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Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction

Yoshinori Miyahara[1,9], Noritoshi Nagaya[1,9], Masaharu Kataoka[1], Bobby Yanagawa[1], Koichi Tanaka[1], Hiroyuki Hao[2], Kozo Ishino[3], Hideyuki Ishida[4], Tatsuya Shimizu[5], Kenji Kangawa[6], Shunji Sano[3], Teruo Okano[5], Soichiro Kitamura[7] & Hidezo Mori[8]

Mesenchymal stem cells are multipotent cells that can differentiate into cardiomyocytes and vascular endothelial cells. Here we show, using cell sheet technology, that monolayered mesenchymal stem cells have multipotent and self-propagating properties after transplantation into infarcted rat hearts. We cultured adipose tissue–derived mesenchymal stem cells characterized by flow cytometry using temperature-responsive culture dishes. Four weeks after coronary ligation, we transplanted the monolayered mesenchymal stem cells onto the scarred myocardium. After transplantation, the engrafted sheet gradually grew to form a thick stratum that included newly formed vessels, undifferentiated cells and few cardiomyocytes. The mesenchymal stem cell sheet also acted through paracrine pathways to trigger angiogenesis. Unlike a fibroblast cell sheet, the monolayered mesenchymal stem cells reversed wall thinning in the scar area and improved cardiac function in rats with myocardial infarction. Thus, transplantation of monolayered mesenchymal stem cells may be a new therapeutic strategy for cardiac tissue regeneration.

Myocardial infarction, a main cause of heart failure, leads to loss of cardiac tissue and impairment of left ventricular function. Therefore, restoring the scarred myocardium is desirable for the treatment of heart failure. Although needle injections of bone marrow cells into the myocardium have been performed for cardiac regeneration[1–5], it is difficult to reconstruct sufficient cardiac mass in the thinned scar area after myocardial infarction.

Recently, our colleagues have developed cell sheets using temperature-responsive culture dishes[6]. These cell sheets allow for cell-to-cell connections and maintain the presence of adhesion proteins because enzymatic digestion is not needed[7–10]. Therefore, cell sheet transplantation may be a promising strategy for partial cardiac tissue reconstruction. Skeletal myoblasts, fetal cardiomyocytes and embryonic stem cells have been considered as candidates for an implantable cell source[11–13]. It is difficult, however, to produce a multilayered construct requiring a vascular network. Thus, autologous somatic stem cells with self-propagating properties that can induce angiogenesis are a desirable cell source for a transplantable sheet.

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that reside within the bone marrow microenvironment[14,15]. MSCs can differentiate not only into osteoblasts, chondrocytes, neurons and skeletal muscle cells, but also into vascular endothelial cells[16] and cardiomyocytes[17–20]. In contrast to their hematopoietic counterparts, MSCs are adherent and can expand in culture. Recently, MSCs have been isolated from adipose tissue[21–24], which is typically abundant in individuals with cardiovascular disease. Here, we investigated the therapeutic potency of monolayered MSCs derived from adipose tissue using cell sheet technology.

RESULTS

Characteristics of adipose tissue–derived MSCs

We isolated MSCs from subcutaneous adipose tissue of male Sprague-Dawley rats on the basis of the adherent properties of these cells. We obtained $1.7 \times 10^5 \pm 0.2 \times 10^5$ cells from 1 g adipose tissue in a 12-h culture. By day 4 of culture of the minced adipose tissue, spindle-shaped adherent cells were apparent and formed symmetric colonies. After approximately three to four passages, most adherent cells expressed CD29 and CD90 (**Supplementary Fig. 1** online). In contrast, the majority of adherent cells were negative for CD34 and CD45. They were also negative for CD31, a marker for vascular endothelial cells, and negative for α smooth muscle actin (αSMA), a marker for smooth muscle cells. A small fraction of adherent cells expressed CD71, CD106 and CD117. These results were similar to those from bone marrow–derived MSCs[15,22,25] (**Supplementary Fig. 1** online). Using previously described methods[16,22,26], we confirmed that these adipose-derived adherent cells, like bone marrow–derived MSCs, were multipotent, as judged by their ability to differentiate into adipocytes, osteoblasts and vascular endothelial cells. Thus, we

[1]Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute and [2]Department of Pathology, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka, 565-8565, Japan. [3]Department of Cardiovascular Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama, 700-8555, Japan. [4]Department of Physiology, School of Medicine, Tokai University, Bohseidai, Isehara, Kanagawa, 259-1193, Japan. [5]Institute of Advanced Biomedical Engineering and Science, Tokyo Woman's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo, 162-8666, Japan. [6]Department of Biochemistry, National Cardiovascular Center Research Institute and [7]Department of Cardiovascular Surgery, National Cardiovascular Center and [8]Department of Cardiac Physiology, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka, 565-8565, Japan. [9]These authors contributed equally to this work. Correspondence should be addressed to N.N. (nnagaya@ri.ncvc.go.jp) or H.M. (hidemori@ri.ncvc.go.jp).

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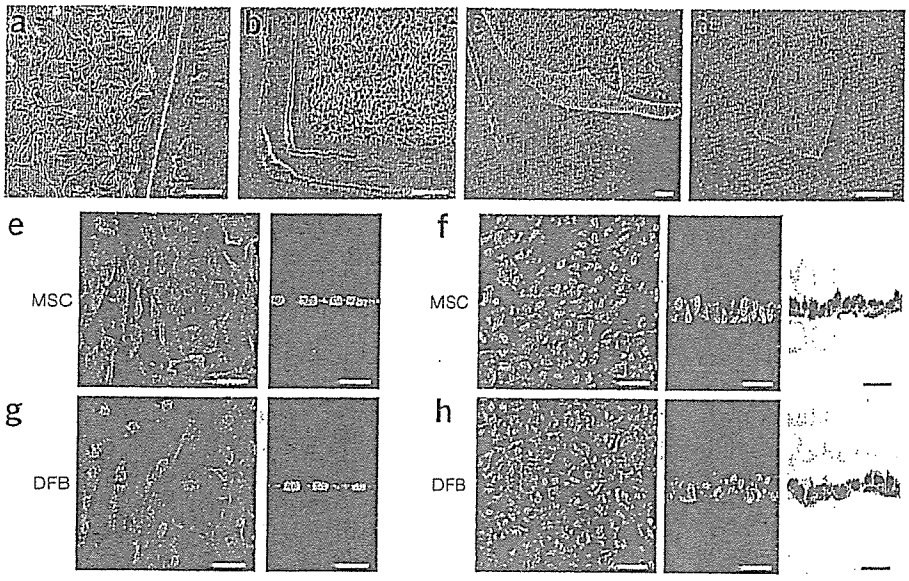

Figure 1 Preparation of monolayered MSCs. (a) MSCs 2 d after seeding on a temperature-responsive dish. (b) Cultured MSCs expanded to confluence within the square area of the dish by day 3. (c) The monolayered MSCs detached easily from the culture dish at 20 °C. (d) The completely detached monolayered MSCs were identified as a 12 × 12 mm square sheet. (e–h) Cross-sectional analysis of GFP-expressing monolayered MSCs and DFBs before detachment (e and g, confocal images) and after detachment (f and h, left and center, confocal images; right, Masson trichrome). The thickness of both monolayers was 3.5-fold greater than the thickness before detachment, and constituent cells were compacted. Scale bars in a–c, 100 μm; in d, 5 mm; in e–h, 20 μm.

confirmed that the majority of adherent cells isolated from adipose tissue were MSCs.

Preparation and transplantation of monolayered MSCs

We cultured adipose tissue–derived MSCs (5×10^5 cells) on temperature-responsive dishes for 3 d until confluent. MSCs were attached on the poly-*N*-isopropylacrylamide (PIPAAm)-grafted area (24 × 24 mm; **Fig. 1a,b**). As the culture temperature was decreased from 37 °C to 20 °C, MSCs detached spontaneously and floated up into the culture medium as a monolayer of MSCs within 40 min (**Fig. 1c,d**). As a control, we prepared dermal fibroblasts (DFBs) by the skin explant technique[27]. DFBs (8×10^5 cells) were cultured on the temperature-responsive dishes, and monolayered DFBs were fabricated as described above. The final cell counts for monolayered MSCs and DFBs before transplantation were $9.4 \pm 0.6 \times 10^5$ and $8.6 \pm 0.6 \times 10^5$ cells, respectively ($n = 6$ each). To identify the thickness of monolayered MSCs, we used green fluorescent protein (GFP)-expressing cell grafts derived from the GFP-transgenic Sprague-Dawley rats. Immediately after detachment, cells became compacted, possibly owing to cytoskeletal tensile reorganization, and the thickness of monolayered MSCs and DFBs was approximately 3.5-fold greater than the thickness before detachment (MSCs, 6.2 ± 0.3 to 21.5 ± 0.8 μm; DFBs, 6.5 ± 0.4 to 22.4 ± 1.1 μm; **Fig. 1e–h**). MSCs on the temperature-responsive dishes were positive for vimentin and slightly positive for collagen type 1, whereas DFBs were positive for both markers (**Fig. 2a**). We transferred detached monolayered MSCs above the myocardial scar (**Fig. 2b**) and then attached them to the surface of the anterior scar (**Fig. 2c**).

Secretion of angiogenic factors from monolayered MSCs

We measured secretion of angiogenic factors from MSCs 24 h after monolayers had formed, equivalent to day 4 after initial cell seeding. The monolayered MSCs secreted significantly larger amounts of angiogenic and antiapoptotic factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) than did the monolayered DFBs ($P < 0.01$; **Fig. 2d**). The control medium supplemented with 10% fetal calf serum contained less than 5 pg/ml of VEGF or HGF. These results suggest that the paracrine effects of monolayered MSCs on host myocardium are greater than those of monolayered DFBs.

Engraftment and growth of monolayered MSCs

To identify the transplanted cells in myocardial sections, we used GFP-expressing cell grafts derived from the GFP-transgenic Sprague-Dawley rats. We grafted monolayered MSCs or DFBs onto the scar area of the anterior wall (**Fig. 3**). Fluorescence microscopy showed that GFP-expressing monolayered MSCs gradually grew *in situ* and developed into a thick stratum, up to ~600 μm thick over the native tissue at 4 weeks (**Fig. 3a–f**). The engrafted MSC tissue tapered off toward the healthy myocardium (**Fig. 3d,e**), although most of the monolayered MSCs were attached only to the scar area in the anterior wall because of the large infarct. We rarely detected TUNEL-positive MSCs in the sheet (<1%) 48 h after transplantation (**Fig. 3g**), implying that cell viability in the sheet was maintained. In contrast, we frequently detected TUNEL-positive cells (15% ± 2%) in the DFB sheet, which was observed as a thin layer above the scar. Subsequently, the DFB sheet was undetectable 1 week later. Masson trichrome staining showed increased thickness of the anterior wall and attenuation of left ventricle enlargement after transplantation of monolayered MSCs (**Fig. 3h**), although the infarct size did not differ significantly among the untreated, DFB and MSC groups (**Supplementary Table 1** online).

Reconstruction of cardiac mass

After growth *in situ*, GFP-expressing MSC tissue contained a number of mature vascular structures that had positive staining for von Willebrand factor (vWF) and αSMA (**Fig. 4a,b**). A small fraction of the MSC tissue had positive staining for cardiac troponin T and desmin (**Fig. 4c,d**). On the other hand, a large proportion of the MSC tissue was positive for vimentin, a marker for mesenchymal lineage cells (**Fig. 4e**). The percentages of graft-derived cells that expressed endothelial (vWF), smooth muscle (αSMA), cardiac (troponin T) and mesenchymal (vimentin) markers were 12.2% ± 0.6%, 5.0% ± 0.3%, 5.3% ± 0.3% and 57.8% ± 2.2%, respectively. Notably, based on expression of these markers, two-thirds of vascular endothelial cells, four-fifths of smooth muscle cells and one-twentieth of cardiomyocytes within the MSC tissue were GFP^- and hence were derived from the host. The MSC tissue stained modestly for collagen type 1 (**Fig. 4f**). Picrosirius red staining showed that collagen deposition was found mainly in the extracellular matrix and the epicardial margin of the MSC tissue (**Fig. 4g**). Excluding staining in blood vessels, the MSC tissue was also negative for αSMA, a marker for myofibroblasts (**Fig. 4b**). This phenotype was consistent with properties of MSCs

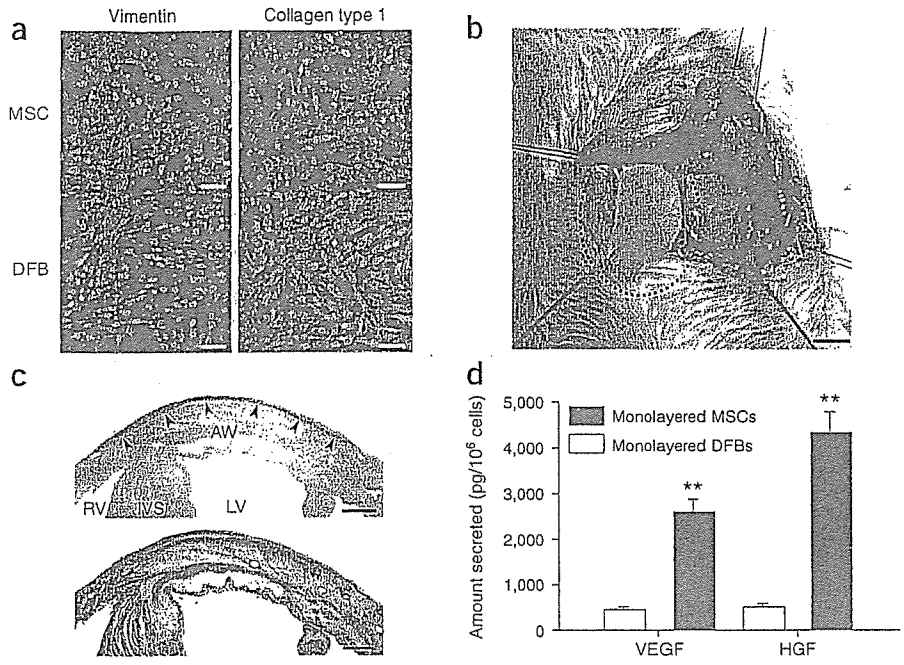

Figure 2 Characteristics of monolayered MSCs. (a) Properties of constituent cells in the monolayered grafts. Compared with DFBs (green), MSCs (green) are positive for vimentin (red) and slightly positive for collagen type 1 (red). (b) Monolayered MSCs (in the dotted circle) transferred to the infarcted heart. (c) Extent of monolayered MSCs 48 h after transplantation (arrows). AW, anterior wall; LV, left ventricle; RV right ventricle; IVS, interventricular septum. (d) Comparison of secretion of growth factors between monolayered MSCs and DFBs. $^{**}P < 0.01$ versus DFBs. Scale bar in a, 20 μm; in b, 5 mm; in c, 100 μm.

before transplantation (Fig. 2a and Supplementary Fig. 1 online), suggesting that the MSC tissue includes a number of undifferentiated MSCs. Taken together, the grown MSC tissue was composed of newly formed blood vessels, undifferentiated MSCs and few cardiomyocytes.

Fluorescence *in situ* hybridization analysis

We performed fluorescence *in situ* hybridization (FISH) to detect X and Y chromosomes after sex-mismatched transplantation of monolayered MSCs. We transplanted GFP-expressing monolayered MSCs derived from male rats to female Sprague-Dawley rats that had suffered an infarct. Four weeks later, newly formed cardiomyocytes that were positive for GFP had only one set of X and Y chromosomes, whereas we detected two X chromosomes exclusively in GFP^- host-derived cells (Fig. 4h). We counted the X and Y chromosomes in male and female control rats and in the MSC sheet–transplanted rats (Supplementary Table 2 online), and we did not detect extra copies of the X or Y chromosome in graft-derived GFP^+ cardiomyocytes. When we compared the frequencies of the occurrence of zero, one, two and more than two X chromosomes in the GFP^+ cardiomyocytes with the frequencies in male control cardiomyocytes, the GFP^+ cardiomyocytes did not show an increased proportion of X chromosomes ($0.25 > P > 0.10$, χ^2 test).

Effects of monolayered MSCs on cardiac function

Heart failure developed 8 weeks after coronary ligation, as indicated by an increase in left ventricle end-diastolic pressure (LVEDP) and attenuation of maximum and minimum rate of change in left ventricular pressure (dP/dt). Autologous transplantation of monolayered MSCs, however, resulted in decreased LVEDP (Fig. 5a). Left ventricle maximum and minimum dP/dt were significantly improved in the MSC group (Fig. 5b,c). We did not observe these hemodynamic improvements in the DFB group. The MSC group also had significantly lower right ventricular weight and lung weight than the DFB and untreated groups 4 weeks after transplantation (Supplementary Table 1 online). These results suggest that transplantation of monolayered MSCs has beneficial hemodynamic effects in rats with chronic heart failure.

Echocardiographic analysis showed that transplantation of monolayered MSCs significantly increased diastolic thickness of the infarcted anterior wall (Fig. 5d). Left ventricle end-diastolic dimension at 8 weeks was significantly smaller in the MSC group than in the DFB and untreated groups (Fig. 5e). Transplantation of the monolayered MSCs significantly increased left ventricle fractional shortening (Fig. 5f). Left ventricle wall stress in diastole was markedly lower in the MSC group than in the DFB and untreated groups (Supplementary Table 3 online). Plasma atrial natriuretic peptide (ANP) in the DFB and untreated groups was markedly elevated 8 weeks after myocardial infarction (Fig. 5g). Transplantation of the monolayered MSCs inhibited the increase in plasma ANP.

Survival analysis

The Kaplan-Meier survival curve showed that 4-week survival after coronary ligation did not differ significantly between the untreated and MSC groups before transplantation (Fig. 5h). Notably, however, no rats died after transplantation of monolayered MSCs. Therefore, the survival rate after transplantation was markedly higher in the MSC group than in the untreated group (4-week survival after transplantation was 100% for the MSC group versus 71% for the untreated group, log-rank test, $P < 0.05$).

DISCUSSION

There are several advantages to monolayered MSC transplantation. First, the self-propagating property of MSCs *in situ* leads to the formation of a thick stratum on the surface of the scarred myocardium. Second, the multipotency of MSCs and their ability to supply angiogenic cytokines allows neovascularization in the MSC tissue. Third, the reconstruction of thick myocardial tissue reduces left ventricle wall stress and results in improvement of cardiac function after myocardial infarction. Finally, a substantial part of the transplanted tissue is composed of undifferentiated MSCs, and it is tempting to speculate that such cells may act against future progressive left ventricle remodeling.

Cellular cardiomyoplasty using needle injections is emerging as a treatment option for individuals with chronic heart failure, but it may be limited by failure to regenerate cardiac mass. The cell sheet allows for cell-to-cell connections owing to the lack a need for enzymatic digestion[6–10]. Thus, the cell sheet has attracted considerable interest as a tool for tissue engineering[28]. Here, we used adipose tissue–derived MSCs as a cellular source for the cell sheet, which resulted in successful autologous transplantation in heterogenic rats without immunological

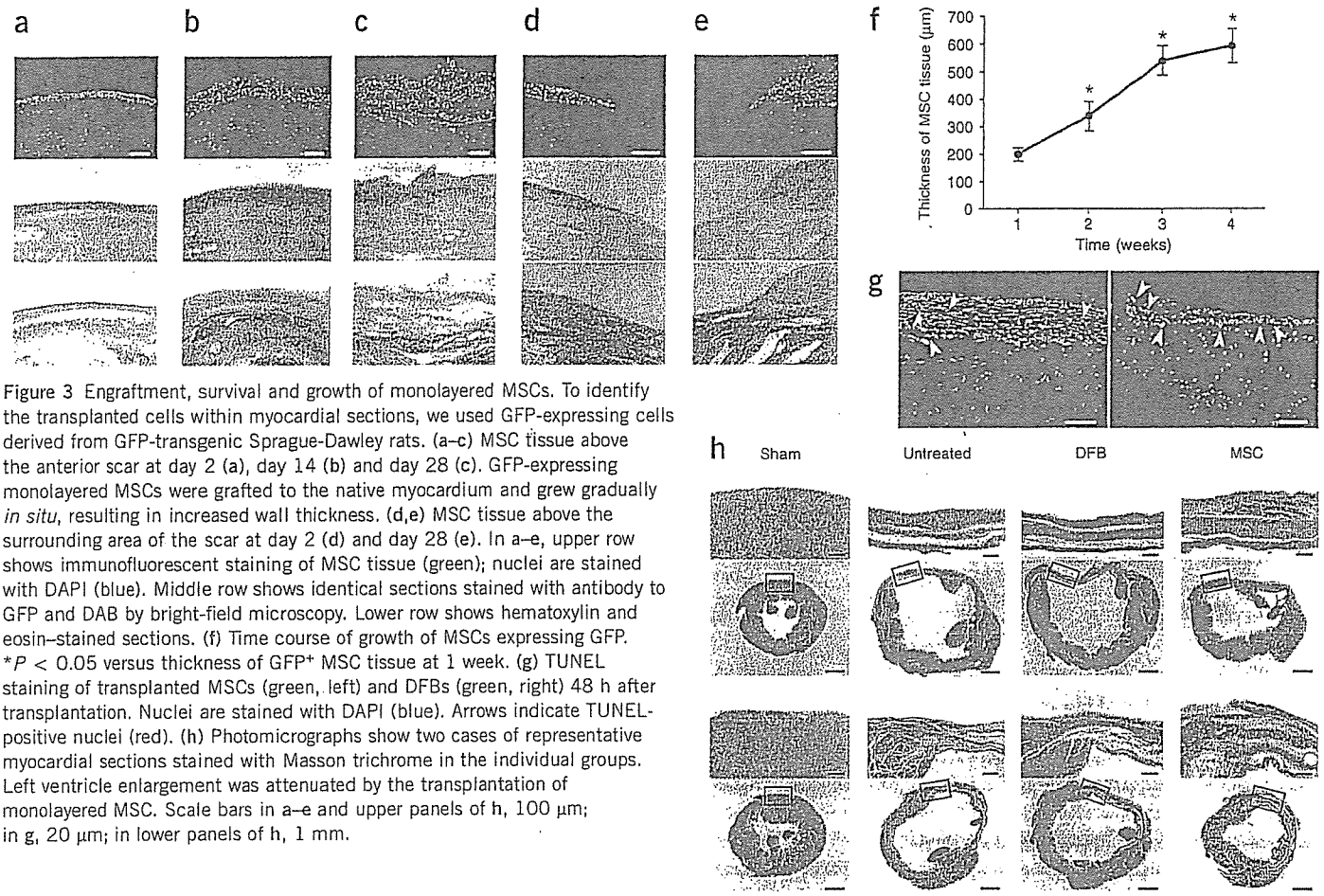

Figure 3 Engraftment, survival and growth of monolayered MSCs. To identify the transplanted cells within myocardial sections, we used GFP-expressing cells derived from GFP-transgenic Sprague-Dawley rats. (a–c) MSC tissue above the anterior scar at day 2 (a), day 14 (b) and day 28 (c). GFP-expressing monolayered MSCs were grafted to the native myocardium and grew gradually *in situ*, resulting in increased wall thickness. (d,e) MSC tissue above the surrounding area of the scar at day 2 (d) and day 28 (e). In a–e, upper row shows immunofluorescent staining of MSC tissue (green); nuclei are stained with DAPI (blue). Middle row shows identical sections stained with antibody to GFP and DAB by bright-field microscopy. Lower row shows hematoxylin and eosin–stained sections. (f) Time course of growth of MSCs expressing GFP. $^*P < 0.05$ versus thickness of GFP$^+$ MSC tissue at 1 week. (g) TUNEL staining of transplanted MSCs (green, left) and DFBs (green, right) 48 h after transplantation. Nuclei are stained with DAPI (blue). Arrows indicate TUNEL-positive nuclei (red). (h) Photomicrographs show two cases of representative myocardial sections stained with Masson trichrome in the individual groups. Left ventricle enlargement was attenuated by the transplantation of monolayered MSC. Scale bars in a–e and upper panels of h, 100 μm; in g, 20 μm; in lower panels of h, 1 mm.

rejection. Using flow cytometry, we did not find any substantial differences between adipose tissue–derived MSCs and bone marrow–derived MSCs, consistent with results from previous studies[22,25]. Adipose-derived MSCs readily attached to and propagated on the temperature-responsive dish. Abdominal subcutaneous adipose tissue is clinically redundant and easily accessible by rapid and minimally invasive surgery such as liposuction. Thus, adipose tissue may serve as a source of stem cells for therapeutic cell sheets.

Here, monolayered MSCs could readily be transferred and grafted to the scarred myocardium without additives or suturing. This may be attributable to cell-to-cell connections as well as extracellular matrix deposits on the basal surface of the monolayered MSCs. Regeneration of myocardial mass is thought to require multilayered constructs of the cell sheet. Unfortunately, however, the lack of a vascular network has limited the formation of a thick construct[10,29]. The transplanted monolayered MSCs thickened gradually, developing into a stratum of up to 600 μm in thickness over the native tissue 4 weeks after transplantation, suggesting that monolayered MSCs have an ability to grow *in situ*. As a result, the transplanted MSC tissue reversed wall thinning of the infarcted myocardium. On the other hand, the fibroblast sheet did not grow *in situ*. It should be noted that the MSC tissue included a large number of newly formed blood vessels. These vessels were composed of graft-derived cells, host-derived cells or both. The MSC sheet secreted a large amount of angiogenic and antiapoptotic cytokines, including VEGF and HGF, as compared with the fibroblast sheet. These results suggest that MSCs induce neovascularization within the sheet not only through their ability to differentiate into vascular cells but also through growth factor–mediated paracrine regulation. Thus, we believe that the angiogenic action of MSCs is important for reconstruction of cardiac mass by the MSC tissue.

Four weeks after transplantation, a small fraction of the engrafted MSCs were positive for cardiac proteins such as cardiac troponin T and desmin, suggesting the presence of cardiomyocytes within the MSC tissue. FISH analysis suggested that the most cardiomyocytes within the MSC tissue were not derived from cell fusion, but we are unable to exclude the possibility that some were. Further studies are necessary to investigate the mechanisms by which MSCs within the MSC tissue regenerate cardiomyocytes. The majority of the MSC tissue was positive for vimentin, a marker for undifferentiated MSCs and fibroblasts. In addition, the majority of MSCs within the graft were negative for collagen type 1 and αSMA, a marker for myofibroblasts. These results suggest that the grown-up MSC tissue is composed of newly formed blood vessels, undifferentiated MSCs and few cardiomyocytes.

We have also shown that transplantation of the monolayered MSCs significantly increased left ventricle maximum dP/dt, decreased LVEDP and inhibited the development of left ventricle enlargement in rats with chronic heart failure secondary to myocardial infarction. These results suggest that transplantation of monolayered MSCs improves cardiac function. But the presence of cardiomyocytes within the MSC tissue seemed to be rare. Thus, this improvement may be explained mainly by growth factor–mediated paracrine effects of the MSC sheet and a decrease in left ventricle wall stress resulting from the thick MSC tissue. Furthermore, no rats treated with the monolayered MSCs died during the study period, although untreated rats died frequently. These results indicate that fatal arrythmogenic problems were not caused by integration of the MSC tissue.

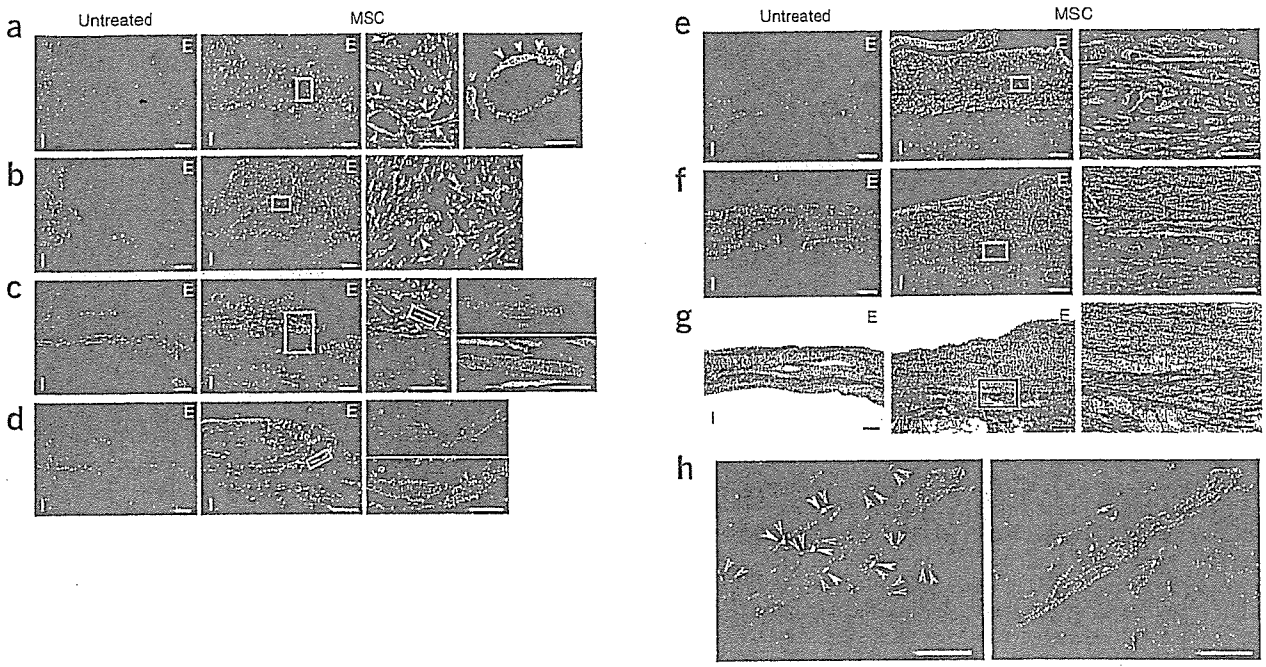

Figure 4 Differentiation of MSCs within the MSC tissue after growth *in situ*. (a,b) GFP-expressing MSCs (green) were identified as a thick stratum at the epicardial side of the myocardium. The MSC tissue contained a number of vascular structures positive for vWF (red, a) and αSMA (red, b). MSCs that did not participate in blood vessel formation were only rarely positive for αSMA, a marker for myofibroblasts. Arrows indicate transplanted MSCs positive for vWF or αSMA. (c,d) Some MSCs within the MSC tissue were positive for cardiac markers cardiac troponin T (red, c) and desmin (red, d). (e) Most of the MSC tissue was positive for vimentin (red). (f) The MSC tissue modestly stained for collagen type 1 (red). (g) Collagen deposition was also detected by picrosirius red staining. (h) FISH analysis. Newly formed cardiomyocytes (desmin, red) that were positive for GFP (green) had only one set of X (purple) and Y chromosomes (white), whereas two X chromosomes were detected exclusively in GFP^- host-derived cells. Nuclei are stained with DAPI (blue, a–f and h). Scale bars in left three panels of a and c and in two left panels of b and d–g, 100 μm; in h and far right panels of a–g, 20 μm. E, epicardial side; I, intimal side.

In summary, adipose tissue–derived monolayered MSCs can be readily engrafted to the scarred myocardium, grow gradually *in situ* and become a thick stratum that includes newly formed vessels, cardiomyocytes and undifferentiated MSCs. The engrafted MSCs reversed wall thinning in the scar area and improved cardiac function and survival in rats with myocardial infarction. Thus, transplantation of monolayered MSCs may be a new therapeutic strategy for cardiac tissue regeneration.

METHODS

Model of heart failure. All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the Japanese National Cardiovascular Center Research Institute. We used male Sprague-Dawley rats (Japan SLC) weighing 187–215 g. A myocardial infarction model was produced by ligation of the left coronary artery, as described previously[30]. Briefly, we anesthetized rats with sodium pentobarbital (30 mg/kg) and ventilated them with a volume-regulated respirator. We exposed hearts by left thoracotomy, and ligated the left coronary artery 2–3 mm from its origin between the pulmonary artery conus and the left atrium with a 6-0 Prolene suture. The sham group underwent thoracotomy and cardiac exposure without coronary ligation. The surviving rats were maintained on standard rat chow.

Study protocol. We randomly placed rats into four groups: rats with chronic heart failure that underwent transplantation of monolayered MSCs (MSC group; $n = 12$), rats with chronic heart failure given monolayered DFBs (DFB group; $n = 12$), rats with chronic heart failure without transplantation (untreated group; $n = 12$) and sham-operated rats without transplantation (sham group; $n = 10$). Four weeks after coronary ligation, the MSC and DFB groups underwent autologous transplantation of each monolayered cell graft onto the anterior wall, including the scar area (**Supplementary Methods** online). The other two groups underwent the same operative procedures without transplantation. We performed hemodynamic studies, echocardiography and histological assessments 4 and 8 weeks after coronary ligation (**Supplementary Methods**). Upon killing at 8 weeks after coronary ligation, only those rats with infarct size >25% of the left ventricle area were included in this study. Therefore, the variation in infarct size between the experimental rats was relatively low (28–41%, average 33.9% ± 1.9%).

Isolation and culture of MSCs from adipose tissue. Immediately after coronary ligation, we acquired subcutaneous adipose tissue (1.1 ± 0.1 g) from the right inguinal region of each rat. We minced adipose tissue with scissors and digested it with 10 ml of type 1 collagenase solution (0.1 mg/ml, Worthington Biochemical) for 1 h in a 37 °C water bath shaker. After filtration with mesh filter (Costar 3480, Corning) and centrifugation at 780*g* for 8 min, we suspended isolated cells in α-MEM supplemented with 10% FCS and antibiotics, plated them onto a 100-mm dish and incubated them at 37 °C with 5% CO_2. A small number of spindle-shaped cells were apparent in visible symmetric colonies by days 5–7.

Preparation of temperature-responsive dishes. Specific procedures for preparation of square-designed PIPAAm-grafted dishes have been previously described[9]. Briefly, we spread IPAAm monomer (Kohjin) in 2-propanol solution onto 60-mm polystyrene culture dishes (Corning). We then subjected the dishes to irradiation (0.25-MGy electron beam dose) using an Area Beam Electron Processing system (Nisshin High-Voltage) to immobilize IPAAm on the dish surface; we then rinsed dishes with cold distilled water and dried them in nitrogen gas. In the second step, we masked the PIPAAm-grafted surface with a square glass coverslip (24 × 24 mm, Matsunami Glass). We spread acrylamide (AAm) monomer solution in 2-propanol onto the masked dish surface. We then irradiated the dish surface with an electron beam and washed it. As a result, the central square area of each dish was PIPAAm grafted (temperature responsive), and the surrounding border was poly-AAm grafted (non–cell adhesive). This PIPAAm-grafted surface is hydrophobic under culture

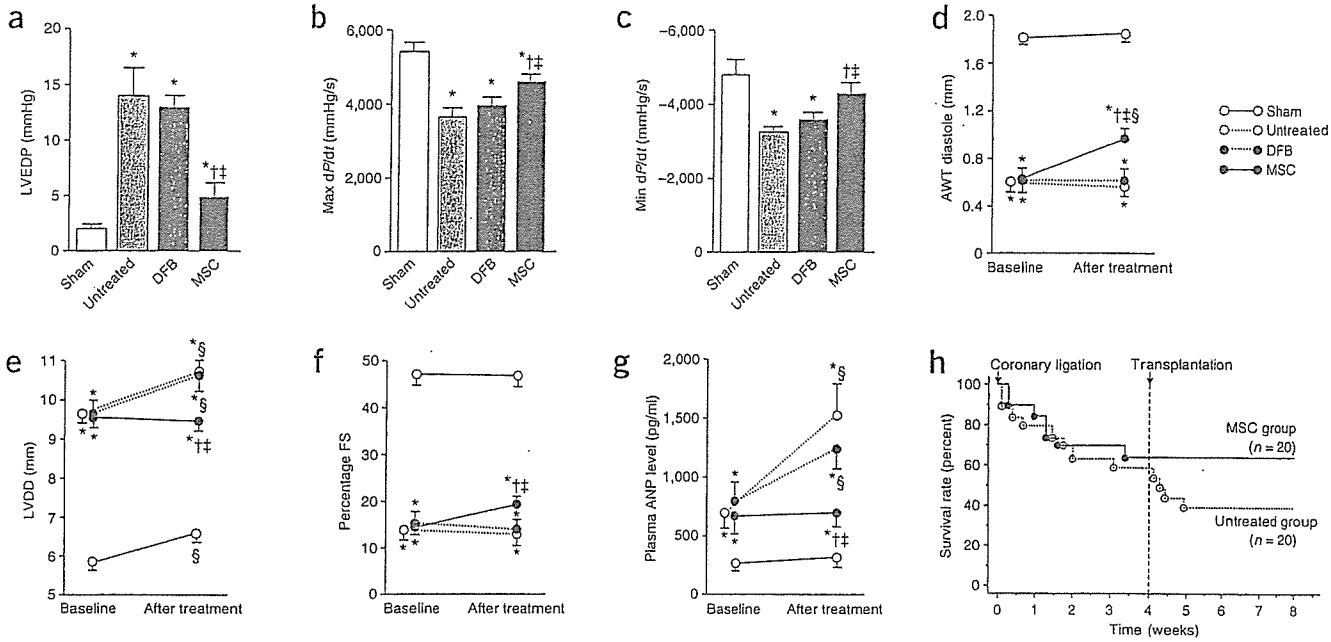

Figure 5 Cardiac structure and function after transplantation of monolayered MSCs. (a–c) Hemodynamic parameters obtained by catheterization. LVEDP, left ventricle end-diastolic pressure. (d–f) Echocardiographic findings. AWT, anterior wall thickness; LVDD, left ventricle end-diastolic dimension; FS, fractional shortening. (g) Plasma atrial natriuretic peptide (ANP) level. Baseline represents measurements 4 weeks after coronary ligation; 'after treatment' represents measurements taken 4 weeks after transplantation (8 weeks after coronary ligation). Data are mean ± s.e.m. $^{*}P < 0.05$ versus sham group; $^{\dagger}P < 0.05$ versus untreated group; $^{\ddagger}P < 0.05$ versus DFB group; $^{\S}P < 0.05$ versus baseline. (h) Survival of rats with chronic heart failure with or without monolayered MSC transplantation. The Kaplan-Meier survival curve demonstrates an 8-week survival rate of 65% for the MSC group versus 45% for the untreated group. Survival rate after transplantation was significantly higher in the MSC group than in the untreated group (100% versus 71% 4-week survival rate after transplantation, log-rank test, $P < 0.05$).

conditions at 37 °C and becomes reversibly hydrophilic below 32 °C. Therefore, cultured cells that adhere to the dish surface spontaneously detach from the grafted surface without enzymatic digestion.

Preparation of monolayered cell grafts. We suspended MSCs at the third or fourth passage from adipose tissue or DFBs at the second passage by trypsinization, and plated the cell suspension containing 3 ml of complete medium onto a 60-mm temperature-responsive dish at 5×10^5 cells per dish (MSCs) or 8×10^5 cells per dish (DFBs) and cultured cells at 37 °C. After 3 d of culture, confluently cultured MSCs or DFBs on the temperature-responsive dishes were incubated at 20 °C. By 40 min, both MSCs and DFBs detached spontaneously and floated up into the medium as monolayered cell grafts. Immediately after detachment, we gently aspirated the monolayered cell grafts using a 1,000 µl pipette tip and transferred them onto an elastic plastic sheet.

Statistical analysis. Numerical values are expressed as mean ± s.e.m. There are four groups of continuous variables in this study. Therefore, for multiple comparisons of more than two groups, we performed one-way analysis of variance (ANOVA). If the ANOVA was significant, we used the Newman-Keul procedure as a *post hoc* test. For repeated measurement such as echocardiographic parameters, we performed two-way repeated ANOVA with the Newman-Keul test. Comparisons of parameters between two groups were made by unpaired Student *t*-test. A value of $P < 0.05$ was considered significant.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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Beraprost sodium enhances neovascularization in ischemic myocardium by mobilizing bone marrow cells in rats

Yoshinori Miyahara [a], Shunsuke Ohnishi [a], Hiroaki Obata [a], Kozo Ishino [b], Shunji Sano [b], Hidezo Mori [c], Kenji Kangawa [d], Soichiro Kitamura [e], Noritoshi Nagaya [a,*]

[a] *Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, Osaka, Japan*
[b] *Department of Cardiovascular Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*
[c] *Department of Cardiac Physiology, National Cardiovascular Center Research Institute, Osaka, Japan*
[d] *Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan*
[e] *Department of Cardiovascular Surgery, National Cardiovascular Center, Osaka, Japan*

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Abstract

Beraprost sodium, an orally active prostacyclin analogue, has vasoprotective effects such as vasodilation and antiplatelet activities. We investigated the therapeutic potential of beraprost for myocardial ischemia. Immediately after coronary ligation of Sprague–Dawley rats, beraprost (200 μg/kg/day) or saline was subcutaneously administered for 28 days. Four weeks after coronary ligation, administration of beraprost increased capillary density in ischemic myocardium, decreased infarct size, and improved cardiac function in rats with myocardial infarction. Beraprost markedly increased the number of CD34-positive cells and c-kit-positive cells in plasma. Also, four weeks after coronary ligation of chimeric rats with GFP-expressing bone marrow, bone marrow-derived cells were incorporated into the infarcted region and its border zone. Treatment with beraprost increased the number of GFP/von Willebrand factor-double-positive cells in the ischemic myocardium. These results suggest that beraprost has beneficial effects on ischemic myocardium partly by its ability to enhance neovascularization in ischemic myocardium by mobilizing bone marrow cells.

Keywords: Prostacyclin analogue; Myocardial infarction; Neovascularization; Bone marrow mobilization

Interruption of myocardial blood flow leads to rapid death of cardiomyocytes and vascular structures, resulting in the development of heart failure [1]. Stem or progenitor cells are mobilized from bone marrow into the peripheral blood in response to tissue ischemia, migrate to sites of injured tissues, and differentiate into endothelial cells and cardiomyocytes [2–4]. However, the compensatory mechanisms are insufficient to heal infarcted myocardium. Earlier studies have shown that bone marrow cells artificially mobilized by cytokines repair the infarcted heart and improve cardiac function after acute myocardial infarction [5,6]. Therefore, enhancement of bone marrow cell mobilization leading to neovascularization following revascularization would be beneficial for the treatment of acute myocardial infarction.

Beraprost sodium (BPS) is a chemically stable prostacyclin analogue owing to its cyclo-pentabenzofuranyl structure [7]. It has been well established that BPS has vasoprotective effects such as vasodilation and antiplatelet activities [8–11]. Thus, BPS has been used in the treatment of peripheral arterial disease [12,13] and pulmonary arterial hypertension [14,15]. Although a limited number of studies suggest therapeutic potential of prostacyclin for the treatment of myocardial ischemia [16–18], the underlying mechanisms still remain unclear. In addition, little information is available regarding the therapeutic potential of prostacyclin analogues such as BPS for myocardial ischemia. A recent study has shown that BPS activates endothelial

* Corresponding author. Fax: +81 6 6833 9865.
E-mail address: nnagaya@ri.ncvc.go.jp (N. Nagaya).

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nitric oxide synthase (eNOS) through the c-AMP/protein kinase A pathway [19]. Activation of eNOS is known to contribute to bone marrow cell mobilization, leading to neovascularization [20]. These results raise the possibility that BPS may have beneficial effects on the ischemic myocardium through enhancement of bone marrow cell mobilization.

Thus, the purposes of this study were: (1) to examine the effect of BPS on mobilization and recruitment of bone marrow cells after acute myocardial infarction, (2) to investigate whether BPS induces neovascularization in the ischemic myocardium, and (3) to investigate whether treatment with BPS improves cardiac function in rats with myocardial infarction.

Methods

Model of myocardial infarction. We used male Sprague–Dawley rats (Japan SLC Inc., Hamamatsu, Japan) weighing 185–215 g. Myocardial infarction was produced by left coronary ligation, as described previously [21]. Briefly, after rats were anesthetized with sodium pentobarbital (30 mg/kg), they were artificially ventilated with a volume-regulated respirator. The heart was exposed via a left thoracotomy incision. Then, the left coronary artery was ligated 2–3 mm from its origin between the pulmonary artery conus and the left atrium with a 6-0 Prolene suture. Finally, the heart was restored to its normal position, and the chest was closed. Experimental protocols were performed in accordance with the "Guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute", which complies NIH Guidelines.

Administration of BPS. Immediately after coronary ligation, BPS (200 μg/kg/day, Astellas Pharma Inc., Tokyo, Japan) was subcutaneously administered to surviving rats using an osmotic mini-pump for 4 weeks (BPS group, $n = 12$). As a control, saline was similarly administered to rats receiving coronary ligation (Control group, $n = 12$).

Echocardiographic studies. Echocardiographic studies were performed 4 weeks after coronary ligation. M-mode tracings were obtained at the level of the papillary muscles using an echocardiographic system equipped with a 7.5-MHz phased-array transducer (HP SONOS 5500; Hewlett Packard Co., Andover, MA). Anterior and posterior end-diastolic and end-systolic wall thickness, LV end-diastolic and end-systolic dimensions, and LV fractional shortening were measured by the American Society for Echocardiography leading-edge method in three consecutive cardiac cycles. LV meridional wall stress was estimated as $0.344 \times \text{LV pressure} \times \{\text{LV dimension}/(1 + \text{PWT/LV dimension})\}$, where PWT is posterior wall thickness [22].

Hemodynamic studies. Hemodynamic studies were performed 4 weeks after coronary ligation, following echocardiography. After anesthesia with pentobarbital sodium, a 1.5F micromanometer-tipped catheter (Millar Instruments Inc., Houston, TX) was advanced into the LV through the right common carotid artery. Hemodynamic variables were measured with a pressure transducer connected to a polygraph. After completion of these measurements, the left and right ventricles and the lungs were excised and weighed. Infarct size was determined as a percentage of the entire LV area ($n = 5$ in each group), as reported previously [23]. Briefly, incisions were made in the posterior LV so that the tissue could be pressed flat. The circumference of the entire flat LV and of the visualized infarcted area, as judged from both the epicardial and endocardial sides, was outlined on a clear plastic sheet. The difference in weight between the two marked areas on the sheet was used to determine infarct size and was expressed as a percentage of LV surface area.

Measurement of plasma ANP level. Blood samples were obtained 4 weeks after coronary ligation. Plasma atrial natriuretic peptide (ANP), a marker for heart failure, was measured by enzyme immunoassay (Peninsula Laboratories Inc., San Carlos, CA).

Mononuclear cell mobilization and FACS analysis. To investigate whether administration of BPS mobilizes bone marrow cells, an additional 12 rats were randomized to receive BPS (200 μg/kg/day, BPS group, $n = 6$) or saline (Control group, $n = 6$). On the third day of BPS or saline treatment, 4 ml of blood was drawn from the inferior vena cava of each rat. Peripheral blood was obtained at the end of infusion. After mononuclear cells were counted, they were incubated for 30 min at 4 °C with fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies against rat CD34 (clone ICO-115, Santa Cruz) and CD45 (clone OX-1), and FITC-conjugated rabbit anti-rat c-Kit polyclonal antibody (clone C-19, Santa Cruz). Immunofluorescence-labeled cells were analyzed by quantitative flow cytometry with a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). Isotype-identical antibodies served as controls.

RT-PCR assay. To investigate whether bone marrow cells express the prostacyclin receptor (IP receptor), we analyzed expression of its mRNA by reverse transcription-polymerase chain reaction (RT-PCR). In brief, total RNA of bone marrow cells was extracted with guanidine isothiocyanate (RNeasy Mini Kit, Qiagen). Then, reverse-transcribed single-stranded cDNA was subjected to PCR (PCR Amplification Kit, Takara) using primer sets for the IP receptor (Hokkaido System Science Co., Ltd., Sapporo, Japan, forward, 5′-GGCACGAGAGGATGAAGTTTACC-3′; reverse, 5′-GTCAGAGGCACAGCAGTCAATGG-3′) and G3PDH (Clonetech Laboratories Inc., Mountain View, CA, forward, 5′-TG AAGGTCGGTGTCAACGGATTTGGC-3′; reverse, 5′-CATGTAGG CCATGAGGTCCACCAC-3′).

Creation of bone marrow-chimeric rats. To assess recruitment of bone marrow cells after BPS administration, bone marrow transplantation was performed by using male normal Sprague–Dawley rats as recipients and male Green fluorescent protein (GFP)-transgenic rats (SD-Tg [Act-EGFP] CZ-004Osb, Japan SLC Inc.) as donors, using a previously described method [24]. Briefly, bone marrow was harvested by flushing the cavity of femurs and tibias from GFP-transgenic rats with phosphate-buffered saline. Then, 3×10^7 GFP-positive bone marrow cells were individually administered to 12 lethally irradiated (900c Gray) rats via the tail vein. Four weeks after transplantation, flowcytometric analysis determined that 90% of peripheral blood mononuclear cells from both donors and 8 of 12 chimeric rats were GFP-positive, suggesting the establishment of stable chimerism. These chimeric rats were subjected to left coronary ligation, followed by administration of BPS (200 μg/kg/day, BPS group, $n = 4$) or saline (Control group, $n = 4$) using an osmotic mini-pump for 4 weeks.

Histological examination. To detect fibrosis in the cardiac muscle, the LV myocardium ($n = 5$, each group) was fixed in 10% formalin, cut transversely in three sections, embedded in paraffin, and stained with Masson's trichrome. To detect capillary endothelial cells in the peri-infarct area, we performed DAB staining (LSAB2 System HRP, Dako Cytomation Co., Denmark) using rabbit polyclonal anti-von Willebrand factor (vWF) antibody (Dako). A total of 10 different fields from three different sections were randomly selected, and the number of capillaries was counted in the peri-infarct area using a light microscope at 200× magnification. Capillary density was expressed as the mean number of capillaries per square millimeter. Also, 4 weeks after coronary ligation in bone marrow-chimeric rats ($n = 4$ in each group), the LV myocardium was excised, embedded in OCT compound, snap-frozen in liquid nitrogen, and cut transversely into 6-μm-thick sections from base to apex. Immunofluorescent staining was performed using rabbit polyclonal anti-vWF antibody (Dako), mouse monoclonal anti-cardiac troponin T antibody (Neomarkers, Fremont, CA), and rabbit polyclonal Alexa 488-conjugated anti-GFP antibody (Molecular Probes Inc., Eugene, OR). The nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). We measured the number of GFP/vWF-double-positive cells incorporated into vascular structures in 10 randomly selected fields in the peri-infarct area per section in a blinded fashion using a fluorescence microscope.

Statistical analysis. Numerical values are expressed as means ± SEM. Comparisons of parameters between two groups were made by unpaired Student's t test. A value of $p < 0.05$ was considered significant.

Results

Cardiac structure

Body weight at 4 weeks after coronary ligation was significantly greater in the BPS group than in the Control group (Table 1). Right ventricular weight and lung weight in the BPS group were significantly smaller than those in the Control group, although LV weight did not differ between the two groups. Moderate to large infarcts were observed in the Control group (Fig. 1A). However, administration of BPS significantly decreased infarct size in rats with myocardial infarction (Fig. 1A and B). BPS significantly decreased LV end-diastolic dimension (LVDD) (Fig. 1C).

Table 1
Physiological profiles of experimental groups

	Control	BPS
Number	12	12
Body weight (g)		
Baseline	198 ± 3	204 ± 3
After treatment	319 ± 6	352 ± 9*
LV wt/body wt (g/kg)	2.28 ± 0.04	2.27 ± 0.04
RV wt/body wt (g/kg)	0.99 ± 0.05	0.61 ± 0.02**
Lung wt/body wt (g/kg)	6.55 ± 0.62	3.88 ± 0.1**
Plasma AND level (pg/ml)	798 ± 99	498 ± 57*

Control, infarct rats without treatment; BPS, infarct rats treated with BPS administration; AND, atrial natriuretic protein. Data are expressed as means ± SEM. *$p < 0.05$, **$p < 0.01$ vs. Control group.

Cardiac function

Neither heart rate nor mean arterial pressure differed between the BPS and Control groups (Table 2). LV fractional shortening and LV maximum dP/dt in the BPS group were significantly greater than those in the Control group (Fig. 2A and B). LV end-diastolic pressure (LVEDP) in the BPS group was significantly lower than that in the Control group (Fig. 2C). LV minimum dP/dt was also improved by BPS (Fig. 2D). Treatment with BPS attenuated the increase in plasma ANP level after myocardial infarction (Table 1). BPS significantly increased anterior wall thickening, although it did not significantly alter posterior wall thickening (Table 2). Thickness of the anterior and posterior walls tended to be greater in the BPS group, but these changes did not reach statistical significance. LV diastolic wall stress in the BPS group was significantly lower than that in the Control group.

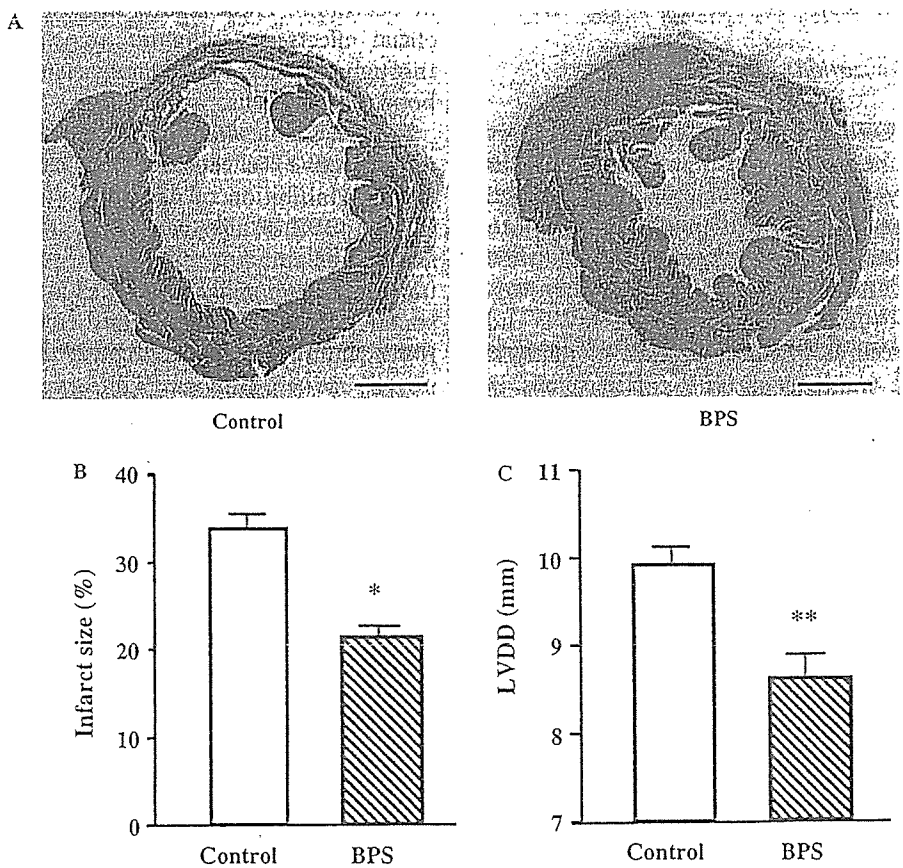

Fig. 1. (A) Representative examples of Masson's trichrome staining of transverse sections of LV myocardium 4 weeks after coronary ligation. Scale bars = 2 mm. (B,C) Quantitative analysis of infarct size and LV end-diastolic dimension (LVDD). Infarcted area and LVDD in the BPS group were significantly smaller than those in the Control group. Data are expressed as means ± SEM. *$p < 0.05$, **$p < 0.01$ vs. Control group.

Table 2
Echocardiographic and hemodynamic data

	Control	BPS
AWT diastole (mm)	0.62 ± 0.04	0.74 ± 0.05
AW thickening (%)	17 ± 3	34 ± 6*
PWT diastole (mm)	1.55 ± 0.07	1.70 ± 0.04
PW thickening (%)	43 ± 4	49 ± 3
Heart rate (bpm)	458 ± 7	471 ± 10
Mean arterial pressure (mmHg)	103 ± 5	115 ± 4
LV systolic pressure (mmHg)	113 ± 4	127 ± 5*
LV diastolic wall stress (kdyne/cm^2)	24 ± 4	5 ± 1**
LV systolic wall stress (kdyne/cm^2)	267 ± 18	225 ± 14

AWT, anterior wall thickness; AW, anterior wall; PWT, posterior wall thickness; PW, posterior wall. Data are expressed as means ± SEM. *$p < 0.05$, **$p < 0.01$ vs. Control group.

Mobilization of bone marrow cells

RT-PCR demonstrated that IP receptor mRNA was expressed in bone marrow cells (Fig. 3A), indicating a direct effect of BPS on these cells. Three-day administration of BPS significantly increased the number of peripheral blood mononuclear cells compared to saline administration (Fig. 3B). Administration of BPS markedly increased the number of circulating progenitor cells such as CD34-positive cells and c-kit-positive cells (Fig. 3C and D). BPS also increased the number of CD45-positive hematopoietic lineage cells (Fig. 3E).

BPS-induced neovascularization

Chimeric rats with GFP-expressing bone marrow were used to assess recruitment of bone marrow cells. Four weeks after coronary ligation, bone marrow-derived GFP-positive cells were incorporated predominantly into the infarcted region and its border zone (Fig. 4A), while these cells were rarely detected in the noninfarcted myocardium. Some of the GFP-positive cells stained for vWF and formed vascular structures. Semi-quantitative analysis demonstrated that the number of GFP-positive cells in the myocardium was significantly greater in the BPS group than in the Control group (Fig. 4B). The number of GFP-vWF double-positive cells (bone marrow-derived endothelial cells) in the ischemic myocardium was significantly greater in the BPS group than in the Control group (Fig. 4C). In addition, a small number of GFP-troponin T-double-positive cells were observed in the BPS group (Fig. 4D).

Capillary density

In the peri-infarct area, clustering of relatively small vessels was seen in BPS-treated hearts, which is indicative of recent endothelial regeneration (Fig. 5A). Semi-quantitative analysis also demonstrated that administration of BPS significantly increased the capillary density in the peri-infarct area compared to the Control group (Fig. 5B).

Discussion

In the present study, we demonstrated that treatment with BPS (1) decreased infarct size and improved cardiac structure and function in rats with acute myocardial infarction, (2) increased the number of circulating progenitor cells such as CD34-positive cells and c-kit-positive cells in rats, and (3) increased the number of bone marrow-derived endothelial cells and the capillary density in the ischemic myocardium. These results suggest that BPS may have beneficial effects on ischemic myocardium at least in part through enhancement of neovascularization by mobilizing bone marrow cells.

Earlier studies have reported that prostacyclin has cardioprotective effects in ischemia–reperfusion injury through inhibition of neutrophil activation and migration [25,26]. BPS is also reported to inhibit chemotaxis and superoxide anion production of neutrophils which contribute to tissue damage by releasing tissue destructive lysosomal enzymes [27]. Infusion of BPS has been shown to reduce infarct size in the dog heart with left coronary occlusion by reducing myocardial oxygen demand and by inhibition of the migration of neutrophils [28]. However, these

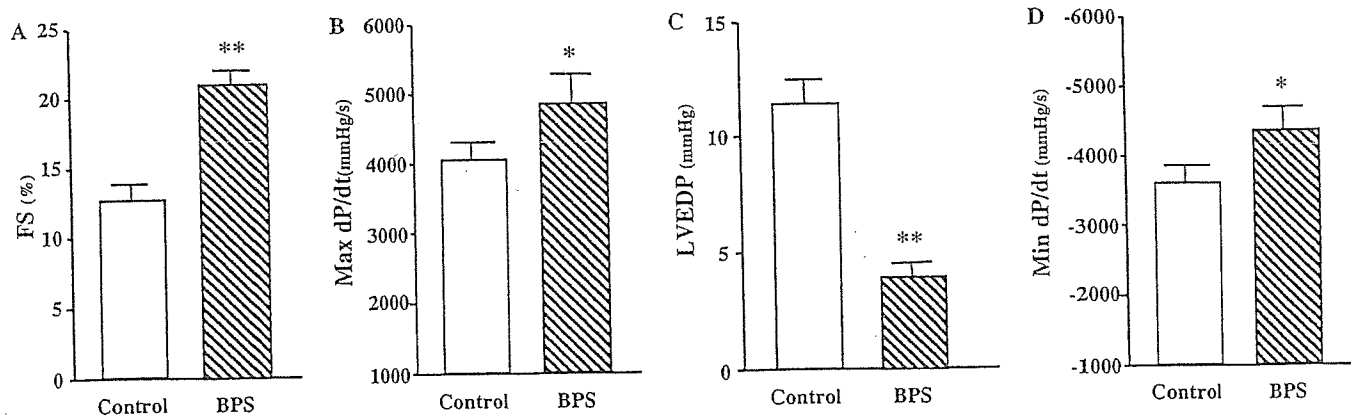

Fig. 2. Cardioprotective effects of BPS on echocardiographic and hemodynamic parameters. FS, fractional shortening; LVEDP, LV end-diastolic pressure; Max and Min dP/dt, maximum and minimum dP/dt. Data are expressed as means ± SEM. *$p < 0.05$, **$p < 0.01$ vs. Control group.

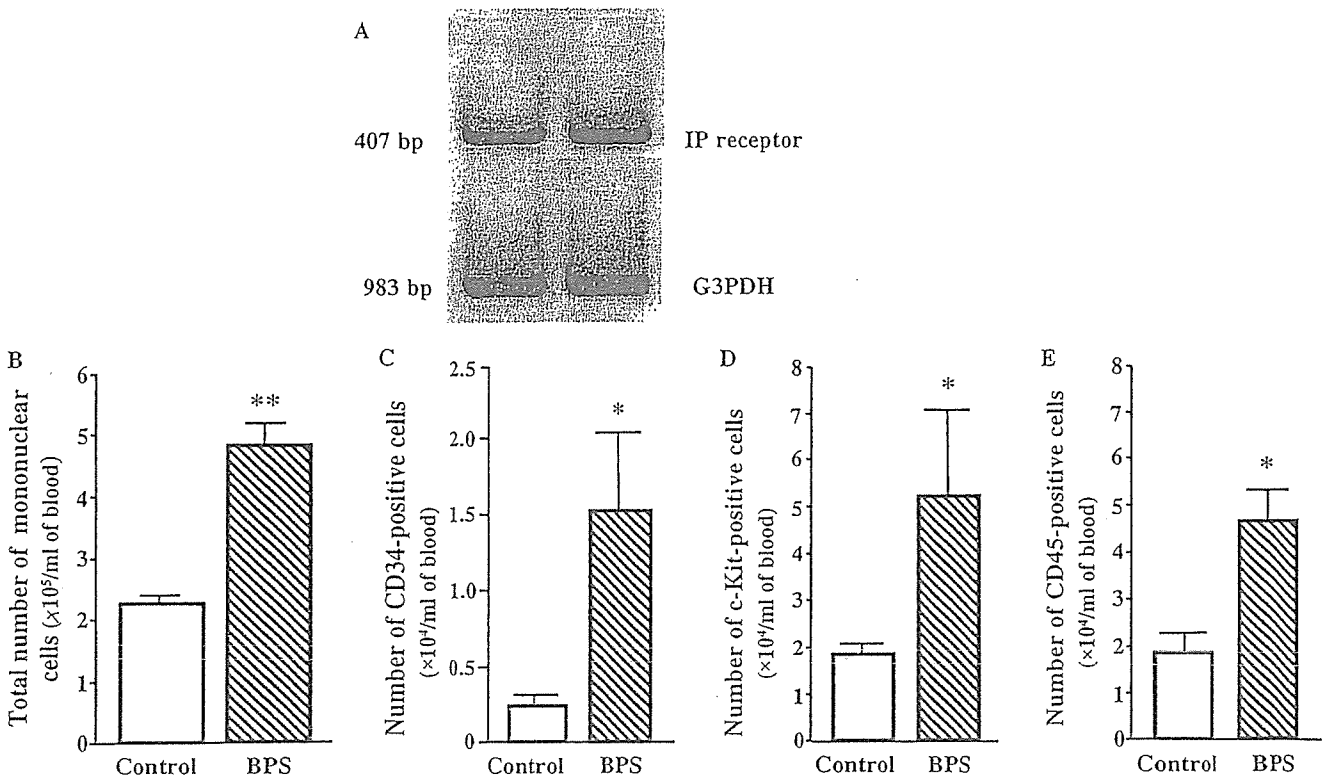

Fig. 3. BPS-induced mobilization of bone marrow cells. (A) Expression of prostacyclin receptor (IP receptor) on bone marrow cells. (B–E) Quantification of BPS-induced MNC mobilization by FACS analysis. Administration of BPS markedly increased the number of circulating progenitor cells such as CD34-positive cells and c-kit-positive cells. BPS also increased the number of CD45-positive hematopoietic lineage cells. Data are expressed as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Control group.

biological activities of BPS appear to be insufficient to explain the decrease in infarct size as well as suppression of LV remodeling.

Recent studies have shown that mobilization of bone marrow cells by cytokines promotes myocardial repair and regeneration after acute myocardial infarction [5,6]. In the present study, three-day administration of BPS markedly increased the number of circulating progenitor cells such as CD34-positive cells and c-kit-positive cells in rats. In addition, treatment with BPS enhanced recruitment of bone marrow cells to the ischemic myocardium and increased capillary density in the peri-infarct area. Earlier studies have shown that CD34-positive cells have angiogenic potential to treat ischemic heart [29–31]. Also, another stem cell fraction, c-kit-positive cells have ability to repair ischemic myocardium by differentiating into vascular endothelial cells [32,33]. These findings suggest that administered BPS induces neovascularization partly via enhancement of bone marrow cell mobilization. RT-PCR demonstrated that IP receptor mRNA was expressed in bone marrow cells, indicating a direct effect of BPS on these cells. A recent study has shown that BPS increases eNOS expression in cultured endothelial cells through activation of c-AMP/Protein kinase A signal transduction [19]. Also, earlier studies have shown that eNOS plays essential role in the recruitment of EPCs to the ischemic myocardium [20]. Taken together, administered BPS may act as a potent stimulator of cell mobilization from bone marrow, although further studies are necessary to examine the underlying mechanisms.

In the present study, treatment with BPS significantly attenuated infarct size after myocardial infarction. BPS improved cardiac function and attenuated the development of LV remodeling after acute myocardial infarction, as indicated by increases in LV fractional shortening and maximum dP/dt, and decreases in LVEDP and LVDD. Taken together, BPS may attenuate myocardial infarction through enhancement of neovascularization via modification of bone marrow kinetics. Interestingly, a small fraction of mobilized bone marrow cells expressed cardiac troponin T in the ischemic myocardium in the BPS group, suggesting that BPS may partially contribute to myocardial regeneration after acute myocardial infarction. Earlier studies have demonstrated that BPS has other beneficial effects for ischemic heart disease including anti-thrombotic activity [34], inhibition of reperfusion injury [35], and prevention of coronary spasm [36], and re-stenosis [37]. These findings suggest that administration of BPS may be a promising therapy for acute myocardial infarction.

Granulocyte colony stimulating factor (G-CSF) is currently used agent for mobilization of bone marrow. Infusion of G-CSF after myocardial infarction improves LV function increasing peripheral stem cell fraction [5,38]. A recent clinical trial, however, claimed the G-CSF therapy

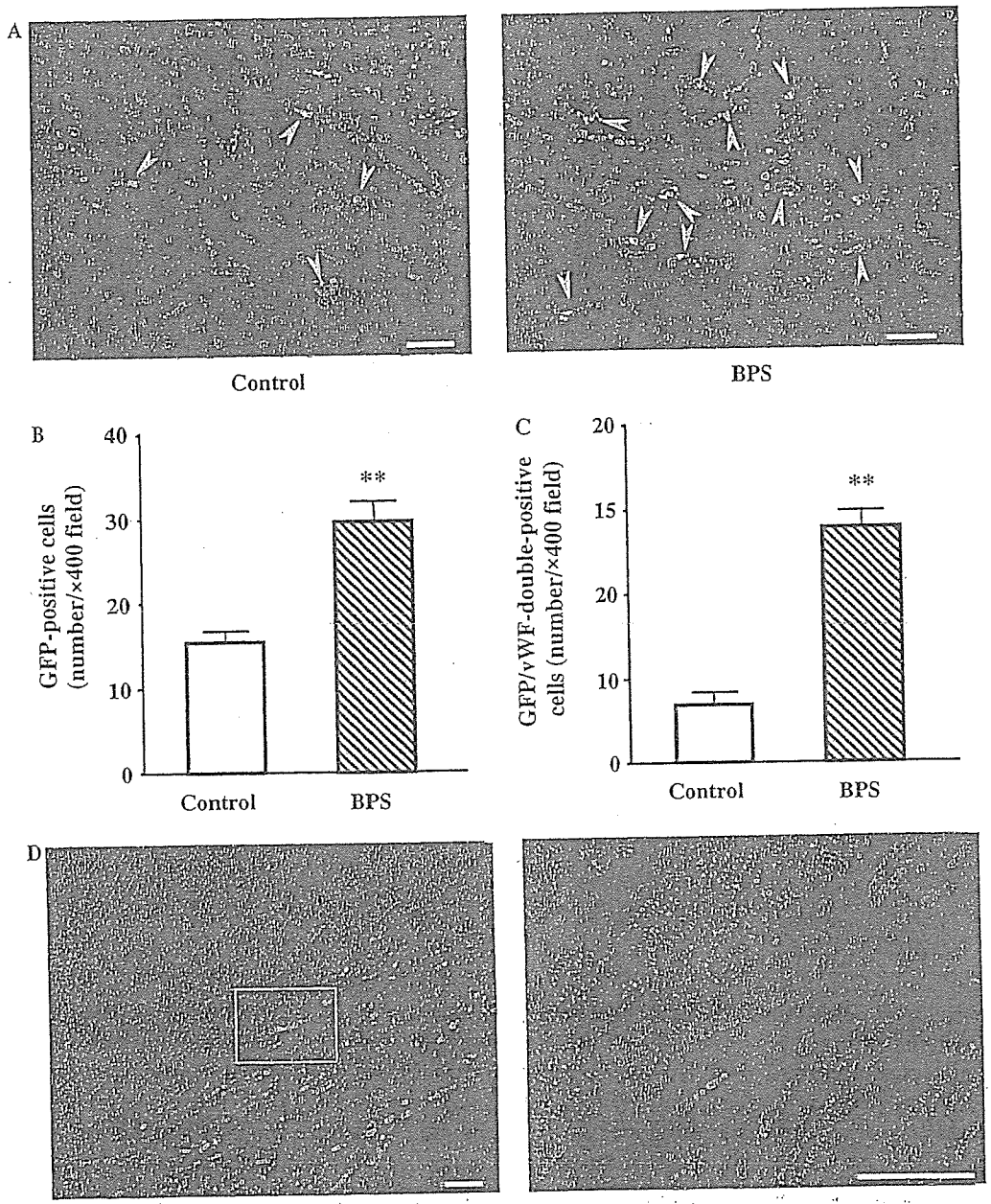

Fig. 4. BPS-induced neovascularization. (A) Representative immunofluorescent images stained with antibodies to von-Willbrand factor (vWF, red) and green fluorescent protein (GFP, green). Nuclei were counterstained with DAPI (blue). (B,C) Semi-quantitative analyses of numbers of GFP-positive cells and GFP-vWF double-positive cells in the peri-infarct area. (D) Representative immunofluorescent image of GFP-positive cells (green) expressing cardiac troponin T (red) observed in the BPS group. Scale bars = 50 μm. Data are expressed as means ± SEM. $^{**}p < 0.01$ vs. Control group.

has serious problem with re-stenosis after recanalization [39]. On the other hand, the safety of BPS has been identified in the treatment of peripheral arterial disease [12,13] and pulmonary arterial hypertension [14,15]. A randomized, controlled clinical trial failed to demonstrate therapeutic potential of prostacyclin for the treatment of severe congestive heart failure [40], which has long discouraged the pursuit of prostacyclin as a therapeutic option for the treatment of acute myocardial infarction. Interestingly, however, double-blinded, randomized, placebo-controlled, large-scale studies showed that treatment with BPS decreased vascular events in patients with peripheral arterial disease [41,42]. Thus, adequate use of BPS for only acute myocardial infarction may have beneficial effects on ischemic myocardium, although further preclinical trials are required to verify the safety and efficacy of BPS.

Conclusion

In summary, administration of BPS improved cardiac structure and function in rats with acute myocardial infarction. This beneficial effect of BPS may be mediated partly by its ability to enhance neovascularization in ischemic myocardium by mobilizing bone marrow cells.

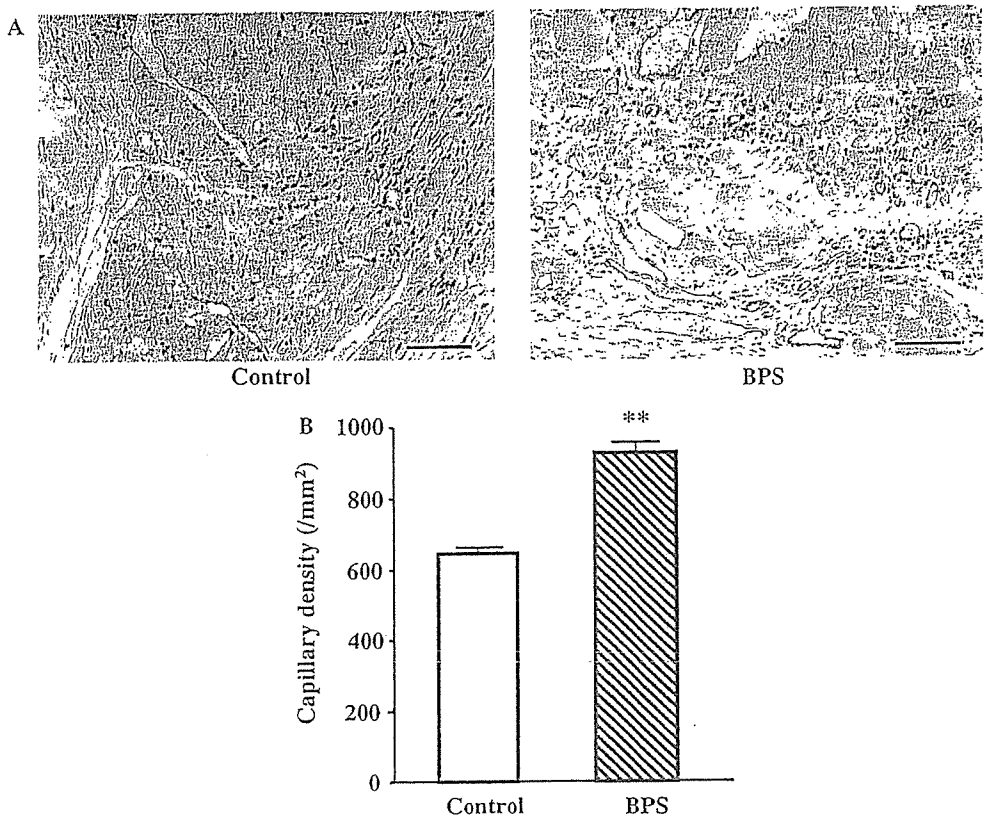

Fig. 5. (A) Representative samples stained with antibody to von Willebrand factor by bright-field DAB. (B) Quantitative analysis of capillary density in peri-infarct area. Administration of BPS increased capillary density by 37%. Scale bars = 50 μm. Data are expressed as means ± SEM. $^{**}p < 0.01$ vs. Control group.

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Unblinded Pilot Study of Autologous Transplantation of Bone Marrow Mononuclear Cells in Patients With Thromboangiitis Obliterans

Koji Miyamoto, MD; Kazuhiro Nishigami, MD; Noritoshi Nagaya, MD; Koichi Akutsu, MD; Masaaki Chiku, MD; Masataka Kamei, MD; Toshihiro Soma, MD; Shigeki Miyata, MD; Masahiro Higashi, MD; Ryoichi Tanaka, MD; Takeshi Nakatani, MD; Hiroshi Nonogi, MD; Satoshi Takeshita, MD

Background—The short-term clinical benefits of bone marrow mononuclear cell transplantation have been shown in patients with critical limb ischemia. The purpose of this study was to assess the long-term safety and efficacy of bone marrow mononuclear cell transplantation in patients with thromboangiitis obliterans.

Methods and Results—Eleven limbs (3 with rest pain and 8 with an ischemic ulcer) of 8 patients were treated by bone marrow mononuclear cell transplantation. The patients were followed up for clinical events for a mean of 684±549 days (range 103 to 1466 days). At 4 weeks, improvement in pain was observed in all 11 limbs, with complete relief in 4 (36%). Pain scale (visual analog scale) score decreased from 5.1±0.7 to 1.5±1.3. An improvement in skin ulcers was observed in all 8 limbs with an ischemic ulcer, with complete healing in 7 (88%). During the follow-up, however, clinical events occurred in 4 of the 8 patients. The first patient suffered sudden death at 20 months after transplantation at 30 years of age. The second patient with an incomplete healing of a skin ulcer showed worsening of the lesion at 4 months. The third patient showed worsening of rest pain at 8 months. The last patient developed an arteriovenous shunt in the foot at 7 months, which spontaneously regressed by 1 year.

Conclusions—In the present unblinded and uncontrolled pilot study, long-term adverse events, including death and unfavorable angiogenesis, were observed in half of the patients receiving bone marrow mononuclear cell transplantation. Given the current incomplete knowledge of the safety and efficacy of this strategy, careful long-term monitoring is required for future patients receiving this treatment. (*Circulation.* **2006;114:2679-2684.**)

Key Words: angiogenesis ■ collateral circulation ■ endothelium ■ peripheral vascular diseases

The clinical consequences of severe peripheral arterial disease or critical limb ischemia include rest pain and the loss of tissue integrity in the distal limb.[1-3] Therapeutic options for such patients are limited. These conditions are often refractory to conservative measures and are typically unresponsive to drug therapy. When vascular obstruction involves a long segment or is widespread, percutaneous revascularization may not be feasible. Surgical therapy, consisting of arterial bypass or amputation, is complicated by variable morbidity and mortality, and its effectiveness depends on the short- and long-term patencies of the conduit employed. Therapeutic angiogenesis thus constitutes a potential alternative treatment strategy for such patients.[4,5]

Previous investigators have suggested that endothelial progenitor cells, originating from bone marrow, circulate in adult peripheral blood and participate in postnatal neovascularization.[6-8] Subsequent experiments have shown that bone marrow or bone marrow–derived cells have the potential to stimulate angiogenesis and thereby modulate the hemodynamic deficit in ischemic limbs in vivo.[9,10] The Therapeutic Angiogenesis by Cell Transplantation (TACT) study first demonstrated that the magnitude of angiogenesis stimulated by these cells is sufficient to constitute a therapeutic benefit in patients with critical limb ischemia.[11] In that study, the investigators injected bone marrow mononuclear cells (BM-MNCs) into the ischemic limb of patients and documented a significant improvement in the hemodynamic deficit as well as the relief of ischemic symptoms. Although the TACT

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From the Departments of Medicine (K.M., K.N., K.A., M.C., H.N., S.T.), Regenerative Medicine and Tissue Engineering (N.N.), Anesthesiology (M.K.), Transfusion Medicine (S.M.), Radiology (M.H., R.T.), and Organ Transplantation (T.N.), National Cardiovascular Center, Osaka; and the Department of Clinical Laboratory (T.S.), Osaka Minami Medical Center, Osaka, Japan.

Correspondence to Dr Satoshi Takeshita, MD, FACC, Department of Medicine (Cardiology), National Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan. E-mail stake@muse.ocn.ne.jp

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study established the concept of using BM-MNCs for therapeutic angiogenesis, limited information is available about the long-term safety and efficacy of this strategy.

The purpose of the present study was to determine the long-term safety and clinical impact of BM-MNC transplantation for "no-option" patients with thromboangiitis obliterans.

Methods

Patients

Eight patients with thromboangiitis obliterans were treated with an autologous transplantation of BM-MNCs between March 2002 and September 2004. The diagnosis of thromboangiitis obliterans was based on the criteria proposed by Olin[12]: (1) onset before age 45; (2) current (recent) history of tobacco use; (3) the presence of distal-extremity ischemia (infrapopliteal or infrabrachial) indicated by claudication, rest pain, ischemic ulcers, or gangrene; (4) exclusion of autoimmune or connective tissue diseases, hypercoagulable states, and diabetes mellitus; (5) exclusion of a proximal source of emboli by echocardiography and arteriography; and (6) consistent arteriographic findings in the clinically involved and noninvolved limbs.

Patients qualified for cell transplantation if they had chronic limb ischemia, with rest pain or a nonhealing ischemic ulcer, present for a minimum of 4 weeks without evidence of improvement in response to conventional drug therapy; showed angiographic evidence of vasculopenia in the affected limb; and were not candidates for percutaneous or surgical revascularization. The exclusion criteria included severe concurrent illness, the presence of proliferative diabetic retinopathy, and a history or clinical evidence of a malignant disorder.

All the patients involved in the present study received continuous medical therapy for >2 months before BM-MNC transplantation to confirm that conventional measures would be insufficient to achieve improvement in rest pain or skin ulcer/gangrene. During this period, no surgical therapies such as bypass grafting, extensive debridement, skin grafting, or limb amputation were performed. In addition, the patients were admitted to the hospital for a minimum of 1 month before BM-MNC transplantation to exclude the likelihood of spontaneous improvement in ischemic symptoms resulting from an enrollment bias. It should be also pointed out that the patients remained in the hospital and received the same therapy for at least 1 month after BM-MNC transplantation to avoid changes in their treatment.

BM-MNC Transplantation

While the patients were under general anesthesia, marrow cells were aspirated from the ileum. BM-MNCs were sorted on an AS-104 blood-cell separator (Fresenius HemoCare, Redmond, Wash) and concentrated to a final volume of ≈50 mL. After bone marrow cells were sorted on the AS-104 blood-cell separator, a small fraction of the cells was used for BM-MNC counting; the concentration of BM-MNCs in the final product was determined by using a microscope counting chamber after May-Giemsa staining. By using another fraction of cells, the number of $CD34^+$ cells in the BM-MNCs was also determined by fluorescence-activated cell sorting (FACS SCAN flow cytometer; Becton Dickinson, San Jose, Calif). The cells were incubated with the FITC-conjugated mouse monoclonal antibody against human CD34 (clone 581; Becton Dickinson) according to manufacturer's instructions.

For each patient, ≈100 aliquots of BM-MNCs (0.5 mL per aliquot) were administered via a syringe with a 27-gauge needle. Injection was performed into 9 lower limbs in 7 of the patients and the bilateral hands in 1. Injection sites were arbitrarily selected according to angiographic findings (ie, the degree of vasculopenia) and included calf muscles such as the soleus and gastrocunemius muscles as well as the sole muscles of the foot. For the patient with hand ischemia, injection was performed in palm muscles.

Assessment of Short-Term Outcome

Ischemic pain was assessed with a visual analog pain scale (VAS) with 10 levels. Ischemic ulcers were documented by color photography. Resting ankle-brachial pressure index (ABI) was calculated as the quotient of absolute ankle pressure and brachial pressure (the patient who received BM-MNC transplantation in his hands was excluded from ABI analysis). Angiographic assessment was performed with magnetic resonance angiography, computed tomographic angiography, or digital subtraction angiography. Adverse events were defined as death, limb amputation, pathological angiogenesis, recurrence/worsening of ischemic symptoms (ie, rest pain, skin ulcer, gangrene), myocardial infarction, stroke, and malignant disease.

Assessment of Long-Term Outcome

The mean length of follow-up was 684±549 days (range 103 to 1466). Patients were followed up by history analysis, physical examination, routine blood testing, ABI, and angiography at prescribed intervals during the first year, after which they were contacted at an outpatient clinic or by telephone to track events.

Data Analysis

All data are presented as mean±SD (range) or frequencies (percentage).

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

Diagnosis

The diagnosis of thromboangiitis obliterans was made according to the criteria described above. Among the 8 patients, only patient 6 did not completely fulfill the criteria; ie, this patient had no history of tobacco use (Table 1). Laboratory screening excluded the possibility of other underlying diseases, however, including autoimmune and connective tissue diseases. It should be also pointed out that patient 6 had diabetes mellitus at the time of cell transplantation but not at the onset of thromboangiitis obliterans. With the typical characteristic angiographic findings of thromboangiitis obliterans, such as multiple segmental arterial involvement (skip lesions) and "cork-screw" collateral vessels, we diagnosed patient 6 as having thromboangiitis obliterans, even though the patient did not have a history of tobacco use.

Patient Characteristics

The demographic and clinical data of the 8 patients are shown in Table 1. The mean age of the patients enrolled was 46±14 years (range 28 to 63). Seven patients (88%) were male. One patient had undergone prior femoral-tibial artery bypass grafting, and 1 had undergone sympathetic ganglion block. These treatments were performed >1 year before BM-MNC transplantation. Seven patients (88%) had a history of smoking, all of whom stopped smoking at least 1 month before transplantation.

Short-Term Outcome

The total volume of cells aspirated from the ileum was 728±72 mL (range 600 to 800) per patient, and the total volume of injected BM-MNCs was 45±7 mL (range 30 to 50) per patient. Total number of injected BM-MNCs was $3.5\pm0.8\times10^9$ (range 2.0 to 4.7×10^9), and that of $CD34^+$ cells was $6.8\pm2.6\times10^7$ (range 2.4 to 9.7×10^7).

TABLE 1. Patient Characteristics

Patient	Age	Sex	Fontaine Stage	Previous Treatment	DM	HT	HLP	Smoking	BM-MNC ($\times10^9$)	CD34$^+$ in BM-MNC ($\times10^7$)	ABI, Baseline	ABI, 1 Month	VAS, Baseline	VAS, 1 Month
1	63	M	III(lt)	Bypass graft	−	−	+	+	3.0	6.6	0.34	0.55	5	0
2	31	M	IV(rt)	Medical	−	−	−	+	4.7	9.7	0.49	0.39	5	1
3	52	M	IV(lt)	Medical	−	−	−	+	4.1	9.0	0.65	0.67	7	2
4	28	M	IV(lt)	Sympathetic ganglion block	−	−	−	+	2.0	6.8	0.50	0.26	5	0
5	32	M	IV(rt)	Medical	−	−	−	+	3.8	2.4	−	−	5	2
			IV(lt)								−	−	5	2
6	55	F	IV(lt)	Medical	+	+	−	−	3.4	4.0	0.53	0.51	4	3
7	63	M	III(rt)	Medical	−	+	−	+	3.0	9.1	1.10	0.91	5	3
			IV(lt)								0.76	0.94	5	3
8	43	M	IV(rt)	Medical	−	+	−	+	3.6	6.8	1.00	1.04	5	0
			III(lt)								1.00	1.07	5	0

DM indicates diabetes mellitus; HT, hypertension; and HLP, hyperlipidemia.

Angiographic assessment at 4 weeks after transplantation revealed an apparent increase in limb vascularity in 3 of the 8 (38%) patients (4 of the 11 limbs) (Figure 1). Hemodynamic assessment also failed to document evidence of improved collateral development. Specifically, an increase in ABI (>0.1) was observed in 2 of 7 (29%) patients (2 of 8 limbs), whereas a decrease in ABI (>0.1) was observed in 2 of 7 (29%) patients (2 of 8 limbs). As a result, mean ABI measured at 4 weeks (0.71±0.30) did not differ from that at the baseline (0.70±0.27). Because 2 patients had sites of arterial occlusion distal to the ankle, they showed normal ABIs before treatment. Even after the exclusion of these 2 patients, ABI showed no changes between before (0.55±0.15) and after transplantation (0.55±0.24).

In contrast to the angiographic and hemodynamic results, improvement in limb status was observed in all 8 patients (100%). Improvement in VAS was observed in all 11 limbs, with a decrease from a mean of 5.1±0.7 to 1.5±1.3. Furthermore, complete pain relief was achieved in 4 of the 11 limbs (36%). Improvement in skin ulcers was also observed in all 8 limbs (100%), with complete healing in 7 (88%). Although surgical amputations of the distal limb were performed in 2 patients at 1 month, these operations were intentionally scheduled to be performed after transplantation with the expectation of sufficiently improving the limb perfusion to distally advance the site of amputation (Table 2; Figure 2A and 2B).

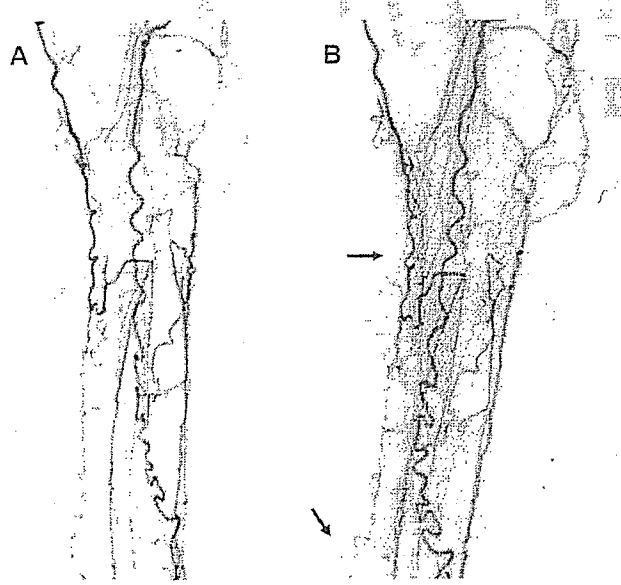

Figure 1. Digital subtraction angiography at (A) baseline and (B) 1 month after cell transplantation. Arrows indicate newly visible collateral vessels at the calf level.

Long-Term Outcome

The mean follow-up period was 684±549 days (range 103 to 1466). At the final follow-up, VAS score remained unchanged from that observed at 1 month after transplantation in 5 of the 8 patients (63%). The mean VAS score at follow-up also remained low (2.3±1.9) compared with that observed at baseline (5.1±0.7).

In contrast to the pain scale results, adverse events were observed in as many as 4 patients (50%) (Table 2). At age 30

TABLE 2. Adverse Outcomes After Autologous Transplantation of BM-MNCs in Patients With Thromboangiitis Obliterans

Adverse Outcomes	30 Days	Final Follow-Up
Death	0	1 (13)
Major amputation	0	0
Minor amputation	2 (25)*	0
Unexpected angiogenesis	0	1 (13)
Recurrence/worsening of skin ulcer/gangrene	0	2 (25)†
Recurrence/worsening of pain	0	1 (13)
Cardiovascular event	0	0
Cerebrovascular event	0	0
Malignancy	0	0

Values are expressed as n (%).

*Amputation was intentionally scheduled to be performed at 1 month after transplantation.

†One patient was the same one who developed unexpected angiogenesis.

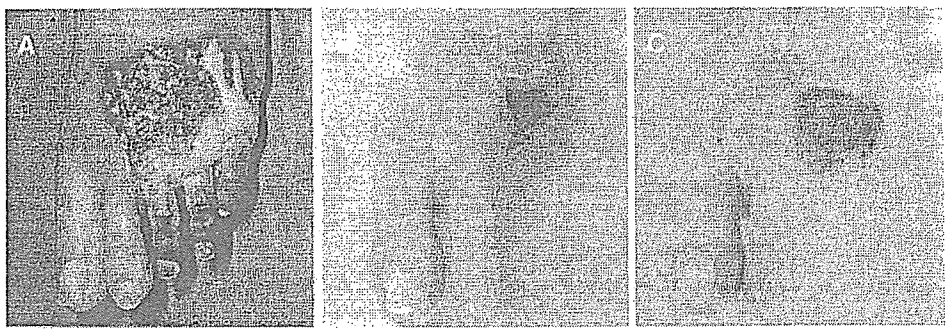

Figure 2. Skin ulcer at (A) 1 month, (B) 2 months, and (C) 4 months after cell transplantation. The patient had far-advanced gangrene, and total limb integrity could not be fully preserved. The patient received a prescheduled amputation of the distal limb at 1 month, and the skin ulcer continued to improve thereafter. At 4 months, however, the skin lesion began to enlarge.

years, patient 4 suddenly died of an unknown cause at 20 months after transplantation. This patient had previously been a smoker, but had stopped smoking before cell transplantation. He had no history of diabetes, hypertension, or hyperlipidemia. Furthermore, ^{201}thallium myocardial scan performed before BM-MNC transplantation showed no signs of myocardial ischemia. After cell transplantation, his limb pain disappeared within 1 week and his skin ulcer resolved by 1 month. Thereafter, he was completely free of limb symptoms. Twenty months after cell transplantation, however, he was found dead at his home. He had never experienced chest pain up to the time of his death. Because no autopsy was performed, the cause of his death remains unknown.

Patient 6 showed worsening of an ischemic ulcer at 4 months. The patient had far-advanced gangrene, and total limb integrity could not be fully preserved. The patient underwent a prescheduled amputation of the distal limb at 1 month (see Short-Term Outcome) (Figure 2A), and the skin ulcer continued to improve thereafter (Figure 2B). At 4 months, however, the skin lesion began to increase in size (Figure 2C). The patient subsequently received a second round of cell therapy.

In patient 7, despite complete healing of the skin ulcer, rest pain did not completely resolve after transplantation, with a VAS score of 3 at 1 month. At 8 months, the patient experienced worsening of rest pain (VAS score=4). After a combination of exercise training and maximal drug therapy, the pain improved and became well tolerated.

Patient 8 experienced swelling and recurrence of the skin ulcer in his foot at 7 months. Computed tomographic angiography documented an early venous return of contrast material in his right limb (Figure 3B) that was not observed at the baseline (Figure 3A). Ultrasound examination disclosed an arterialized waveform in the dorsal vein at the base of his third toe, suggesting the presence of an arteriovenous shunt. By 1 year, the swelling and skin ulcer had spontaneously regressed. The systolic pulsatile component in the venous waveform was found to be diminished on ultrasound examination, and early venous filling had disappeared on computed tomographic angiography (Figure 3C).

Discussion

In the present unblinded and uncontrolled pilot study, we documented that the transplantation of BM-MNCs was associated with an improvement in ischemic symptoms for up to 4 years. Indeed, VAS scores improved from 5.1±0.7 to 2.3±1.9 at follow-up. Furthermore, skin ulcers remained completely healed in 6 of 7 patients. In this regard, the present findings extend previous observations[11] by establishing the potential long-term benefit of BM-MNC transplantation for the treatment of arterial insufficiency.

It should be noted, however, that half of the patients suffered adverse events during follow-up. Such a high rate of adverse events cannot be explained by the natural course of the disease itself. In general, the prognosis of patients with thromboangiitis obliterans is directly related to tobacco

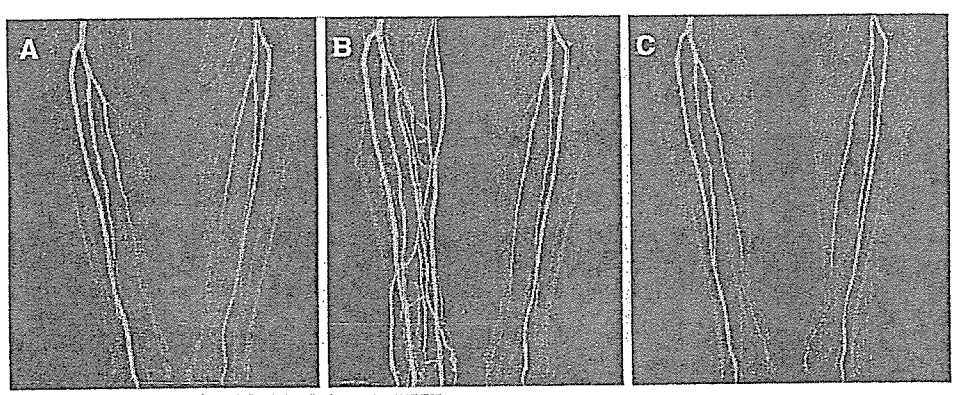

Figure 3. Computed tomographic angiography at (A) baseline, (B) 7 months, and (C) 1 year after cell transplantation. Early venous return of contrast material observed at 7 months spontaneously regressed by 1 year.