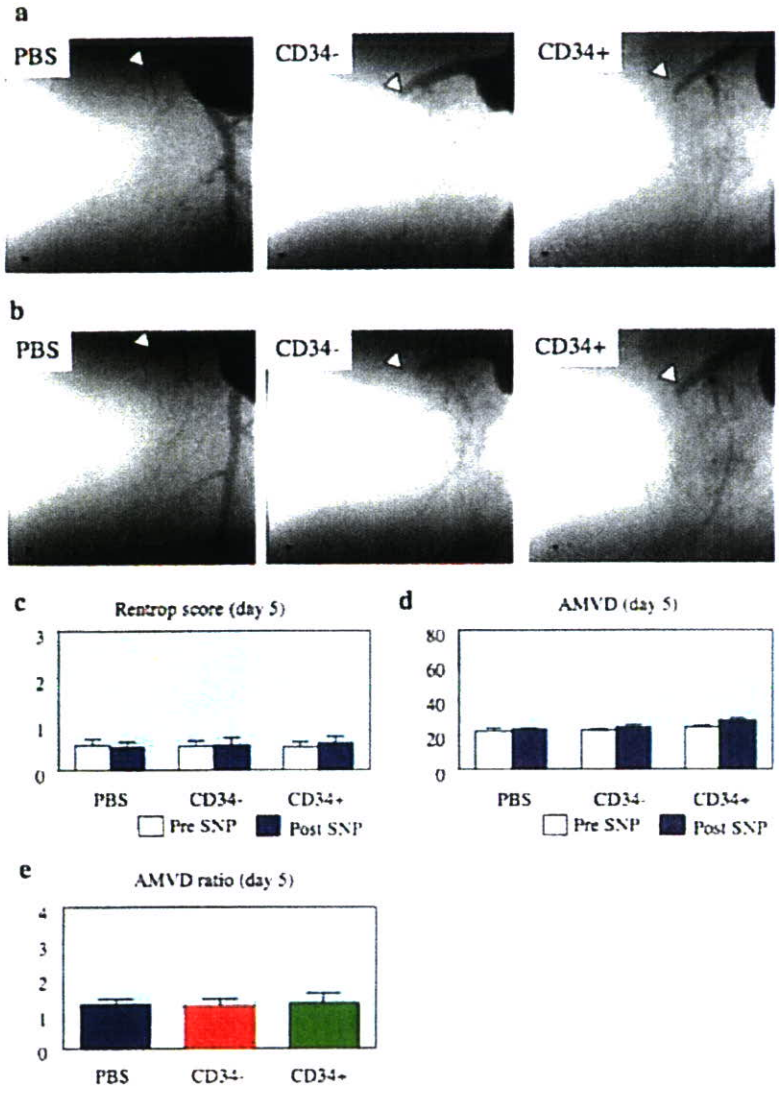

Figure 1. a, Representative images of synchrotron radiation microangiography (SRM) 5 days after PBS, CD34−, or CD34+ cell transplantation (pre sodium nitroprusside [SNP]; 7.0×7.0 mm). Collateral vessels were poorly visualized in all groups. Arrowhead shows ligation point (scale bar; 100 μm). b, Representative SRM images post SNP at day 5 (7.0×7.0 mm). Collateral vessels were poorly visualized in all groups (scale bar; 100 μm). c, Rentrop score of collateral development pre and post SNP in each group at day 5. d, Angiographic microvessel density (AMVD) pre and post SNP in each group at day 5. e, Ratio of AMVD post SNP to pre SNP (AMVD ratio) in each group at day 5.

SRM post SNP was similarly performed to evaluate the augmentation of new microvasculature 5 and 28 days after transplantation. SRM on day 5 revealed slightly better visualization of the new microvasculature post SNP compared with pre SNP in each group (Figure 1b). However, Rentrop score post SNP at day 5 was not significantly different in each group (Figure 1c). SRM on day 28 in CD34+ cell group, not in CD34− and PBS groups, revealed that new microvasculature in the occluded LAD area was better visualized post SNP than pre SNP (Figure 2a and 2b). Rentrop score at day 28 in CD34+ cell group was significantly greater post SNP than pre SNP (CD34+ post SNP, 1.8±0.1; CD34+ pre SNP, 1.6±0.2, $P<0.05$). However, in PBS or CD34− cell group, Rentrop score at day 28 post SNP was not significantly different from that pre SNP (Figure 2c).

SRM post SNP at day 28 revealed better visualization of collateral vessels into the distal LAD area in CD34+ cell group compared with both CD34− cell and PBS groups (Figure 2b). Rentrop score post SNP at day 28 was significantly greater in CD34+ cell group than either CD34− cell or PBS group (CD34+, 1.8±0.1; CD34−, 0.7±0.2; PBS, 0.5±0.2, $P<0.01$ for CD34+ versus CD34− and PBS) (Figure 2c).

Thus, the new generation SRM system enabled visualization and evaluation of new microvasculature created by CD34+ cell transplantation in the fast beating rat hearts. These results suggest that CD34+ cell transplantation may enhance collateral blood flow in the ischemic myocardium and may also improve collateral vascular function in response to SNP infusion.

Angiographic Microvessel Density (AMVD) in SRM

To quantify the activity of collateral vascular formation in the occluded LAD area, we measured the AMVD in SRM by computed analysis. AMVD pre and post SNP on day 5 was not significantly different in CD34+ cell group from that in other groups (Figure 1d). The ratio of AMVD post SNP to pre SNP (AMVD ratio) on day 5 was similar in all groups (Figure 1e).

AMVD pre SNP on day 28 was not significantly different in CD34+ cell group from that in other groups (CD34+, 27.3±3.2; CD34−, 23.2±0.8, PBS, 21.6±2.7). However, in CD34+ cell group, not in other groups, AMVD on day 28 was significantly greater post SNP than

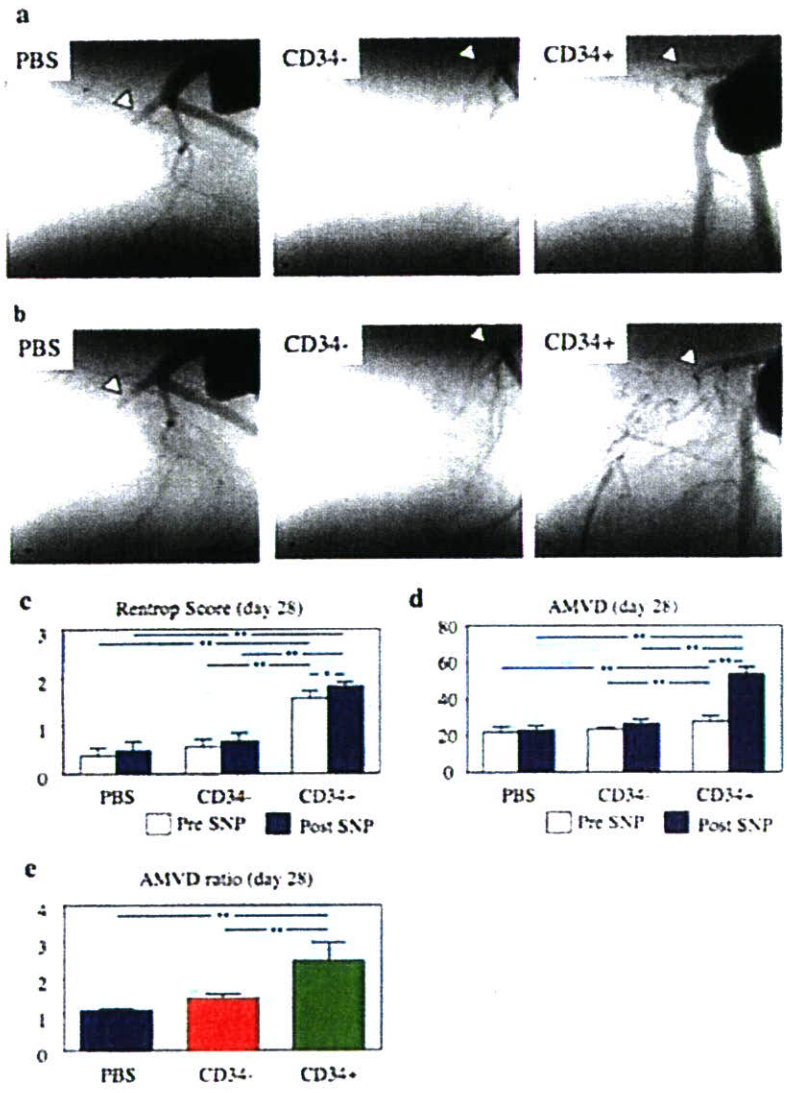

Figure 2. a, Representative microangiographic images pre SNP 28 days after PBS, CD34−, or CD34+ cell transplantation (7.0×7.0 mm). Collateral vessels were better developed in rat receiving CD34+ cells compared with rats receiving PBS and CD34− cells (scale bar, 100 μm). b, Representative microangiographic images post SNP at day 28 (7.0×7.0 mm). Augmentation of collateral microvessel development into distal portion of LAD area was further visualized in rat receiving CD34+ cells compared with rats receiving PBS and CD34− cells (scale bar, 100 μm). c, Rentrop score pre and post SNP at day 28 in each group. *$P<0.05$; **$P<0.01$. d, AMVD pre and post SNP at day 28 in each group. **$P<0.01$. e, AMVD ratio at day 28 in each group. **$P<0.01$.

pre SNP (post SNP, 53.2±3.8; pre SNP, 27.3±3.2, $P<0.05$). AMVD post SNP on day 28 was significantly greater in CD34+ cell group compared with CD34− cell and PBS groups (CD34+, 53.2±3.8; CD34−, 26.5±2.0, PBS, 23.0±2.0, $P<0.01$ for CD34+ versus CD34− and PBS) (Figure 2d). AMVD ratio on day 28 was also significantly greater in CD34+ cell group than either CD34− cell or PBS group (CD34+, 2.5±0.5; CD34−, 1.4±0.1, PBS, 1.1±0.1, $P<0.01$ for CD34+ versus CD34− or PBS). AMVD ratio on day 28 was similar in CD34− cell and PBS groups (Figure 2e).

These results indicate that AMVD analysis may be useful to quantify the effect of therapeutic neovascularization by CD34+ cell transplantation. Similarly as the Rentrop grade examination, AMVD assessment suggests contribution of CD34+ cell transplantation to improvement of collateral vessel function in response to SNP.

Histological Evaluation of Capillary Density

Histochemical staining for isolectin B4 was performed to identify capillaries in ischemic myocardium 4 weeks after cell transplantation (Figure 3a). Histological capillary density was significantly greater in CD34+ cell group than in CD34− cell and PBS groups. Histological capillary density in CD34− cell group was not significantly different from that in PBS group (CD34+, 711±15; CD34−, 365±23; PBS, 294±17/mm^2, $P<0.01$ for CD34+ versus CD34− and PBS) (Figure 3b).

Correlation Between SRM and Histological Assessments

To confirm whether AMVD is precise assessment of vascular development by CD34+ cell transplantation, we investigated correlation between AMVD and histological capillary density on day 28. AMVD pre SNP did not significantly correlate with histological capillary density ($R=0.16$, $P=0.42$), however AMVD post SNP closely correlated with histological capillary density ($R=0.82$, $P<0.0001$) (Figure 3c).

These results suggest that AMVD post SNP may be accurate and useful for precise evaluation of collateral and vascular formation following SCTx.

Discussion

Many investigators have demonstrated efficacy of various stem/progenitor cell transplantation against ischemic disease

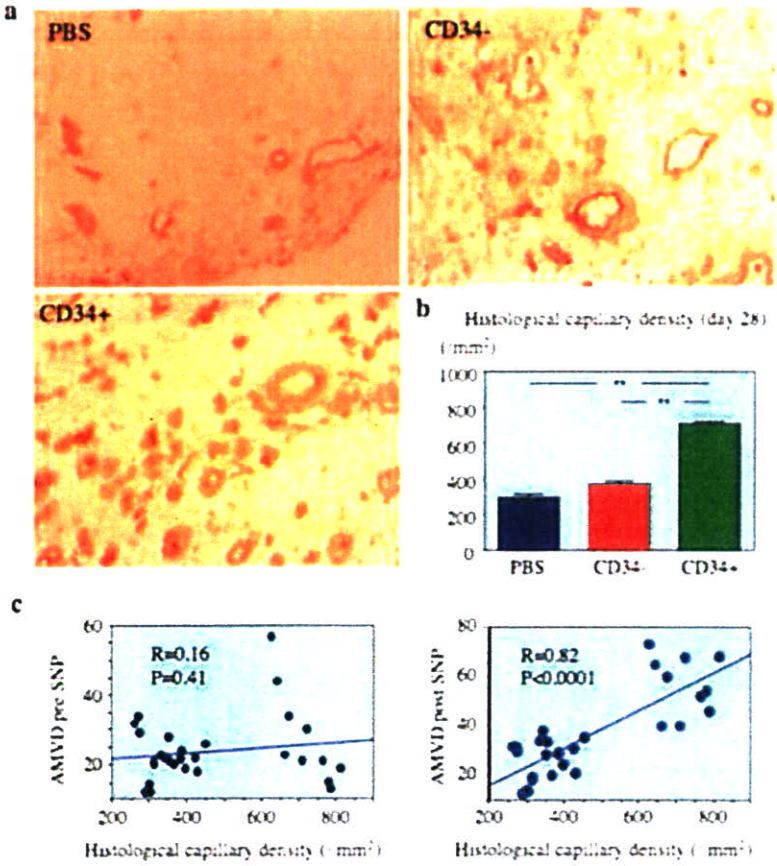

Figure 3. Histological and physiological evaluation of myocardial neovascularization after MI. a, Representative immunostaining for isolectin B4 in each group at day 28 (×200). b, Histological capillary density in rats receiving CD34+ cells, CD34− cells, or PBS at day 28. Ischemic neovascularization was significantly enhanced after CD34+ cell transplantation. $^{**}P<0.01$. c, Correlations of AMVD pre SNP or post SNP with histological capillary density 4weeks after the treatment. AMVD post SNP, but not pre SNP, closely correlated with histological capillary density.

such as MI and limb ischemia in vivo.[30,31] Although immunodeficient rats/mice provide enormous information about regenerative property of human stem/progenitor cells in the animal models of tissue ischemia, the vasculogenic/angiogenic effect of the human cells has been mainly evaluated by histological assessments because of technical limitation for physiological examinations in small animals.[15] Recently, Toyota et al[32] reported critical role of VEGF for coronary collateral growth by using micro CT. However, the micro CT can be performed only for postmortem examination, ie, not for fast beating hearts, and the spatial resolution of this method was 18 μm, which is 3 times larger than that in our novel SRM system and is not considered to be ideal for visualization of the collateral vessels.

In our SRM system, monochromatic SR is used as an x-ray source, and high speed and resolution imaging system, which has the potential to visualize blood vessels as small as 20 μm in diameter (spatial resolution: 6 μm), is also used. In the present study, we demonstrated usefulness of the SRM imaging to evaluate therapeutic neovascularization by cell-based therapy in small animals. Similarly as the previous reports,[4,7,8] histological and molecular examinations in this study confirmed endothelial differentiation and therapeutic efficacy of the transplanted CD34+ cells for augmentation of myocardial neovascularization. The SRM examination revealed that diameter of the collateral vessels was generally 20 to 120 μm, which is apparently invisible size in conventional angiography, and the collaterals were better visualized after SNP-induced vasodilatation than pre SNP. In comparison with postmortem studies such as histology, corrosion casts infusion and micro CT, it may be a great advantage of the SRM to elucidate physiology of the microvessels in response to vasoactive agents under the fast beating condition. To our knowledge, this is the first report demonstrating coronary microangiography under fast beating condition in both acute and chronic phases after MI and SCTx. Extent of collateral development was evaluated by conventional Rentrop score and novel assessment of AMVD. Although Rentrop score has been widely used in preclinical and clinical fields,[7] the examination has several limitations: (1) The scoring is semi-quantitative; (2) The system is to indirectly evaluate collateral development by grading collateral filling into the occluded coronary artery, and not to directly examine developed vascularization. Therefore, we assessed AMVD to quantitatively and directly evaluate blood vessel development as angiographic vessel density independent of blood flow in the occluded arteries. In the present study, both conventional and novel assessments revealed that collateral development and vascularization in ischemic myocardium was similar in all groups on day 5, but was significantly augmented in CD34+ cell group than other groups on day 28. Interestingly, the intergroup difference in AMVD was observed only post SNP, not pre SNP. Similarly, AMVD post SNP, not pre SNP, closely correlated with histological capillary density, which has been used for morphological evaluation of neovascularization in small animal studies. These results indicate accuracy and usefulness of AMVD post SNP for elucidating preserved vascular volume created by SCTx in fast beating

hearts of small animals, and also suggest that even in SRM with high imaging resolution, SNP infusion may be essential to avoid underestimation of the angiographic vascular density. The correlation between histological capillary density and AMVD post SNP proves the quality of AMVD to identify capillary vascular volume regenerated by SCTx. SNP infusion may increase the diameter of not only already visible vessels but also invisible capillaries (diameter <20 μm) pre SNP up to detectable size, thereby represents significant augmentation of blood perfusion in ischemic myocardium following CD34+ cell transplantation.

Present Limitations and Future Plans

The microangiographic imaging system requires a high shutter speed (short exposure time) to produce sharp and blur-free images of fast-moving hearts. In the current SRM system, the rotating disk X-ray shutter has been developed to produce X-ray pulses with the minimum pulse length of 0.1 ms, because even the beating heart is to remain almost motionless during the exposure time for ideal imaging. However, the exposure time was adjusted to around 2.0 ms in this experiment, because X-ray flux was not sufficient for the 0.1 ms shutter operation. A speed of the coronary arteries in rats is a few μm/ms at the end of diastole, ie, the movement of the arteries in 2.0 ms is several μm in the present system. On the other hand, the limiting spatial resolution of the image detector is approximately 6 μm, when digital images are acquired with a 1024×1024 pixel format, an input field of view of 4.5 mm×4.5 mm and pixel size of 4.5 μm. These facts indicate that the detector's spatial resolution is comparable to the motion blur amount in the present rat heart imaging, however there is still some room for improvement of the image quality. We are planning to develop a new X-ray optical system used for SR to increase the X-ray flux for the 0.1 ms shutter operation. Another limitation of the present study is that despite of the high quality of SRM for visualization of coronary arteries and the microvascular bed of fast beating hearts, we cannot take serial images in each individual at days 5 and 28, because they have to be examined ex vivo not in vivo. Future establishment of in vivo SRM imaging would be also warranted.

Conclusions

The present results indicate that the SRM may be useful to both morphologically and physiologically evaluate therapeutic neovascularization by SCTx in small animals. The novel imaging system may be not only an essential tool in future translational research of stem cell biology but also useful assessment of microvascular beds in small animal models of various diseases such as hypertension, diabetes mellitus, and cardiomyopathy. Further development of in vivo imaging system in future may lead to clinical application of the SRM, which is expected to be useful for assessment of microangiopathy, elucidation of therapeutic neovascularization, and determination of optimal treatment strategies in both preclinical and clinical trials.

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Disclosures

None.

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Intramyocardial Transplantation of Autologous CD34$^+$ Stem Cells for Intractable Angina

A Phase I/IIa Double-Blind, Randomized Controlled Trial

Douglas W. Losordo, MD; Richard A. Schatz, MD; Christopher J. White, MD; James E. Udelson, MD; Vimal Veereshwarayya, PhD; Michelle Durgin, MHA; Kian Keong Poh, MD; Robert Weinstein, MD; Marianne Kearney, BS; Muqtada Chaudhry, MD; Aaron Burg, BS; Liz Eaton, BS; Lindsay Heyd, BS; Tina Thorne, BS; Leon Shturman, MD; Peter Hoffmeister, MD; Ken Story, PhD; Victor Zak, PhD; Douglas Dowling, BS; Jay H. Traverse, MD; Rachel E. Olson, MS; Janice Flanagan, RN; Donata Sodano, RN; Toshinori Murayama, MD; Atsuhiko Kawamoto, MD; Kengo Fukushima Kusano, MD; Jill Wollins, MD; Frederick Welt, MD; Pinak Shah, MD; Peter Soukas, MD; Takayuki Asahara, MD; Timothy D. Henry, MD

Background—A growing population of patients with coronary artery disease experiences angina that is not amenable to revascularization and is refractory to medical therapy. Preclinical studies have indicated that human CD34$^+$ stem cells induce neovascularization in ischemic myocardium, which enhances perfusion and function.

Methods and Results—Twenty-four patients (19 men and 5 women aged 48 to 84 years) with Canadian Cardiovascular Society class 3 or 4 angina who were undergoing optimal medical treatment and who were not candidates for mechanical revascularization were enrolled in a double-blind, randomized (3:1), placebo-controlled dose-escalating study. Patients received granulocyte colony-stimulating factor 5 $\mu g \cdot kg^{-1} \cdot d^{-1}$ for 5 days with leukapheresis on the fifth day. Selection of CD34$^+$ cells was performed with a Food and Drug Administration–approved device. Electromechanical mapping was performed to identify ischemic but viable regions of myocardium for injection of cells (versus saline). The total dose of cells was distributed in 10 intramyocardial, transendocardial injections. Patients were required to have an implantable cardioverter-defibrillator or to temporarily wear a LifeVest wearable defibrillator. No incidence was observed of myocardial infarction induced by mobilization or intramyocardial injection. The intramyocardial injection of cells or saline did not result in cardiac enzyme elevation, perforation, or pericardial effusion. No incidence of ventricular tachycardia or ventricular fibrillation occurred during the administration of granulocyte colony-stimulating factor or intramyocardial injections. One patient with a history of sudden cardiac death/ventricular tachycardia/ventricular fibrillation had catheter-induced ventricular tachycardia during mapping that required cardioversion. Serious adverse events were evenly distributed. Efficacy parameters including angina frequency, nitroglycerine usage, exercise time, and Canadian Cardiovascular Society class showed trends that favored CD34$^+$ cell–treated patients versus control subjects given placebo.

Conclusions—A randomized trial of intramyocardial injection of autologous CD34$^+$ cells in patients with intractable angina was completed that provides evidence for feasibility, safety, and bioactivity. A larger phase IIb study is currently under way to further evaluate this therapy. (***Circulation.*** **2007;115:3165-3172.)**

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

Despite the optimal use of antianginal medications and mechanical revascularization, a large number of patients with coronary artery disease remain severely symptomatic with disabling angina. It is estimated that 300 000 to 900 000 patients exist in the United States alone who have exhausted conventional medical therapies and continue to experience

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From Feinberg Cardiovascular Research Institute and Program in Cardiovascular Regenerative Medicine (D.W.L., V.V., M.D., T.T.), Division of Cardiovascular Medicine, Department of Medicine, Northwestern University Feinberg School of Medicine and Northwestern Memorial Hospital, Chicago, Ill; Division of Cardiovascular Medicine (D.W.L., V.V., M.D., K.K., R.W., M.K., M.C., A.B., L.E., L.H., T.T., L.S., P.H., V.Z., J.F., D.S., T.M., A.K., K.F.K., J.W., F.W., P. Shah, P. Soukas, T.A.), Department of Medicine, Caritas St. Elizabeth's Medical Center, Boston, Mass; Division of Cardiovascular Research (R.A.S.), Scripps Green Hospital, La Jolla, Calif; Department of Medicine (C.J.W.), Ochsner Clinic, New Orleans, La; Division of Cardiology (J.E.U.), Tufts-New England Medical Center, Boston, Mass; Baxter Healthcare (K.S.), Deerfield, Ill; Division of Cardiology (J.H.T., R.E.O., T.D.H.), Minneapolis Heart Institute, Minneapolis, Minn; and Cardiovascular Core Laboratories Inc (D.D.), Boston, Mass.

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Correspondence to Douglas W. Losordo, MD, Feinberg Cardiovascular Research Institute and Northwestern Memorial Hospital, Tarry 12-703, 303 E Chicago Ave, Chicago, IL 60611. E-mail d-losordo@northwestern.edu

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angina,[1] with 100 000 to 200 000 new cases per year.[2] The development of new therapies for this patient population therefore represents a suitable therapeutic target.

Laboratory and preclinical studies have provided evidence of the safety and potential efficacy of a strategy of intramyocardial transplantation of autologous CD34$^+$ stem cells for neovascularization of chronically ischemic myocardium.[3–6] Accordingly, we performed a pilot, first-in-human study to evaluate the safety and bioactivity of this approach in patients with coronary artery disease and intractable angina.

Methods

Study Design

This was a phase I/IIa, double-blind, placebo-controlled, randomized clinical trial. Patients were enrolled into 1 of 3 dose cohorts (5×10^4, 1×10^5, and 5×10^5 CD34$^+$ cells/kg) versus placebo. All patients in all treatment groups, including placebo, received granulocyte colony-stimulating factor (GCSF) at a dose of 5 $\mu g \cdot kg^{-1} \cdot d^{-1}$ for 5 days. We chose a low dose in the present study on the basis of the published dose-response relationship of the drug, which showed good mobilization at the 5-μg/kg dose, and out of a desire to approach these patients with extensive coronary artery disease as conservatively as possible given prior data indicating that higher doses could be associated with an increased possibility of significant side effects.[7] Leukoapheresis was performed on the fifth day (Amicus, Baxter Healthcare, Deerfield, Ill) for collection of mononuclear cells. The cells were stored overnight at 4°C, and the following morning, the CD34$^+$ fraction was purified on a commercially available device (Isolex 300i, Baxter Healthcare) according to the manufacturer's instructions. Cells were then subjected to testing and were required to meet lot-release criteria that included sterility, viability, absence of endotoxin, and CD34 cell content. Once cells passed lot-release criteria, the patients underwent NOGA electromechanical mapping and intramyocardial injection of CD34$^+$ cells (suspended in saline plus 5% autologous serum; serum was added to support cell viability) versus cell diluent (saline plus 5% autologous serum) using the NOGA Myostar catheter (Biosense Webster, Diamond Bar, Calif).[8] Randomization codes were established by the study statistician and were revealed only to the stem cell laboratory technician responsible for separating the cells into aliquots or preparing the placebo material. The dose was divided into 10 injections of 0.2 mL per injection. Patients were discharged from the hospital the day after the injection procedure. Follow-up occurred at 1, 2, and 4 weeks and at 2, 3, 6, 9, and 12 months. Crossover was permitted if the patient met study entry criteria after 6 months of follow-up. The present report is restricted to the results of the initial randomized study population.

Patient Population

Patients included for enrollment were required to be >21 years old with functional Canadian Cardiovascular Society (CCS) class III or IV angina; to have attempted "best" medical therapy, including long-acting nitrates, maximal use of β-adrenergic blocking agents, and calcium channel agents, without control of symptoms; and to be taking at least 2 antianginal medications. Patients were required to be considered noncandidates for conventional revascularization by the referring cardiologist, and an independent interventional cardiologist and cardiac surgeon reviewed the most recent (within 6 months) angiogram to verify ineligibility for revascularization. Patients were also required to have ischemia on nuclear perfusion imaging, to complete at least 1 minute but no more than 6 minutes of a standard Bruce protocol, and to experience angina/angina equivalent during the baseline exercise test. Key exclusion criteria included myocardial infarction within 30 days of treatment; successful coronary revascularization within 3 months of enrollment; documented transient ischemic attack within 60 days of treatment; severe aortic stenosis (aortic valve area <1.0 cm^2) or insufficiency (>2+); severe mitral stenosis or severe mitral insufficiency; predominant congestive heart failure symptoms; severe comorbidities associated with a reduction in life expectancy to less than 1 year; uncontrolled hypertension; joint disease, peripheral vascular disease, or chronic obstructive pulmonary disease that would limit walking on the treadmill; and patients with clinical evidence of a neoplasm within the last 5 years (other than nonmelanoma skin cancer or in situ cervical carcinoma).

End Points

Safety

An independent data safety monitoring board was assembled to review safety data in a timely manner. In addition to routine physical examination and laboratory testing, patients were also monitored with ECG and transthoracic echocardiography at routine intervals. Echocardiography was performed immediately after the injection procedure and before hospital discharge in all subjects.

Arrhythmia Monitoring

All patients were required to have in implanted cardioverter-defibrillator already in place or to wear a temporary device (LifeVest) for 1 week before and 4 weeks after the injection procedure. Patients also underwent 24-hour Holter monitoring before and 1 week and 3, 6, and 12 months after injection. Patients with LifeVests did not have 1-week Holter monitoring, and those with implantable cardioverter-defibrillators did not undergo Holter monitoring.

Efficacy

Bioactivity was assessed according to the following parameters: angina frequency, nitroglycerine (NTG) use, exercise tolerance (standard Bruce protocol), CCS class, single-photon emission computed tomography (SPECT) imaging (assessed at a core laboratory by J.U.), and quality-of-life testing.

Statistical Analysis

Because this was a first-in-human study, no prior data were available on which to base power calculations. Accordingly, results are presented as change from baseline in patients assigned to placebo versus those assigned to cell injection. Because power calculations to determine sample size were not done, we do not show probability values, which would imply that a goal of this study was to document efficacy in this study. As a phase I/IIa study, a statistical assessment of efficacy is not the goal, and even if certain parameters revealed "significant" improvement, we believed that it would not be correct to display them, because these were not the prespecified aims of the study. Because crossover of placebo-assigned patients was permitted after 6 months, the analysis of efficacy parameters is restricted to 6 months after initial treatment assignment.

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

Results

Patients

Twenty-four patients, including 5 women and 19 men with a mean age of 62.4 (range 48 to 84) years, were enrolled at 3 centers in the United States from December 2003 through March 2005. Because no dose-response effect was observed, data are presented as CD34$^+$ stem cell–treated patients versus controls (Table 1).

Safety Analysis

Thirteen patients (54.2%) reported that their angina was transiently increased in frequency after the administration of GCSF. Patients were instructed that an increase in angina might occur after administration of GCSF as a result of increasing blood viscosity, metabolic demand, or increased platelet counts. The patient-reported increase in frequency and severity

TABLE 1. Baseline Characteristics by Treatment Group

		CD34⁺ Cells/kg		
	Placebo	5×10^4	1×10^5	5×10^5
Hyperlipidemia	4 (66)	6 (100)	5 (83)	6 (100)
Hypertension	3 (50)	5 (83)	5 (83)	4 (66)
Smoking history	5 (83)	6 (100)	3 (50)	4 (66)
Congestive heart failure	2 (33)	3 (50)	1 (17)	2 (33)
Myocardial infarction	3 (50)	3 (50)	4 (66)	4 (66)
Peripheral vascular disease	2 (33)	3 (50)	1 (17)	3 (50)
Diabetes mellitus	3 (50)	1 (17)	2 (33)	4 (66)
ACE inhibitor	3 (50)	3 (50)	5 (83)	4 (67)
β-Blocker	6 (100)	6 (100)	5 (83)	6 (100)
Statin	5 (83)	6 (100)	6 (100)	4 (67)
Prior PCI procedure(s)	6 (100)	4 (66)	6 (100)	5 (83)
Prior CABG surgery	4 (66)	6 (100)	6 (100)	5 (83)
Prior AICD implantation	2 (33)	1 (17)	2 (33)	0 (0)
Prior TMR or EECP	0 (0)	0 (0)	1 (17)	5 (83)

Values are expressed as n (%). ACE indicates angiotensin-converting enzyme; PCI, percutaneous coronary intervention; CABG, coronary artery bypass grafting; AICD, automated implantable cardioverter-defibrillator; TMR, transmyocardial revascularization; and EECP, enhanced external counterpulsation.

of angina occurred as early 1 day after the initiation of GCSF and persisted in some individuals until the day of apheresis. The only pattern that emerged was the return to baseline symptom frequency after apheresis. The increase in angina was manageable in all patients with the use of sublingual NTG. There were no cardiac enzyme elevations, myocardial infarctions, acute coronary syndromes, or deaths, some of which had occurred after administration of higher doses of GCSF in similar patient populations.[9] Because no GCSF control was available, we are unable to determine whether the reported change in symptom pattern was specifically drug related.

Serious adverse events (Table 2) were distributed among the treatment groups. One patient in the placebo group developed ventricular tachycardia during the mapping procedure. This individual had a history of sudden cardiac death and had a previously implanted cardioverter-defibrillator. The patient was cardioverted successfully, and the remainder of the mapping procedure and injections were performed without incident. No further sustained ventricular arrhythmia occurred in this patient, and no arrhythmias were detected by implantable cardioverter-defibrillator, LifeVest, or Holter monitoring in any patient during or after the injection procedure.

TABLE 2. Incidence of Serious Adverse Events

		CD34⁺ Cells/kg		
	Placebo	5×10^4	1×10^5	5×10^5
Atrial arrhythmia	2 (33)	1 (17)	1 (17)	0
Ventricular arrhythmia	1 (17)	0	0	0
Angina exacerbation	3 (50)	2 (33)	3 (50)	2 (33)
Congestive heart failure	0	0	3 (50)	0
Respiratory arrest	0	2 (33)	0	0
Cerebrovascular accident	0	0	0	1 (17)
Peripheral vascular claudication	0	1 (17)	1 (17)	0
Bleeding/anemia	0	2 (33)	1 (17)	0
Renal insufficiency	2 (33)	0	2 (33)	0
Gastrointestinal complication	1 (17)	0	1 (17)	0
Endocrine/electrolyte disorder	1 (17)	0	2 (33)	1 (17)

Values are n (%).

Efficacy Analysis

Angina Frequency

At baseline, patients in the placebo group were experiencing 20.5±11.5 episodes of angina compared with 21.2±16.1 episodes of angina per week in the treatment group. At 3 months after injection, the frequency of angina was increased in the placebo group and decreased in the active treatment group (3 months: 27.0±23.8 episodes in the placebo group versus 9.6±13.3 in the treated group). At 6 months after injection, the frequency of angina was reduced in both placebo and CD34⁺ cell–treated patients (6 months: 16.0±19.3 versus 8.6±10.3 episodes). At both time points, the CD34⁺ stem cell–treated patients experienced a greater magnitude of reduction of symptoms (change from baseline: control, 3 months 6.5±15.2 and 6 months −4.5±20.1; CD34⁺ cell treatment, 3 months −11.6±19.5 and 6 months −12.6±18.2; Table 3; Figure 1A).

NTG Use

At 3 and 6 months after injection, NTG use in the placebo group was increased compared with baseline (+8.8±20.7 and +4.8±37.9), whereas the CD34⁺ stem cell–treated patients used less NTG at both time points (−9.8±10.8 and −8.1±14.7; Figure 1B; Table 3). The increase in NTG use in the control population, juxtaposed against a decrease in angina, may be a reflection of better utilization of NTG as a result of patient education in the course of trial participation.

Exercise Tolerance

At 3 months after injection, exercise time on the standard Bruce protocol was improved in placebo and active treatment groups compared with baseline (+0.3±2.1 and +0.5±1.3 minutes, respectively; Figure 1C). The CD34+ stem cell–treated patients experienced a slightly greater magnitude of improvement in exercise time.

CCS Class

At 3 and 6 months after injection, the mean CCS class was reduced in the placebo and active treatment groups (3 months: −0.05±1.2 placebo versus −1.1±0.8 treated; 6 months: −0.8±1.7 placebo versus −1.4±1.0 treated; Figure 1D; Table 3). The CD34+ stem cell–treated patients experienced a greater magnitude of reduction of CCS class at both time points. In addition, the percentage of patients experiencing at least a 2–CCS class decrease was greater in the CD34⁺ cell–treated patients than in controls (16.7% decrease in placebo group versus 27.8% in treated group at 3 months and 33.3% decrease in placebo group versus 50% in treated group at 6 months).

SPECT Perfusion Imaging

SPECT imaging at 3 and 6 months after injection yielded inconsistent findings. For example, the automated summed

TABLE 3. Change in Angina, NTG, and CCS at 3 and 6 Months After Injection

Treatment Group and Maximum CD34⁺ Count/μL*	Maximum WBC Count ×10³/mm³	Angina Change From Baseline		NTG Change From Baseline		CCS Change From Baseline		ETT Change From Baseline at 3 Months
		3 Months	6 Months	3 Months	6 Months	3 Months	6 Months	
Placebo								
11	11.4	0	25	0	25	0	1	−0.3
10	25.5	−15	−15	−25	−25	−3	−3	4.1
13	35.8	15	−34	15	−34	0	−3	1.2
13	13.2	2	−1	4	−3	0	0	−2.1
51	33.8	7	7	31	−4	0	0	−0.9
39	24.7	30	−9	28	70	0	0	−0.3
50 000 C/kg								
41	36.6	−45	−43	−20	−25	−1	−2	1.2
38	36.6	−6	−7	−2	−4	−2	−2	0.0
21	34.7	−6	−9	−6	−9	−1	−1	−0.4
32	29	−11	−8	0	4	−1	−1	0.6
.	17.8	−8	−14	−14	−20	0	−3	2.0
8	20.8	−9	−12	−12	−13	−1	−2	3.1
100 000 C/kg								
24	16.7	−34	−28	−34	−27	−1	−1	−0.5
20	22.9	−10	−10	−10	−10	−1	−1	−0.3
40	45.8	−7	−7	0	0	−2	−2	−0.4
47	27.3	−3	7	−1	9	−1	−2	−0.6
67	10.2	−21	−18	−5	23	−2	0	2.8
45	33.6	31	17	−2	−1.5	0	0	−2.0
500 000 C/kg								
6	16.8	−24	−23	−24	−23	−1	−1	−0.3
38	27.4	−11	−11	−8	−8	−3	−3	0.8
31	20.9	24	24	0	2	0	0	0.0
37	15.2	.	.	.	.	−2	−2	2.2
28	24.9	−43	−45	0	0	0	0	−0.6
28	29.3	−14	−28	−28	−35	−1	−2	1.3

WBC indicates white blood cell; ETT, exercise tolerance test; and C/kg, CD34⁺ cells per kilogram of body weight dose administered by intramyocardial injection.
*Days 4 and 5 of mobilization.

difference score was improved in both treatment groups at both time points, with a slightly greater improvement noted in the cell- versus placebo-treated patients at both time points (−1.5±1.6 in placebo versus −2.4±4.4 in CD34⁺ cell–treated patients at 3 months and −0.7±2.0 versus −2.3±3.1 at 6 months; Figure 2). In contrast, the visually estimated summed difference score revealed improvements compared with baseline in both treatment groups and a slightly greater improvement in CD34⁺ cell–treated patients at 3 months(−0.8±1.5 placebo versus −2.5±3.3 treated), with this trend being reversed at 6 months (−2.2±3.4 versus −1.5±4.1; Figure 2).

Quality-of-Life Testing

Seattle Angina Questionnaire assessment of physical limitation, angina stability, angina frequency, disease perception, and treatment satisfaction revealed improvements in both treatment groups (Figure 3). At 3 months, all 5 parameters favored the CD34⁺ cell–treated patients, and at 6 months, 4 of 5 parameters showed trends favoring the cell- versus placebo-treated patients compared with baseline.

Discussion

This double-blind, placebo-controlled trial of intramyocardial injection of autologous CD34⁺ stem cells in patients with intractable angina provides preliminary evidence for the safety of this approach.[10] Evaluation of bioactivity reveals trends in favor of the cell-treated patient in most of the outcome measures. Together, these outcomes describe a successful first-in-human study and serve as the basis for proceeding with a larger phase IIb study, which is under way. The findings also raise many questions, most of which cannot be answered but which are nevertheless important to pose as the field of stem cell therapy continues to evolve.

Two parameters appeared worse in the placebo group at the 3-month follow-up visit: angina frequency and NTG use. At 6 months, angina frequency in the placebo-treated patients was reduced compared with baseline, whereas NTG use

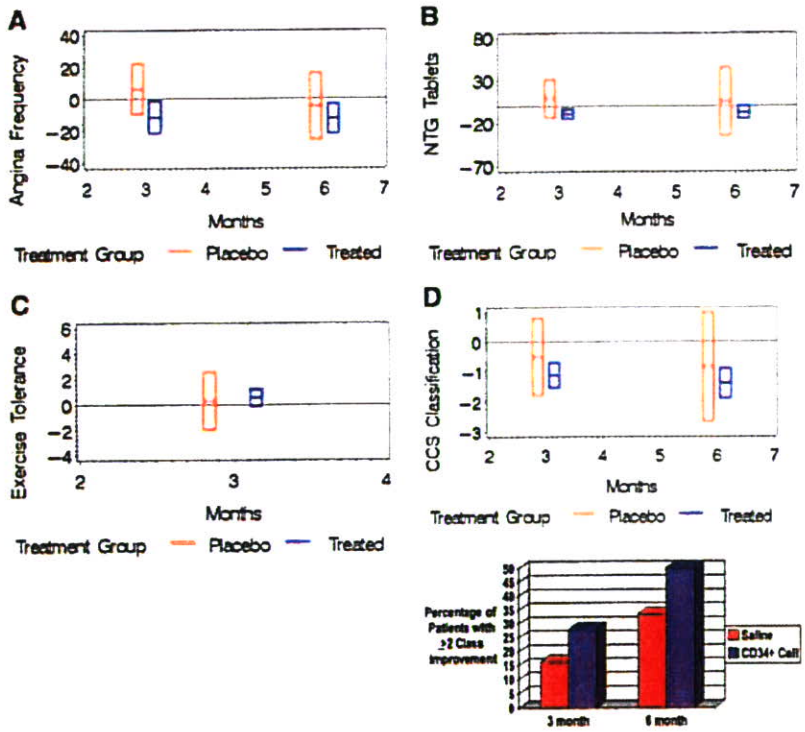

Figure 1. Clinical symptomatic end points. All data are shown as means and 95% confidence limits. A, Angina frequency: patient-reported frequency of angina, at baseline and 3 and 6 months after injection of placebo (red) or $CD34^+$ cells (blue). B, NTG consumption: tablets per week, at baseline and 3 and 6 months after injection of placebo (red) or $CD34^+$ cells (blue). C, Exercise tolerance: total exercise time on the standard Bruce protocol in placebo (red)-injected or $CD34^+$ cell (blue)–injected patients. D, CCS angina classification. Top, CCS class at baseline and 3 and 6 months after injection. Bottom, Percentage of patients with a ≥2 class improvement compared with baseline.

remained higher. In contrast, exercise tolerance, CCS class, and the Seattle Angina Questionnaire parameters of angina frequency, physical limitation, disease perception, and treatment satisfaction all improved in the placebo group at 3 months. Taking into account the small sample size, we must be vigilant for the possibility that placebo patients may experience an exacerbation in symptoms during the study, although most of the evidence suggests a positive placebo effect. The placebo protocol involves administration of GCSF and intramyocardial injection of a solution containing autologous serum, either of which could present theoretical risks.[9]

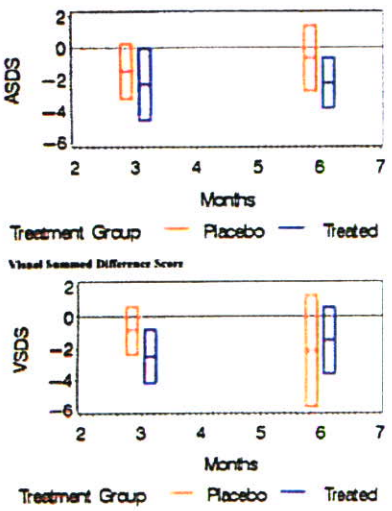

Figure 2. SPECT perfusion imaging. All data are shown as means with 95% confidence limits. Top, Change in automated summed difference score (ASDS) from baseline to 3- and 6-month follow-up. Bottom, Change in visually estimated summed difference score (VSDS) from baseline to 3- and 6-month follow-up.

Two fundamental questions must be addressed: What is the mechanism of the disease, and what is the proposed mechanism of $CD34^+$ stem cell therapy? The answer to the first question is critical before answering the second question and is not as self-evident as it might initially seem. Epicardial coronary disease is the underlying disease process in the target patient population; however, progressive angina and heart failure are known to occur in patients despite no apparent change in the epicardial coronary anatomy. Mounting evidence, both old and new, suggests that attrition of the myocardial microvasculature occurs progressively in the ischemic myocardium, which compounds the loss of macrovascular supply, and that interventions that protect or restore microvascular integrity can improve perfusion and function without altering the macrovascular circulation.[11–16] Data from preclinical models indicate that $CD34^+$ cells restore the microcirculation and improve myocardial tissue perfusion and do so despite the permanent occlusion of the epicardial vessel.[5,6]

The next question about the mechanism of cell therapy revolves around whether the cells participate in new vessel formation or induce neovascularization by elements within the tissue and circulating blood via paracrine effects. The literature and data from our laboratory provide evidence for both phenomena.[17] Most interesting among these data are the evidence that the endothelial progenitor cell (EPC) has a phenotype that drives its potency for neovascularization.[18,19] These data indicate that cells from patients with severe cardiovascular disease are not as functional as those collected from healthy volunteers. These findings have several implications. Clinicians will focus on identifying the specifics of the cell phenotype that define potency, thereby providing the opportunity to enhance outcome in cell-based strategies. Preclinical studies have already revealed the potential of this

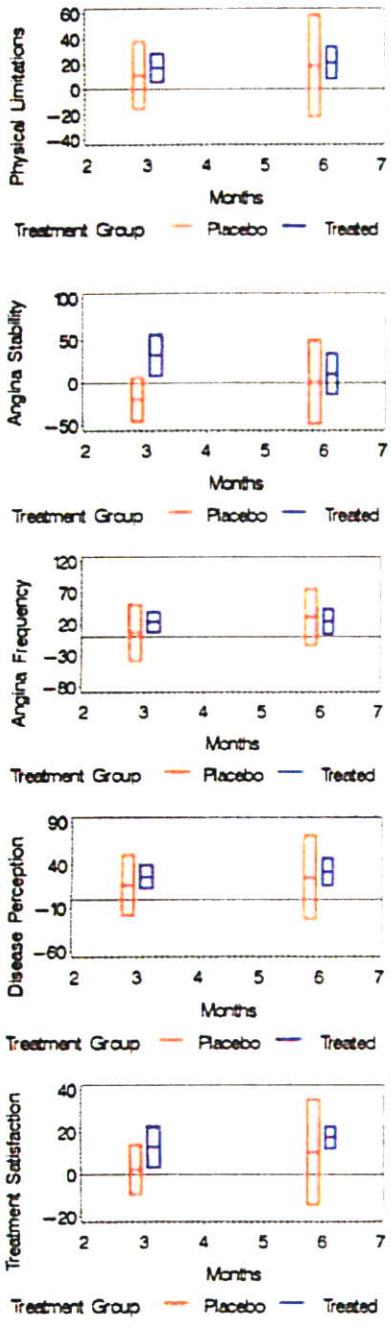

Figure 3. Seattle Angina Questionnaire assessment: change from baseline at 3 and 6 months for the Seattle Angina Questionnaire assessment of physical limitations, angina stability, angina frequency, disease perception, and treatment satisfaction. An increased score indicates functional improvement. Data are represented as means with 95% confidence limits.

strategy.[20] The broader scientific appeal of the link between the EPC and endothelial function is the access that these circulating cells provide to a virtually unattainable material: human endothelium. Multiple investigators have already taken advantage of the EPC as a surrogate for the human endothelium, making observations linking the kinetics and biology of these cells with important cardiovascular disease risk factors and outcome. It is probably only a matter of time before an EPC-based metric will be used as part of cardiovascular disease risk profiling.

The choice of the $CD34^+$ for this therapy was based on extensive preclinical data indicating that surface expression of CD34 identified a population of cells with enhanced potency for neovascularization of ischemic tissue.[3,6,21] Other methods of enriching the population of EPCs have been established, most notably the use of cell culture, with efficacy shown in preclinical studies and more recently in clinical trials.[4,22–24] We chose $CD34^+$ selection on the basis of our preclinical data that indicated enhanced efficacy and safety with $CD34^+$ versus unselected cells[5,6] and because a device was commercially available that could be used to purify the $CD34^+$ cells from patients, thereby obviating the requirement for a current Good Manufacturing Practices facility at each treatment site. Accordingly, the availability of an autologous stem cell with potency for therapeutic neovascularization that could be produced practically at any hospital indicated that this strategy, if successful, could be applied practically on a large scale. We considered this last feature (ie, broad applicability), to be a key feature before considering the initiation of clinical trials. In addition, the possibility that mobilization via administration of GCSF could have an independent therapeutic effect must be considered, although GCSF alone did not improve perfusion in preclinical models.[25] This possibility, along with the desire to maintain study blinding, was part of the reason that GCSF was administered to the control population. A relationship was not detected between the degree of $CD34^+$ cell mobilization and any outcome measures; however, this possibility must be considered as studies with larger sample sizes are performed.

The selection of doses of $CD34^+$ cells was based on the results of preclinical studies.[6] The lowest dose was not optimally therapeutic in the animal studies and was chosen as the starting dose in this first-in-human study to begin to establish a safety profile in a conservative manner. The higher doses represented the doses at which the benefit in the animal models appeared to plateau (ie, higher doses did not provide evidence of enhanced benefit). In our pilot study, no dose-response effect was observed. Setting aside for the moment the obvious fact that the study was not powered to detect a dose response, a discussion of dosing is important. Preclinical models from which data substantiating safety and efficacy are derived are performed in young, healthy animals in which the disease is induced and treated in the course of weeks. Thus, although it is a necessary guidepost for the initiation of clinical trials, the calculation of dose must be reexamined continuously in the context of the patient population. It appears unlikely that a single administration of any dose of cells will completely reverse a disease process that has progressed over the course of decades. A single administration is a necessary starting point for a safety evaluation, but it appears likely to be replaced by an incremental approach if single administration provides evidence of partial improvement.

The present study approaches symptom relief in a patient population for whom a successful therapy has not yet been developed. Accordingly, no precedent exists on which to base clinical trial design. A significant reduction in symptoms in

treated versus control patients is imperative, but thus far, symptom relief has not been sufficient for approval of antianginal therapies. This philosophy is applied inconsistently (analgesics are routinely approved on the basis of relief of pain) and perhaps must be challenged as the population with severe coronary disease continues to expand. Nevertheless, another measure of efficacy, providing evidence of a biological activity, would be desirable. Exercise testing has been widely used as a surrogate for symptomatic improvement in studies of antianginal therapies; however, several characteristics distinguish these prior studies and investigations of the intractable angina population. Antianginal medications were developed in the preangioplasty era and were therefore tested in a younger, healthier population, many of whom had single-vessel disease. The target population of intractable angina therapies is older and has a preponderance of multivessel disease, prior myocardial infarction, and prior (sometimes multiple) bypass surgeries. Accordingly, the ability to increase exercise performance in this population may be restricted by other factors, which limits the potential utility of the exercise tolerance test. In addition, some prior studies in the intractable angina population have shown increases in exercise time in the placebo group of nearly 1 minute.[26] This fact is particularly interesting when viewed in the context of a randomized, controlled trial of PTCA versus best medical therapy.[27] In an unblinded study of relatively young, single-vessel disease patients, the PTCA-treated patients experienced a 90-second improvement in total exercise time compared with those randomized to the control arm.[27] If we factor in the placebo effect, documented to increase exercise time by 30 to 60 seconds in blinded studies, the impact of PTCA (a therapy that is applied as a standard of care to alleviate angina) on *total* exercise time, even in relatively healthy patients, is apparently quite modest.

SPECT imaging would appear to be a logical candidate to provide objective evidence for neovascularization. Our expectations in this regard are tempered, however, by the fact that SPECT imaging has been validated primarily for detection of epicardial disease and is particularly suited for detecting gradients in perfusion that result from disease of 1 or 2 vessels. Thus, although SPECT imaging, as a standard clinical modality, is being applied in most clinical trials of neovascularization, the tool is being scrutinized to determine its suitability for accurately assessing outcome.

Both positron emission tomography–computed tomography and magnetic resonance imaging offer theoretical advantages for assessment of perfusion and function but remain experimental in these applications. In addition, we are hopeful that molecular imaging techniques, capable, for example, of noninvasively quantifying new vessel formation, may offer the precise assessment of a meaningful biological end point that would enable confident assessment of our attempts at microvascular regeneration.[28]

Recently, published studies by Assmus[23] and Schachinger[24] have provided evidence for the therapeutic potency of cultured EPCs for the treatment of ischemic disease. The present data add to this evidence that the $CD34^+$ stem cell, isolated from the circulating blood, can be safely transplanted via intramyocardial injection and may improve perfusion and reduce symptoms in patients with advanced coronary disease who have exhausted the currently available therapeutic armamentarium.

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Disclosures

The study protocol was submitted to the Food and Drug Administration by Dr Losordo as the study sponsor. The data were collected and stored in a database at Caritas St. Elizabeth's Medical Center, and the investigators had full access to the data at all times. Baxter assisted with analysis of the locked data set. Drs Losordo, Henry, and Schatz are consultants to Baxter. Ken Story is an employee of Baxter Healthcare.

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CLINICAL PERSPECTIVE

This report details a first-in-human experience with intramyocardial, transendocardial transplantation of autologous CD34$^+$ cells for intractable angina. The study design was based on preclinical data that provided evidence that selected CD34$^+$ cells were safer and more effective for revascularization of ischemic myocardium. The target population in this study, with a condition estimated to have an annual incidence in the United States of 150 000 to 250 000 per year, included patients with class III and class IV angina refractory to medical treatment and not amenable to revascularization. Features that distinguish this trial from previous reports include the fact that (1) it was performed in the United States, (2) its patients had chronic ischemia, and (3) selected stem cells collected from the peripheral circulation were used (versus bone marrow mononuclear cells in most other reports). This pilot study provided evidence for safety and feasibility and also revealed trends that favor the treatment versus control in most of the parameters assessed. As a pilot study, the trial was not powered for efficacy assessment. Together, these findings supported the initiation of a phase IIb study of 150 patients that is under way. In that study, planned for 150 patients, the lowest dose used here was not included, and the trial is powered, on the basis of the data from the present study, to detect statistically significant differences in reduction in angina.

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VASCULAR BIOLOGY

Basic Science Review

Role of Progenitor Endothelial Cells in Cardiovascular Disease and Upcoming Therapies

Atsuhiko Kawamoto,[1,2*] MD and Takayuki Asahara,[1,2,3*] MD

The field of cell-based transplantation has expanded considerably and is poised to become an established cardiovascular therapy in the near future. In this review, we will focus on endothelial progenitor cells (EPCs), which are immature cells capable of differentiating into mature endothelial cells. EPCs share many surface marker antigens such as CD34, AC133, Flk-1, etc. with hematopoietic stem cells (HSCs) and the major source of EPCs as well as HSCs is the bone marrow (BM). BM-derived EPCs are mobilized into peripheral blood and recruited to the foci of pathophysiological neovascularization and reendothelialization, thereby contributing to vascular regeneration. Severe EPC dysfunction is an indicator of poor prognosis and severe endothelial dysfunction. Indeed, number of circulating EPCs and their migratory activity are reduced in patients with diabetes, coronary artery disease (CAD), or subjects with multiple coronary risk factors. Effective neovascularization induced by EPC transplantation for hindlimb, myocardial, and cerebral ischemia has been demonstrated in many preclinical studies, and early clinical trials of EPC transplantation in chronic and acute CAD indicate safety and feasibility of myocardial cell-based therapies. For therapeutic reendothelialization in patients undergoing percutaneous coronary intervention, CD34 antibody-coated stents have been used clinically to capture circulating EPCs at the injury sites and enhance reendothelialization and safety of stents. Further development in cell processing technology for efficient isolation, expansion, mobilization, recruitment, and transplantation of EPCs into target tissues are underway and expected to be tested in clinical trials in the near future. © 2007 Wiley-Liss, Inc.

Key words: endothelial progenitor cell; regeneration; vasculogenesis

INTRODUCTION

The field of cell-based transplantation has expanded considerably over the recent years and is poised to become an established cardiovascular therapy in the near future. Cell-based therapy to promote angiogenesis and myogenesis has already reached catheterization laboratories worldwide in the format of clinical studies involving patients with both cardiac and peripheral vascular disease. The aim of this review is to familiarize clinicians with this upcoming therapeutic modality. We will focus on endothelial progenitor cells (EPCs), which are immature cells capable of differentiating into mature endothelial cells (ECs). These progenitors have been subject of intense experimental and clinical investigation.

EPCs IN THE ADULT

EPCs are immature cells, which have the capacity to proliferate, migrate, and differentiate into endothelial lineage cells but have not yet acquired characteristics of mature ECs. Developmental biology research disclosed that embryonic hematopoietic stem cells (HSCs) and EPCs are derived from a common precursor (hemangioblast) and share many surface marker antigens such as

[1]Vascular Regeneration Research Group, Institute of Biomedical Research and Innovation, Kobe, Japan
[2]Laboratory for Stem Cell Translational Research, RIKEN Center for Developmental Biology, Kobe, Japan
[3]Department of Regenerative Medicine Science, Tokai University School of Medicine, Isehara, Japan

*Correspondence to: Atsuhiko Kawamoto, MD or Takayuki Asahara, MD, Vascular Regeneration Research Group, Institute of Biomedical Research and Innovation, 2-2 Minatojima Minamimachi, Chuo-Ku, Kobe 650-0047, Japan. E-mail: akawamoto@cdb.riken.jp or asa777@aol.com

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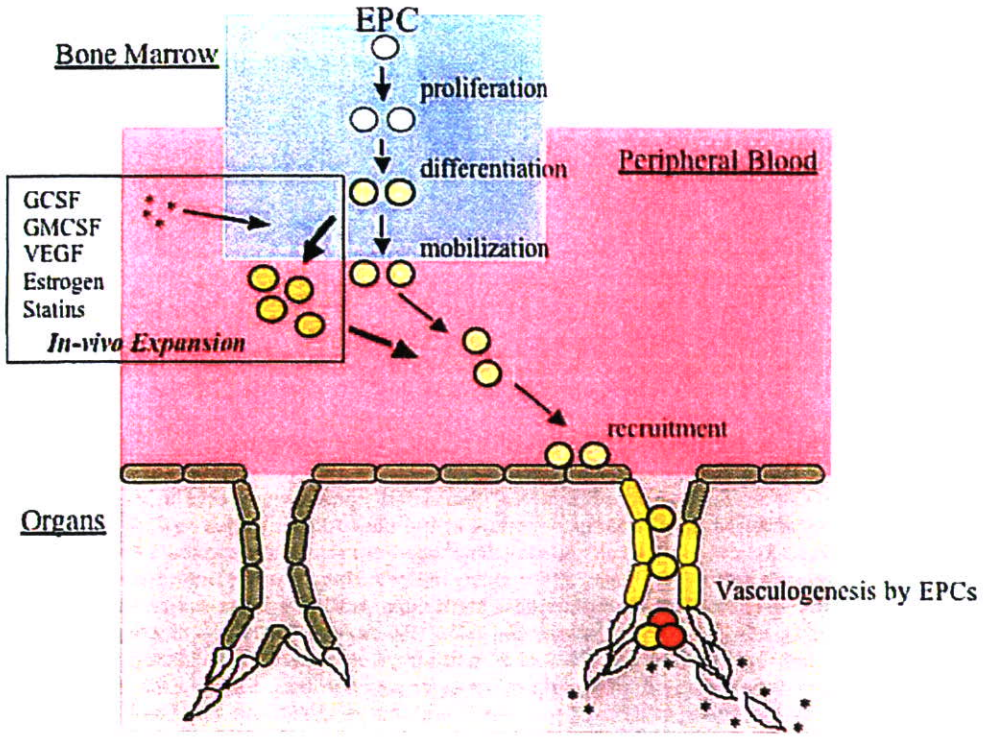

Fig. 1. Kinetics of EPCs for postnatal neovascularization. Circulating EPCs mobilized from BM are recruited into foci of neovascularization and contribute to new blood vessel formation. The mobilization process can be augmented by certain cytokines, growth factors and pharmaceutical agents. GCSF, granulocyte colony stimulating factor; GMCSF, granulocyte macrophage colony stimulating factor; VEGF, vascular endothelial growth factor.

Flk-1, Tie-2, c-Kit, Sca-1, AC133, and CD34 [1–3]. A decade ago, postnatal EPCs were first identified as CD34 antigen-positive ($CD34^+$) mononuclear cells (MNCs) in adult human peripheral blood (proportion of $CD34^+$ cells is generally 0.1–2% of total MNCs in bone marrow (BM), peripheral blood or cord blood) [4]. In vitro, these cells differentiated into endothelial lineage cells, and in animal models of ischemia, heterologous, homologous, and autologous EPCs were shown to incorporate into sites of active neovascularization. This finding was followed by diverse identifications of EPCs using equivalent or different methodologies by several groups [5–9]. Because adult EPCs share many markers with HSCs as in the embryonic situation, no simple definition of EPCs by strictly specific marker expression exists. The term EPC may therefore encompass a group of cells that exist in a variety of stages ranging from hemangioblasts to fully differentiated ECs. Their high proliferation rate distinguishes circulating EPCs in the adult from mature ECs shed from the vessel wall.

EPC KINETICS AND ITS MODULATION FOR PATHOPHYSIOLOGICAL NEOVASCULARIZATION

Following identification of adult EPCs, physiological and pathological role of EPCs have been studied. Given the result of common antigenicity, BM has been considered to be the origin of EPCs as well as HSCs in the adult. BM transplantation (BMT) experiments have demonstrated the incorporation of BM-derived EPCs into foci of pathological neovascularization such as growing tumor, healing wound, ischemic skeletal and cardiac muscles, and cornea receiving micropocket surgery. Similar incorporation was observed in physiological neovascularization in uterus endometrial formation following induced ovulation as well as estrogen administration [10]. These findings suggest that one of the main source of adult EPCs is BM, and EPCs are mobilized from BM into peripheral blood as an endogenous response to pathophysiological demand of neovascularization. Mobilized EPCs in the circulation are recruited into foci of neovascularization and contribute to new blood vessel formation (Fig. 1). These basic findings were comfirmed by clinical observation of EPC mobilization in patients undergoing coronary artery bypass grafting and patients with burns [11] and acute myocardial infarction [12].

DRUG-INDUCED EPC MOBILIZATION

Having demonstrated the potential for endogenous mobilization of BM-derived EPCs, we considered that

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iatrogenic expansion and mobilization of this putative EC precursor population might represent an effective means to augment postnatal neovascularization. Granulocyte macrophage-colony stimulating factor, which stimulates hematopoietic progenitor cells and myeloid lineage cells, as well as nonhematopoietic cells including BM stromal cells and ECs, has been shown to exert a potent stimulatory effect on EPC kinetics: mobilization from BM, incorporation into sites of neovascularization, and proliferation and differentiation in culture. Such cytokine-induced EPC mobilization could enhance neovascularization of severely ischemic tissues as well as de novo corneal vascularization [13]. Recent data indicate that vascular endothelial growth factor (VEGF), the most-critical factor for vasculogenesis and angiogenesis [14–16], is an important factor for the mobilization of EPCs from BM as well. Our studies performed first in mice [17] and subsequently in patients undergoing VEGF gene transfer for critical limb ischemia [18] and myocardial ischemia [19] established that a previously unappreciated mechanism by which VEGF contributes to neovascularization is via mobilization of BM-derived EPCs.

EPC kinetics modulation has been observed in response to other hematopoietic stimulators, such as granulocyte-colony stimulating factor (G-CSF) [9], angiopoietin-1 [20], and stromal cell derived factor-1 [7]. The therapeutic strategy of EPC mobilization has recently been implicated not only by natural hamatopoietic or angiogenic stimulants but also by recombinant pharmaceuticals. Statins inhibit the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the synthesis of mevalonate, a rate-limiting step in cholesterol biosynthesis. Statins rapidly activate Akt signaling in ECs, thereby stimulate EC bioactivity in vitro and enhance angiogenesis in vivo [21]. Our group [22] and Dimmeler et al. [23,24] demonstrated novel function of HMG-CoA reductase inhibitors that contributes to postnatal neovascularization by augmented mobilization of BM-derived EPCs through stimulation of the Akt signaling pathway. The use of G-CSF to mobilize EPCs is routinely used in hematology and oncology practices, and has also been tested safely in patients undergoing primary angioplasty for ST elevation myocardial infarction [25].

EPCs IN CARDIOVASCULAR DISEASES

Recent experimental data raised important questions regarding fundamental concepts of blood vessel growth and development in adult subjects. Does the differentiation of EPCs in situ (vasculogenesis) play an important role in adult neovascularization, and would impairments in this process lead to clinical diseases? There is now a strong body of evidence suggesting that vasculogenesis does in fact make a significant contribution to postnatal neovascularization. Recent studies with animal BMT models in which BM (donor)-derived EPCs could be distinguished have shown that the contribution of EPCs to neovessel formation may range from 5 to 25% in response to granulation tissue formation [26] or growth factor-induced neovascularization [27].

There are no means to precisely track the kinetics of BM-derived EPCs for pathophysiological neovascularization in humans; however, several observations indicate the importance of EPCs in the pathogenesis and prognosis of cardiovascular diseases. Vasa et al. [28] reported that number and migratory activity of circulating EPCs is decreased in patients with stable coronary artery disease (CAD) compared with age-matched control subjects. Interestingly, the decreased number and impaired migratory activity of EPCs inversely correlated with number of coronary risk factors in each CAD patient. Tepper et al. [29] reported that proliferation of EPCs obtained from patients with type II diabetes was decreased by 48% compared with controls and inversely correlated with hemoglobin A1C level. In a Matrigel assay, diabetic EPCs were 2.5 times less likely to participate in tubule formation compared with control EPCs. Hill et al. [30] investigated the relationship between EPC number and cardiovascular risk in subjects with certain risk factors but no history of cardiovascular disease. The number of circulating EPCs was strongly correlated with the combined Framingham risk factor score in each subject. Measurement of flow-mediated brachial-artery reactivity also revealed a significant relation between endothelial function and the number of EPCs. In addition, EPCs from subjects at high risk for cardiovascular events had higher rates of in vitro senescence than cells from subjects at low risk.

These clinical findings indicate that decreased number and impaired function of EPCs may be a sensitive indicator of high risk of atherosclerotic diseases. Considering the classical theory that damage in EC layer could be an initial step of atherosclerotic change in the vessel wall, BM-derived and circulating EPCs might play an important role for repairing the damaged ECs, thereby inhibiting the progression of cardiovascular disease.

PRECLINICAL APPLICATION OF EPC TRANSPLANTATION IN ISCHEMIC DISEASES

It has been well documented that the development of a collateral circulation can attenuate tissue ischemia

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in peripheral, cerebral and CADs. Given the clarification of pathophysiological role of postnatal EPCs, therapeutic impact of EPC transplantation has been reported in various ischemic diseases. Kalka et al. [31] intravenously administered ex vivo expanded EPCs obtained from healthy human peripheral blood into immunodeficient mice with hindlimb ischemia. Histological evidence of the cell incorporation and in situ differentiation into EC lineage as well as physiological evidence of enhancement of limb blood flow recovery was clearly demonstrated following EPC transplantation compared with control treatment including mature EC transplantation. Limb salvage ratio, one of the most important endpoints in the clinical trials for critical limb ischemia, was also dramatically reduced by EPC transplantation. Murohara et al. [32] reported similar therapeutic potential of cord blood EPCs in nude rat model of hindlimb ischemia. We also tested the intravenous administration of ex vivo expanded human EPCs into nude rats with acute myocardial infarction. Similarly as in the hindlimb ischemia model, transplanted EPCs contributed to ischemic neovascularization following recruitment into the ischemic area. The EPC therapy also inhibited left ventricular (LV) fibrosis and preserved LV function [33]. Kocher et al. [34] reported similar potential of intravenous infusion of freshly-isolated (not cultured) $CD34^+$ cells in rats with acute myocardial infarction. Taguchi et al. [35] reported effectiveness of systemic infusion of human cord blood $CD34^+$ cells for neovascularization in nude mice with cerebral infarction. Augmented vascularity resulted in intrinsic nerve regeneration, thereby contributing to the further cerebral tissue repair.

Essential scarcity of EPCs in BM or peripheral blood would be one of the critical issues to overcome in clinical application. As an efficient method to reduce the effective dose of EPCs for therapeutic neovascularization, we tested local transplantation rather than systemic infusion of human circulating $CD34^+$ cells into nude rats with acute myocardial ischemia. The local administration was effective for neocapillary formation, infarct size reduction, and LV functional preservation at the dose of only 5–10% of systemic infusion in the previous studies [36]. To simulate clinical situation, we also tested NOGA electromechanical mapping-guided intramyocardial transplantation of autologous EPCs in swine model of chronic myocardial ischemia. The clinical-relevant EPC therapy resulted in significant attenuation of myocardial ischemia [36].

Recently, we reported the multilineage plasticity of human $CD34^+$ cells for cardiovascular regeneration. Immunohistochemical and molecular analyses enabled us to identify human $CD34^+$ cell-derived cardiomyocytes and smooth muscle cells in the rat infarcted myocardium [37] (Fig. 2). This finding strongly indicates that mechanisms underlying preservation of LV structural integrity following EPC transplantation not only improves myocardial perfusion via neovascularization but may also increase cardiac muscle mass through cardiomyogenesis, although proportional contribution of both mechanisms remains to be clarified.

CLINICAL APPLICATION OF EPCs FOR ISCHEMIC NEOVASCULARIZATION

On the basis of the promising outcomes in the preclinical studies, clinical application of EPCs for ischemic diseases has been started, although these clinical trials are still in the early stage. Stamm et al. [38] performed direct intramyocardial injection of autologous BM $AC133^+$ cells into six patients with chronic myocardial infarction at the time of coronary artery bypass grafting surgeries as an initial pilot study. Improvement of LV global function and cardiac perfusion was observed in most patients 3–9 months later, although sole effect of the EPC transplantation was unclear in that study design. They also reported tendency of superior potency of the combination therapy over the sole bypass surgery for improvement of LV ejection fraction [39]. Klein et al. [40] demonstrated safety, feasibility, and efficacy of intramyocardial transplantation of BM-$AC133^+$ cells without bypass surgery in patients with chronic ischemic cardiomyopathy. Recently, Losordo et al. [41] reported the result of a phase I/II, randomized, placebo-controlled, dose-ranging clinical trial of intramyocardial transplantation of GCSF-mobilized $CD34^+$ cells in 24 patients with intractable angina pectoris. In this study, GCSF injection, leukoapheresis for cell harvest, magnetic cell sorting for $CD34^+$ cell isolation, and NOGA mapping-guided cell injection were well tolerated without any severe adverse events. Favorable trends in efficacy endpoints such as reduction of angina frequency, increase in exercise tolerance, decrease in perfusion defect, etc. were observed in patients receiving $CD34^+$ cells compared with those receiving placebo. Following the promising results of the initial study, a multicenter phase II trial of $CD34^+$ cell transplantation for CAD has been started in the United States.

As for the application of EPC therapy for acute myocardial infarction, Assmus et al. [42] performed intracoronary infusion of BM-MNCs in nine patients and cultured circulating progenitor cells, an EPC-enriched population, in 11 patients with ST elevation myocardial infarction. Cell infusion following successful revascularization similarly improved LV function

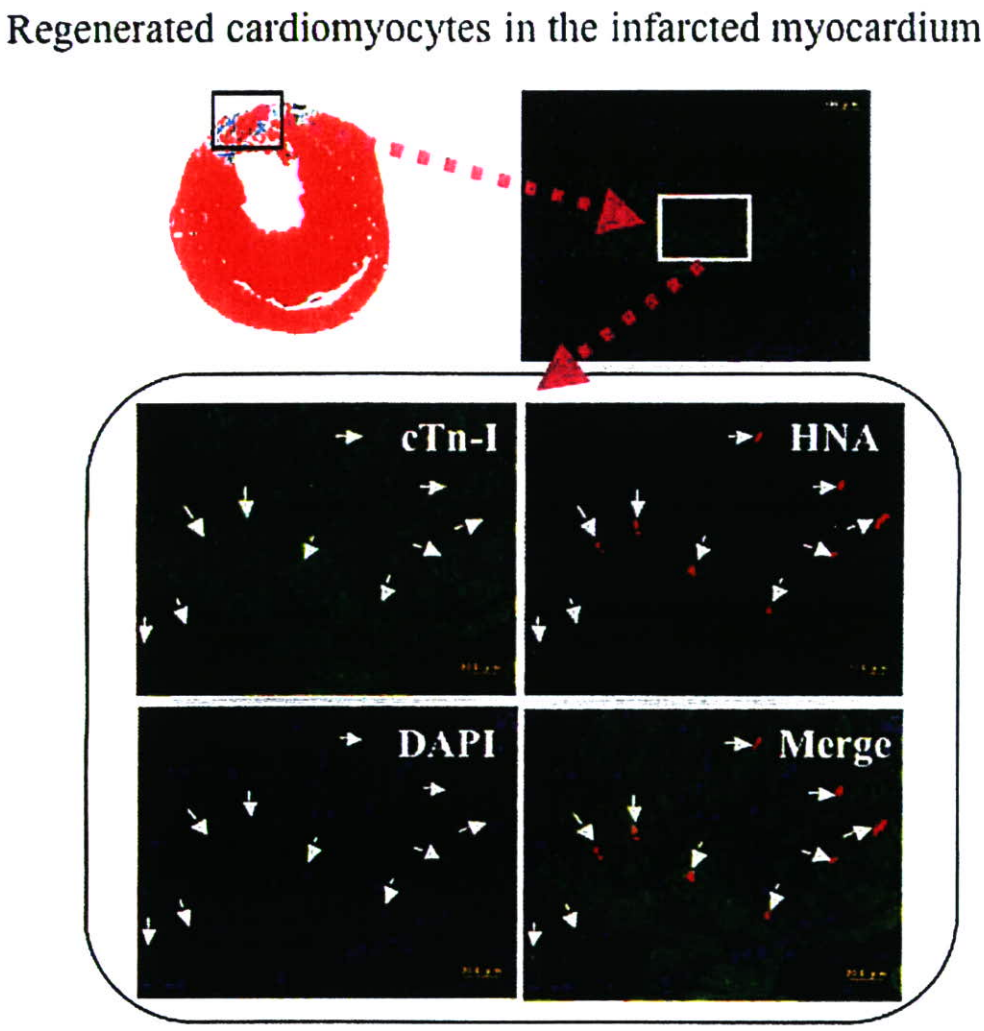

Fig. 2. Immunohistochemical identification of cardiomyogenic regeneration in the rat infarcted myocardium by human CD34⁺ cell transplantation. Cardiac troponin-I (cTn-I)-positive cells with human nuclear antigen (HNA)-positive nuclei are CD34⁺ cell-derived cardiomyocytes (arrows).

TABLE I. Transplantation of Endothelial Progenitor-Enriched Cell Population in Clinical Trials

Reference	Cell type	Patients	Injection route	Results
Stamm [38]	BM-AC133+	chronic MI (n = 6)	direct I.M. with CABG	LVEF↑, Perfusion↑
Stamm [39]	BM-AC133+	chronic MI (n = 40)	direct I.M. with CABG (vs. CABG alone)	LVEF↑, Perfusion↑
Klein [40]	BM-AC133+	chronic ICM (n = 10)	direct I.M.	LVEF↑, NYHA class↓
Losordo [41]	GCSF-mobilized CD34+ cells	chronic CAD (n = 24)	I.M./NOGA (vs. Placebo)	CCS class↓, Perfusion↑
Assmus [42]	Cultured CPCs	acute MI (n = 20)	Intracoronary (vs. BM-MNCs)	LVEF↑, Viability↑
Bartunek [43]	BM-AC133+	recent MI (n = 35)	Intracoronary (vs. standard therapy)	LVEF↑, Perfusion↑

BM, bone marrow; MI, myocardial infarction; I.M., intramyocardial injection; CABG, coronary artery bypass grafting; LVEF, left ventricular ejection fraction; ICM, ischemic cardiomyopathy; NYHA, New York Heart Association; GCSF, granulocyte colony stimulating factor; CAD, coronary artery disease; CCS, Canada Cardiovascular Society; CPCs, circulating progenitor cells; MNCs, mononuclear cells.

and viability in both groups. Although there was technical limitation in their culture method regarding purity and the expansion efficiency of EPCs, these favorable results encourage future controlled, randomized, clinical trials in the acute coronary syndrome setting. Bartunek et al. [43] also reported efficacy of intracoronary infusion of the AC133⁺ cells in patients with recent myocardial infarction in a pilot clinical trial (Table I).