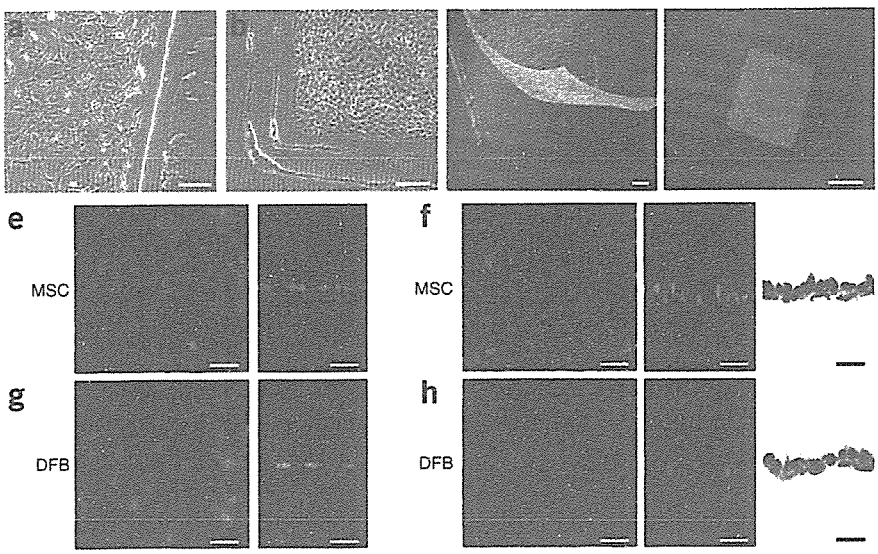

Figure 1 Preparation of monolayered MSCs. (**a**) MSCs 2 d after seeding on a temperature-responsive dish. (**b**) Cultured MSCs expanded to confluence within the square area of the dish by day 3. (**c**) The monolayered MSCs detached easily from the culture dish at 20 °C. (**d**) The completely detached 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

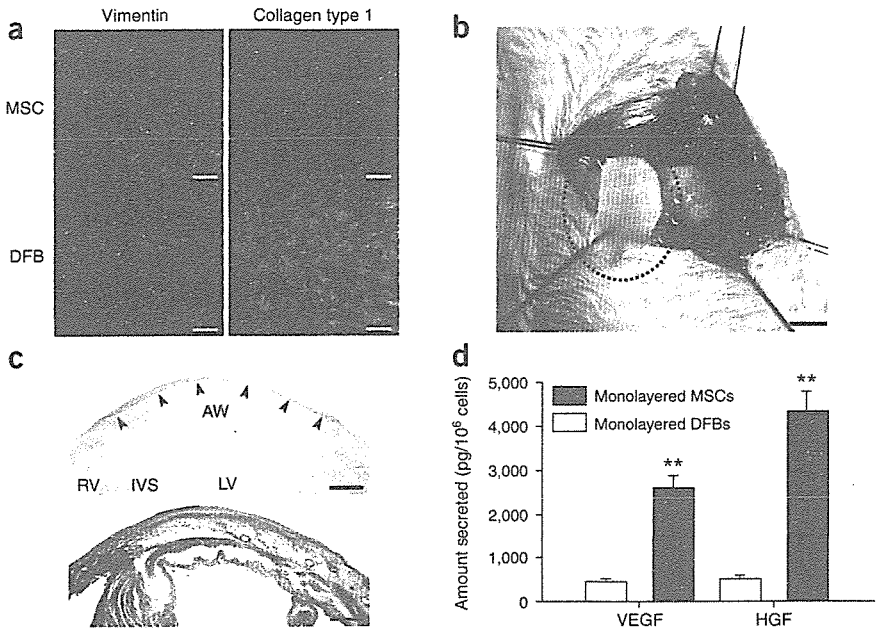

Figure 2 Characteristics of monolayered MSCs. (**a**) Properties of constituent cells in the monolayered grafts. Compared with DFBs (green), MSCs (green) are positive for vimentin (red) and slightly positive for collagen type 1 (red). (**b**) Monolayered MSCs (in the dotted circle) transferred to the 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

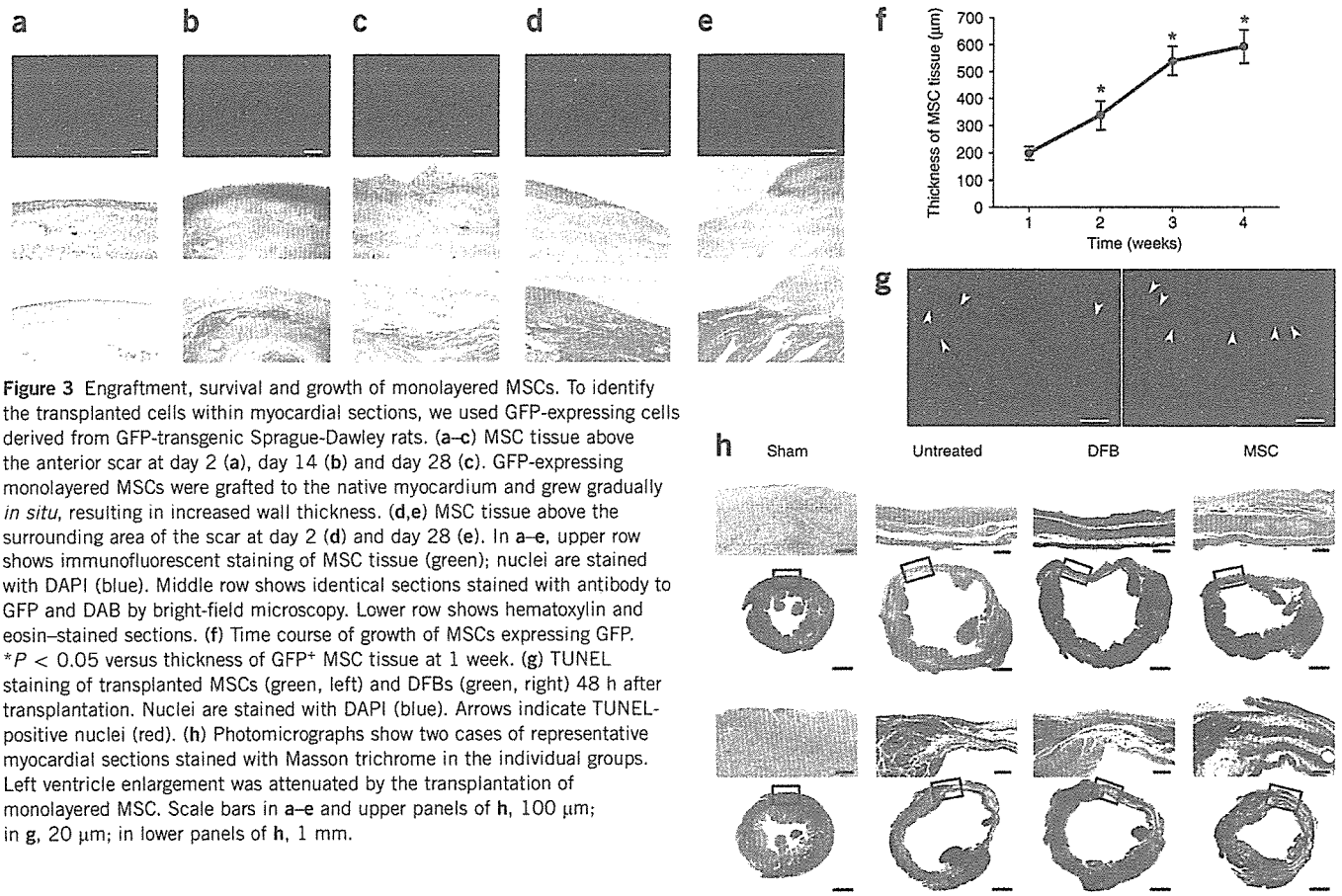

Figure 3 Engraftment, survival and growth of monolayered MSCs. To identify the transplanted cells within myocardial sections, we used GFP-expressing cells derived from GFP-transgenic Sprague-Dawley rats. (**a–c**) MSC tissue above the anterior scar at day 2 (**a**), day 14 (**b**) and day 28 (**c**). 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.

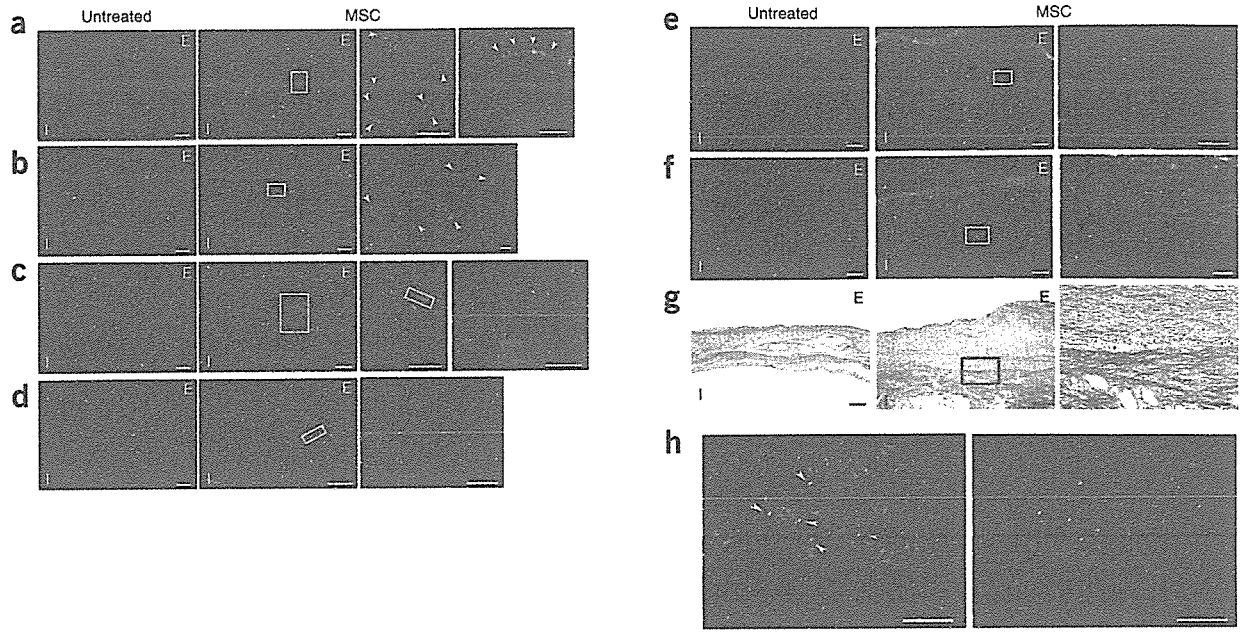

Figure 4 Differentiation of MSCs within the MSC tissue after growth *in situ*. (**a**,**b**) GFP-expressing MSCs (green) were identified as a thick stratum at the epicardial side of the myocardium. The MSC tissue contained a number of vascular structures positive for vWF (red, **a**) and αSMA (red, **b**). 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

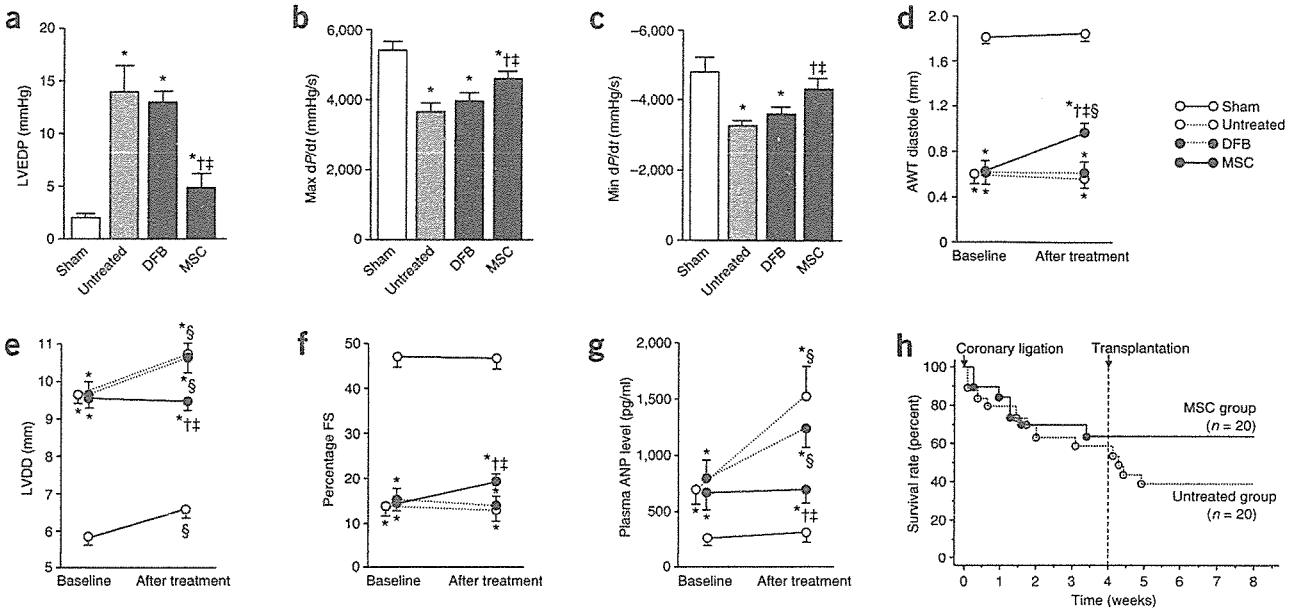

Figure 5 Cardiac structure and function after transplantation of monolayered MSCs. (**a–c**) Hemodynamic parameters obtained by catheterization. LVEDP, left ventricle end-diastolic pressure. (**d–f**) Echocardiographic findings. AWT, anterior wall thickness; LVDD, left ventricle end-diastolic dimension; 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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Technical Report

Assessment of Viability and Osteogenic Ability of Human Mesenchymal Stem Cells After Being Stored in Suspension for Clinical Transplantation

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ABSTRACT

Human mesenchymal stem cells (MSCs) were suspended in phosphate-buffered saline (PBS) and stored up to 24 h at 4°C, 24°C, and 37°C. More than 80% viability was maintained at any temperature for at least 1 h, then gradually decreased over time. After 24 h, the viabilities at 4°C, 24°C, and 37°C were about 81%, 70%, and 62%, respectively. The MSCs suspended/stored in PBS at 4°C for 24 h also exhibited *in vitro* osteogenic differentiation capability as evidenced by mineralized matrix formation as well as high alkaline phosphatase activity when cultured in an osteogenic medium. Furthermore, *in vivo* implantation experiments using the MSCs also demonstrated new bone formation. Because MSCs are known to possess multipotential stem cell characteristics, these data indicate that human MSCs stored in PBS at 4°C could be delivered to distant medical facilities for the purpose of hard tissue and other types of tissue regeneration therapy.

INTRODUCTION

MARROW STROMAL STEM CELLS,[1] recently termed "mesenchymal stem cells (MSCs)"[2] are highly proliferative cells that can be maintained in culture and readily differentiated into mesodermal cell types (fat, bone, cartilage and muscle).[1–3] Many researchers including us have previously reported that human MSCs derived from bone marrow differentiate into osteoblasts by *in vivo* implantation.[4,5] The osteogenic potential of MSCs has already been applied in clinical situations. Horwitz used allogenic MSCs for the treatment of osteogenesis imperfecta,[6] and Quarto *et al.*[7] used autogenous MSCs/ceramic composites for treatments of fractures. The MSCs can also differentiate into osteoblasts by *in vitro* culture with dexamethasone (Dex).[8–10] The *in vitro* differentiation resulted in osteoblastic phenotype expression of MSCs, and the osteoblasts formed extracellular bone matrices with abundant minerals on various culture substrata including ceramic surfaces.[11,12] The *in vitro*-formed osteoblasts/bone matrix on ceramic surface has been used for the treatment of osteoarthritic cases.[13,14] Recent studies have

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also shown the possibility that MSCs can differentiate not only into the variety of mesodermal cells, but also into either ectodermal or endodermal cells.[15–20] This ability indicates the usefulness of MSCs for tissue engineering.

As therapies advanced from research laboratories to actual clinical applications, tissue regeneration using MSCs should be considered from practical viewpoints such as the maintenance and transportation of highly functional MSCs. However, optimal storage conditions have not been extensively investigated, especially for MSCs stored in liquid suspension, which could be available as ready-to-use cellular devices.

In this study, we investigated the viability of MSCs suspended in phosphate-buffered saline (PBS) at three different temperatures after storage from 0 to 24 h. We also observed the cell surface antigens and further examined the *in vitro* as well as *in vivo* osteogenic differentiation capability of the MSCs.

MATERIALS AND METHODS

Preparation and culture of marrow cells

Human bone marrow was aspirated from the iliac crest of three donors (16-year-old girl, 26-year-old man, and 29-year-old woman) with informed consent. Three milliliters of the marrow aspirates were immediately collected into a syringe containing 3 mL of PBS (phosphate buffered saline minus Ca^{2+} and Mg^{2+}, Invitrogen Corp., Carlsbad, CA) composed of 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8.0 g/L NaCl, and 2.16 g/L Na_2HPO_4 and 30 IU of heparin. After centrifugation at 140 ×g for 10 min at 4°C, the supernatants of the plasma and fat layers were discarded. The remaining nucleated cells with the red blood cell layer were seeded/divided into two T-75 flasks (Becton Dickinson Co., NJ) with 15 mL of medium. The culture medium was Eagle's minimum essential medium alpha (α-MEM, Invitrogen Corp.) containing 15% fetal bovine serum (FBS, JRH Biosciences Inc., KS) and antibiotics (100 U/mL penicillin G, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B; Sigma-Aldrich Corp., MO). Primary cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Culture media were renewed two or three times per week. At each medium change, nonadherent red blood cells and hematopoietic cells could be removed. After 10 days, adherent cells became almost confluent.[14]

The adherent cells showed high capability for proliferation and differentiation. Analyses of the cell surface antigens revealed the absence of hematopoietic markers but the presence of mesenchymal cell markers.[21] Therefore, we refer to the cells as mesenchymal stem cells (MSCs) in this article. The cells were released from the substrates using a solution of 0.05 (w/v)% trypsin/0.53 mM EDTA (Invitrogen Corp). The harvested cells were centrifuged to concentrate at a density of 5×10^5 cells/mL in solution for cryopreservation (Cell Banker, Juji Field, Inc., Tokyo, Japan) and frozen at –80°C before use. After thawing, the cryopreserved MSCs attached and proliferated well on the culture dish surface, and their proliferation/differentiation capability was comparable to that of noncryopreserved MSCs.[22]

Cell viability assay

The viability of MSCs was assayed using a NucleoCounter (ChemoMetec, Allerød, Denmark), an instrument for counting mammalian cells, which was equipped with a fluorescence microscope. The cell count system is based on propidium iodide (PI) staining[23] to detect nonviable cells, because the PI only penetrates cells having damaged membranes and binds to DNA. After an additional procedure of complete cell lyses, the PI can bind to DNA in all cells, thus enabling determination of the total number of cells.

After immediate thawing of the frozen MSCs, 5×10^5 of the cells were cultured in a 90-mm^2 tissue culture dish for 7 to 10 days to reach near confluence. After the cells were released by trypsin digestion, the cells at a concentration of 1×10^6 cells/mL were suspended in 600 μL of PBS, and stored for periods from 0 h (immediately used) to 24 h at 4°C, 24°C, and 37°C. To determine viability, 40 μL of each sample diluted five times with PBS was analyzed using the NucleoCounter, giving an estimate of nonviable and total cells.

Although the NucleoCounter provides information of nonviable and total cell number, direct visualization of viable and nonviable cells at the cellular level cannot be

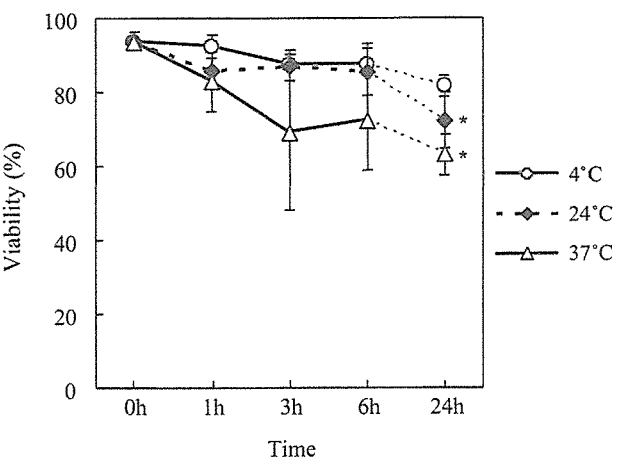

FIG. 1. Percent of viable MSCs stored in PBS at 4°C, 24°C, and 37°C from 0 h to 24 h. The data represent the mean ± SD of the six samples. *$p < 0.05$; significant difference against the viability of the cells stored in PBS at 4°C.

done. To visualize the cells, a LIVE/DEAD Viability Assay Kit (Molecular Probes, Inc., OR) was used to determine the numbers of living and dead cells at the same moment with calcein-AM for intracellular esterase and ethidium homodimer-1(EthD-1) for plasma membrane integrity.

MSCs suspended/stored in PBS at 4°C, 24°C, and 37°C were sampled in the dishes at each time point (0 h, 1 h, 3 h, 6 h, and 24 h), and 2 mM of calcein-AM and 5 mM of EthD-1 in PBS were directly added to the sampled cells at room temperature. After 15 min, the samples were observed by using a fluorescence microscope (IX70, OLYMPUS Co. Ltd., Tokyo, Japan).

Immunostaining and FACS analysis

MSCs suspended/stored in PBS at 4°C, 24°C or 37°C for 24 h were diluted into 1.5-mL centrifuge tubes and incubated with antibodies on ice for 15 min. The cells were pelleted, washed twice in PBS, and analyzed by a FACSCalibur flow cytometer (Becton Dickinson Co.). The antibodies used were CD13-FITC (fluorescein isothiocyanate), CD45-FITC (BIOCARTA Europe, Hamburg, Germany), and CD34-FITC (CARTAG Laboratories, CA).

Osteogenic differentiation assay

MSCs suspended/stored in PBS at 4°C for each time period (0, 1, 3, 6, and 24 h) were seeded at a cell density of 1×10^4 cells/cm^2 in a 12-well culture plate and cultured in media supplemented with 15% FBS, 10 mM β-glycerophosphate (affiliate of Merck KGaA, Darmstadt, Germany), disodium salt, 0.07 mM L-ascorbic acid phosphate (Sigma-Aldrich Corp.), and 0.1 μM dexamethasone (Dex) (Sigma-Aldrich Corp.) for 2 or 3 weeks. Because Dex is known as an osteogenic factor to MSCs,[8,9,21,22] cultures without Dex were used as a negative control. To enable the detection of the mineralized extracellular matrix, 1 μg/mL of calcein (Dojindo Laboratories, Kumamoto, Japan) was added at every media change. After culture for 14 days, the cell layers were washed twice with

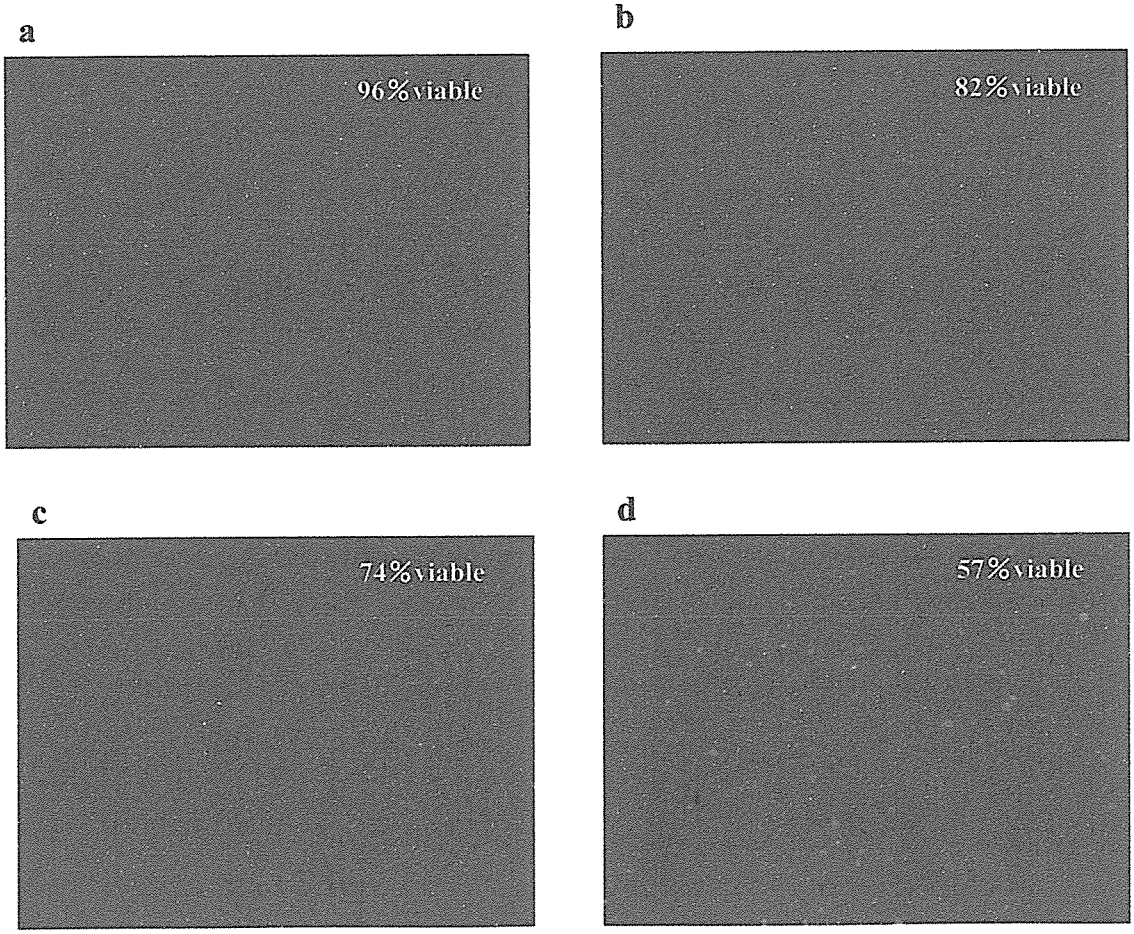

FIG. 2. Fluorescence microscopic view of MSCs stored in PBS after LIVE/DEAD Viability assay. **(a)** Immediately visualized with no storage, **(b)** stored at 4°C for 24 h, **(c)** stored at 24°C for 24 h, and **(d)** stored at 37°C for 24 h. Green fluorescent signal indicates living cells and red fluorescent signal indicates dead cells.

PBS, and then the fluorescence of the deposited calcein was visualized and quantified by using an image analyzer (Typhoon 8600, Molecular Dynamics, Inc., Sunnyvale, CA).[24] The fluorescent intensities parallel well the contents of calcium in the mineralized matrix.[24] Human fibroblasts (2F0-C25, Cell Systems, CA) were also used as negative controls against MSCs. Human fibroblasts stored at 4°C for 24 h were seeded at a cell density of 1×10^4 cells/cm^2 in a 12-well culture plate and cultured in media comprising 15% FBS, 10 mM β-glycerophosphate, disodium salt, 0.07 mM L-ascorbic acid phosphate, 0.1 μM Dex, and 1 μg/mL calcein. After culture for 21 days, the cell layers were washed twice with PBS, and the fluorescence of the deposited calcein was visualized and quantified by using an image analyzer.

To measure ALP activity, the cell layers from each well were collected into 0.5 mL of 10 mM Tris-buffer (pH 7.4, 1 mM EDTA, 100 mM NaCl) by scraping. The scraped cells were then sonicated and centrifuged at 13,000 ×g for 1 min at 4°C. An aliquot (20 μL) of the supernatant was assayed for ALP (alkaline phosphatase) activity using a *p*-nitrophenyl phosphate substrate (Zymed Laboratories Inc., CA).[9,11] The ALP activity was represented by *p*-nitrophenol, which was released after incubation for 30 min at 37°C. In the case of *in vivo* study, implants as described below were homogenized and sonicated in the above buffer solution prior to the ALP activity assay.

Implantation of MSCs/ HA constructs into athymic nude rats

MSCs suspended/stored in PBS at 4°C for 0 h or 24 h at a cell density of 1×10^6 cells/mL were added to porous hydroxyapatite (HA) disks of 5 mm in diameter (Apaceram, Pentax Corp., Tokyo, Japan) and incubated at 37°C for 3 h. The HA disks were transferred to 24-well Falcon tissue culture dishes and subcultured in media containing 15% FBS, 10 mM β-glycerophosphate, disodium salt, 0.07 mM L-ascorbic acid phosphate, and 0.1 μM Dex for 2 weeks for fabrication of MSCs/HA constructs. Seven-week-old male athymic nude Fischer 344 rats (Fischer 344/N Jcl-rnu, Clea Japan, Inc., Tokyo, Japan) were anesthetized by intramuscular injection of pentobarbital (Nembutal, Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) at a final concentration of 3.5 mg/100 g body weight. Six MSCs/HA constructs and two HA disks without cells were implanted subcutaneously into the back of each athymic nude rat; three constructs fabricated from MSCs stored for 0 h and one HA disk without cells were implanted in the right side, and the other three constructs fabricated from MSCs stored for 24 h and one HA disk without cells were separately implanted in the left side. The recipient rats used were two. HA disks without cells were used as negative controls. Animal experiments were carried out in compliance with Japanese Law (No. 105) on animal protection and administration as well as the Regulation on the Imple-

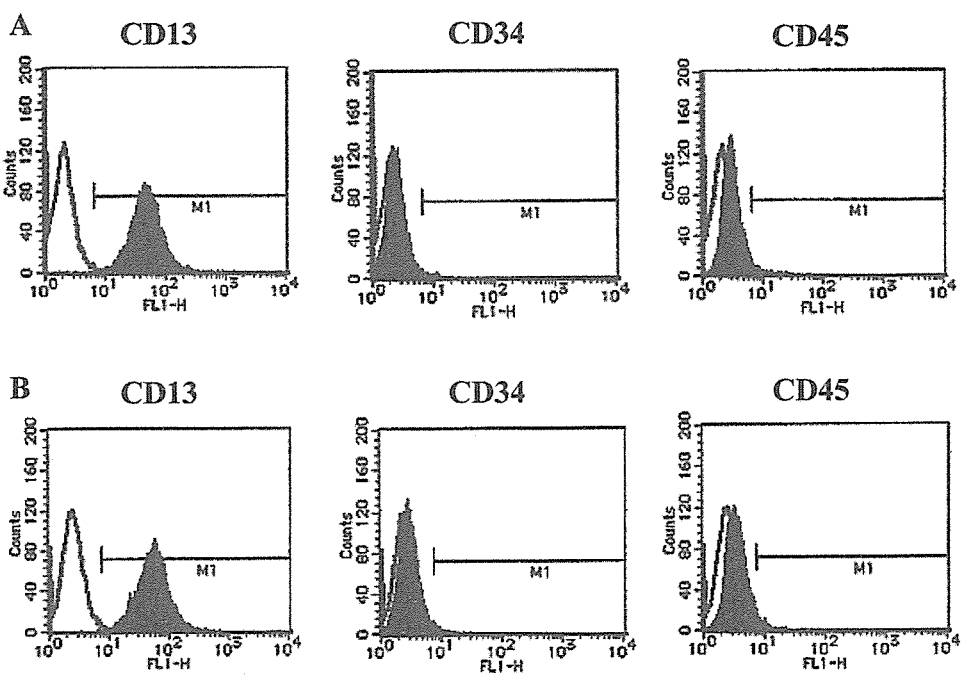

FIG. 3. FACS analysis of MSCs (**A**) and MSCs stored in PBS at 4°C for 24 h (**B**). Both cell types were reacted with each CD antibody and then loaded into a flow cytometer. The open histograms show the fluorescence intensity of the cells with negative control IgG. The closed histograms show the fluorescence intensity of the cells with each CD antibody.

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Analysis of the implants

All implants (MSCs/HA constructs and HA disks without cells) were harvested 6 weeks postimplantation. Each of four constructs fabricated from MSCs stored for 0 h and 24 h, respectively, was used for ALP activity assay as described above. The other constructs and HA disks without cells were fixed with 10% buffered formalin, decalcified with K-CX solution (Falma Co., Tokyo, Japan), and then stained with hematoxylin and eosin. These specimens were examined by light microscopy.

Statistics

All the data were analyzed for statistical significance using Student's *t*-test computed by JMP 5.0 software (SAS Institute, Inc.), and statistical significance was accepted at $p < 0.05$. Experimental results were expressed as the means ± standard deviation (SD) of the mean.

RESULTS

Viability of MSCs suspended/stored in PBS

After culturing MSCs in 90-mm^2 tissue culture dishes for about 10 days, the cells were trypsinized and suspended with PBS at a concentration of 1×10^6 cells/mL and stored at 4°C, 24°C, and 37°C. The viability (percent of total cells that were viable) of the MSCs in the PBS was assayed by a NucleoCounter. The assay was done at different storage intervals (from 0 to 24 h). Cell viability was maintained at more than 80% at any temperature of 4°C, 24°C, and 37°C after 1 h, then gradually decreased over time. The decrease was most apparent for the MSCs stored at 37°C, showing about 61% viability after 24 h. At 4°C and 24°C after 6 h, viabilities were both about 85% and those after 24 h were about 81% and 70%, respectively (Fig. 1). Concerning the cells stored for 24 h, statistical differences are seen among the percent viabilities at 4°C, 24°C, and 37°C.

The viabilities at the single-cell level were visualized by a LIVE/DEAD Viability Assay Kit. As shown in Fig. 2, most cells were green (viable cells) and only a few

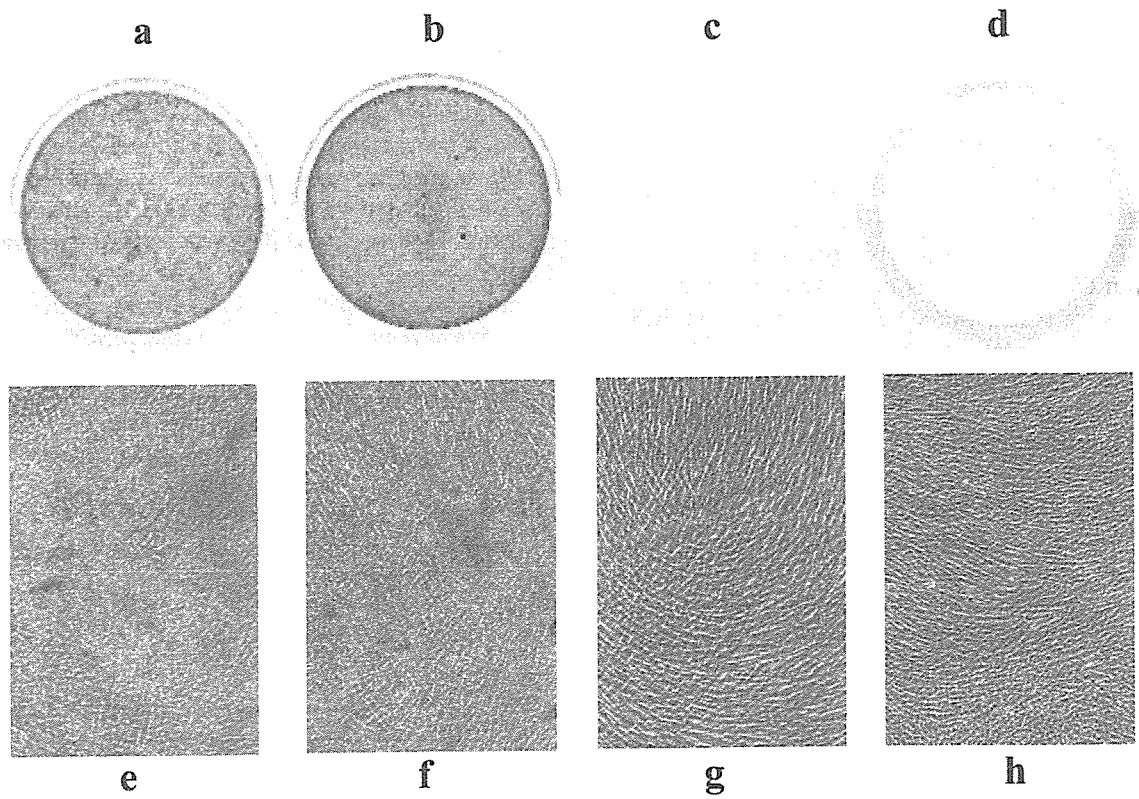

FIG. 4. Mineralization of MSCs on culture dishes. MSCs (**a, b, c, e, f,** and **g**) and human fibroblasts (d and h) were cultured in the presence of Dex (a, b, **d,** e, f, and **h**) or absence of Dex (c and g) for 21 days in 12-well plates. Culture medium contained glycerophosphate and calcium binding fluorescent dye of calcein. After being washed with PBS, the culture dish was visualized using an image analyzer. Fluorescence uptake (a, b, c, and d) and phase contrast view of culture (e, f, g, and h) are shown. MSCs stored in PBS at 4°C for 0 h (a and e) and 24 h (b, c, f, and g) were used for the culture. Black dots (a and b) indicate calcium deposition by fluorescence intensity. (Color images are available at <www.liebertpub.com/ten>.)

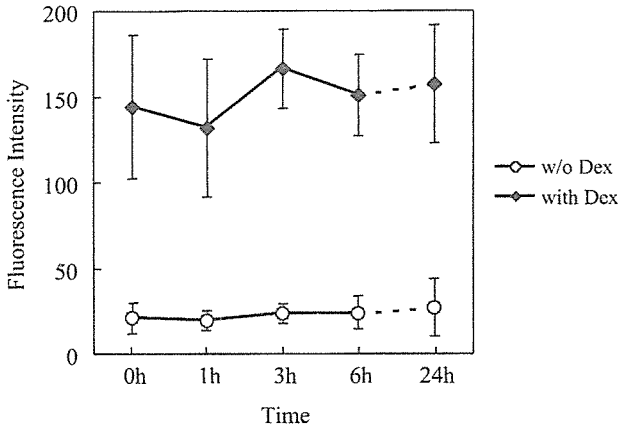

FIG. 5. Fluorescence intensity of cultured MSCs after being stored in PBS at 4°C from 0 h (immediately seeded) to 24 h. The culture occurred in the presence or absence of Dex for 14 days. The data represent the mean ± SD of the six samples.

cells were red (nonviable cells) in cultured MSCs before suspension in PBS. After the MSCs suspension/storage in PBS for 24 h at 4°C, 24°C, and 37°C, the number of green cells decreased, indicating a loss of viability. The percents of green cells (viable) at 4°C, 24°C, and 37°C were 82, 74, and 57, respectively. These data were comparable with those assayed by NucleoCounter (Fig. 1).

Immunostaining and FACS analysis

MSCs suspended/stored in PBS at 4°C after 24 h were analyzed by a flow cytometer for the expressions of CD13, CD34, and CD45 surface antigens and were compared with these expressions of MSCs without storage. Representative results are shown in Fig. 3. Both MSC samples showed similar patterns, that is, cells from both samples were not positive for the expression of hematopoietic markers of CD34 and 45, but strongly positive for CD13 (Aminopeptidase N), which is known to be present in MSCs.[25]

MSCs suspended/stored in PBS at 24°C and 37°C for 24 h also showed similar expression patterns (data are not shown). These results showed that the cell surface expression patterns had not been changed after being suspended/stored for 24 h. Only living cells were gated and analyzed.

Differentiation assay

MSCs suspended/stored in PBS at 4°C for 0 h (immediately used) to 24 h were seeded on a 12-well culture plate and cultured in osteogenic medium. The medium contained β-glycerophosphate, L-ascorbic acid 2-phosphate and, importantly, Dex, which is known to induce undifferentiated MSCs into osteoblasts,[8,21,22] resulting in the formation of a mineralized matrix. As we previously reported, the mineralization (bone matrix formation) could be detected after about 10 to 14 days of culture and more obviously after 21 days.[8,21] As shown in Fig. 4, the culture with Dex for 21 days showed mineralization (amorphous brown color in Fig. 4e and f), which was confirmed by calcein uptake (black areas in Fig. 4a and b). After storage in PBS, the mineralization capacity of MSCs (Fig. 4b and f) was comparable to that without storage (Fig. 4a and e). In contrast, the culture without Dex showed no mineralization (Fig. 4c and g).

Because fibroblasts never exhibit mineralization even culturing with Dex,[24] we also cultured human fibroblasts in the presence Dex as negative controls of differentiation assays. As shown in Fig. 4d and g, human fibroblasts stored at 4°C for 24 h before culture did not show mineralization. These qualitative results indicate that storage at 4°C for 24 h does not affect the osteoblastic differentiation capability of MSCs.

To demonstrate quantitative data, we measured the amount of mineralization and ALP activity, which localizes at the cell membranes of osteoblasts.[26] As shown in Fig. 5, the amounts of mineralization detected by fluorescent uptake of calcein were much higher for the culture in the presence of Dex compared with the culture in the absence of Dex. The high uptake was seen by culturing MSCs suspended/stored in PBS for 0 h to 24 h. The data coincided well with the data of ALP (Fig. 6), which also demonstrated the high ALP activity of MSCs cultured in the presence of Dex. The high level was also detected by culturing MSCs suspended/stored in PBS for 0 to 24 h. As shown in the results, MSCs maintained a high level of osteogenic ability *in vitro* after storage at 4°C for 24 h.

Next, we addressed the *in vivo* ability of MSCs after storage. We have previously reported that MSCs/HA con-

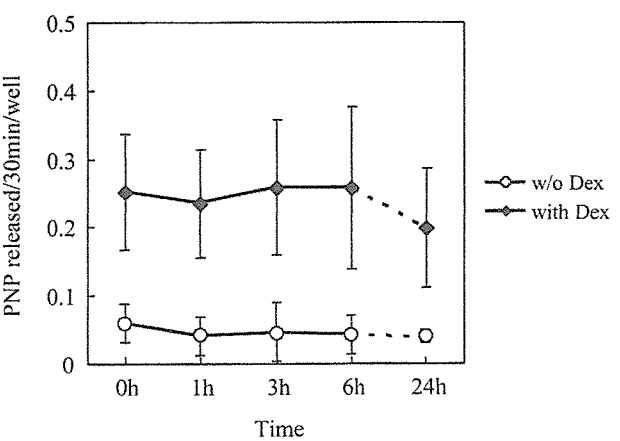

FIG. 6. ALP activity of cultured MSCs after being stored in PBS at 4°C from 0 h (immediately seeded) to 24 h. The culture occurred in the presence or absence of Dex for 14 days. The data represent the mean ± SD of the six samples (*p*-nitrophenol release/30 min/well).

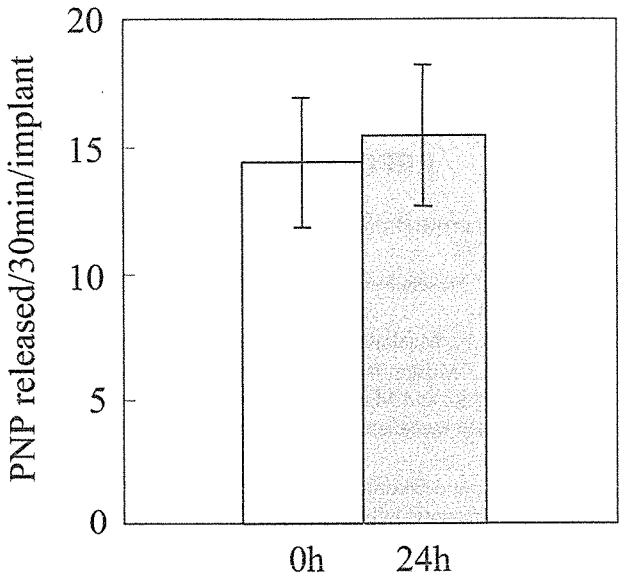

FIG. 7. ALP activity of MSCs/HA constructs after 6-week implantation. MSCs stored at 4°C for 0 h (open bar) or 24 h (closed bar) were cultured on HA disks with Dex for 2 weeks. The MSCs/HA constructs were implanted into athymic nude rats. After 6-week implantation, ALP activity of the constructs was measured. The data represent the mean ± SD of four samples (*p*-nitrophenol released/30 min/implant). (Color images are available at <www.liebertpub.com/ten>.)

structs treated with Dex could show a high level of osteogenic ability after *in vivo* implantation.[27] According to the methods, we prepared the constructs fabricated from MSCs stored at 4°C for 0 h or for 24 h and also HA disks without cells as negative controls, then implanted at subcutaneous sites of athymic nude rats. After 6-week implantation, both constructs showed similar high ALP activity (Fig. 7). Histologic appearance also demonstrated that both constructs showed newly formed bone with active osteoblasts (Fig. 8). However, implants of HA disks without cells did not show any bone formation (Fig. 8).

From these results, we concluded that MSCs suspended/stored in PBS at 4°C up to 24 h could maintain a high level of viability and capability of differentiation *in vitro* as well as *in vivo*.

DISCUSSION

Mesenchymal stem cells (MSCs) can differentiate into osteogenic lineage, and the osteogenic potential of MSCs has already been applied in clinical situations.[6,7,14] Recent studies have also demonstrated the possibility that MSCs can differentiate into other types of tissue-specific cells such as cardiac myoblasts,[15,16] vascular endothelial cells,[17,18] hepatocytes,[19] and neural cells.[20] These results indicate the usefulness of multipotential MSCs for a wide range of tissue engineering purposes in regenerative medicine. In considering the clinical applications of MSCs, the number of MSCs in bone marrow is extremely low,[2,13] and thus a procedure for culture expansion of MSCs is needed. However, such procedures introduce risks for bacterial/fungal contamination. To avoid these risks, biologically safe areas such as a cell processing center (CPC) having clean rooms and careful/safe handling are required. However, it is difficult to provide these facilities in many hospitals, primarily because of limited finances. Therefore, there has been an attempt to establish CPCs, which are available upon request by hospitals. This situation requires delivery of the cultured MSCs from the CPC to the hospital in need. The MSCs must be stored in optimal conditions that guarantee their viability as well as differentiation capability for a specific period.

When hematopoietic cells are stored in suspension, a temperature of around 4°C is recommended to maintain cell viability and function.[28] However, there is no simi-

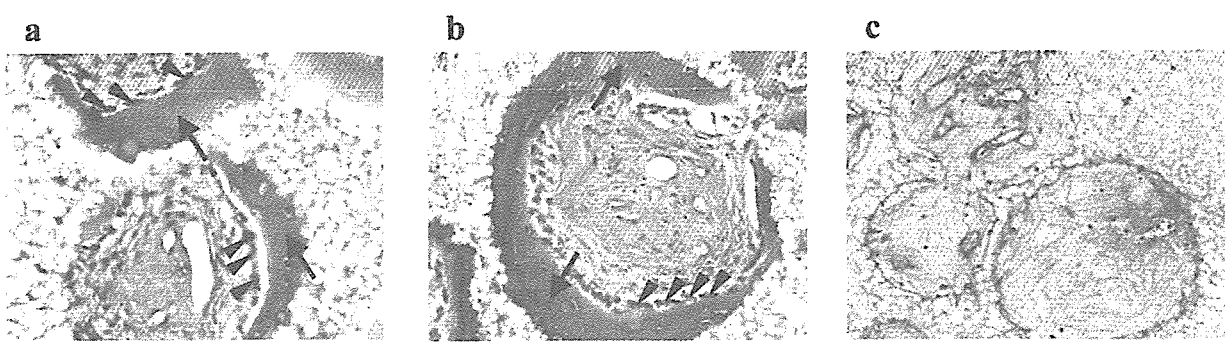

FIG. 8. Histological appearance of MSCs/HA constructs after 6-week implantation. MSCs stored at 4°C for 0 h (**a**) or 24 h (**b**) were cultured on HA disks with Dex for 2 weeks. The MSCs/HA constructs were implanted into athymic nude rats. After 6-week implantation, the constructs were decalcified and stained with hematoxylin and eosin. HA disks without MSCs were implanted as negative controls (**c**). The light microscopy was performed. Arrows show bone matrices in porous areas of HA. Arrowheads show the bone forming active osteoblasts. Original magnification ×200.

lar information about the optimal temperature and conditions for maintaining the viability of adherent cell MSCs. This study focused on investigating viability assessment for successful transportation of MSCs in suspension based on three major factors: incubation time, type of storage medium, and temperature. We also examined osteogenic differentiation capability of MSCs stored in suspension.

Our preliminary data showed that there was no difference of MSC viability in the three kinds of media, PBS, saline, and α-MEM (data not shown). Among them, the composition of PBS and saline are simpler than that of α-MEM. Saline may cause pH changes because there is no buffering action. For these reasons, in the present study we selected PBS as the storage medium for MSCs.

As shown in Fig. 1, MSCs maintained more than 85% viability at both 4°C and 24°C for 6 h, and more than 80% viability at 4°C for 24 h, whereas the viability of the cells stored at 24°C and 37°C for 24 h was significantly decreased. Because the viability at 4°C was most acceptable and it is relatively easy to regulate/maintain the temperature at 4°C, we further performed osteogenic differentiation assay *in vitro* as well as *in vivo* using the cell stored at 4°C. The MSCs suspended/stored in PBS at 4°C for 24 h did not lose their capability for *in vitro* osteogenic differentiation. This is confirmed by the high degree of mineralization and ALP activity of the MSCs after culturing in the presence of Dex (Figs. 4–6). Importantly, the mineralization and ALP activities were comparable to those of control MSCs (nonstored MSCs). The expression of cell surface antigen patterns was similar for the MSCs stored for 24 h and the control MSCs. Consistent with the *in vitro* findings, *in vivo* implants of MSCs/HA construct demonstrated high ALP activity and bone-forming capability using MSCs after the storage. These results thus demonstrated that MSCs can maintain cell viability and osteogenic capability even after 24 h when stored in PBS at 4°C. These conditions enable the delivery of MSCs to distant medical facilities without serious loss of MSC function for the various purposes of tissue regeneration, especially hard tissue regeneration.

The present data clearly showed the durability of MSCs suspended/stored in PBS and that MSCs can be available not only locally but also in distant areas without significant loss of viability as well as differentiation (especially osteogenic differentiation) capability. Therefore, this process represents an opportunity for developing new therapeutic strategies using MSCs.

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Is Extracorporeal Life Support Contraindicated in Elderly Patients?

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Background. Extracorporeal life support (ECLS) using percutaneous extracorporeal membrane oxygenation (ECMO) is now considered an important means of resuscitation for patients suffering from refractory cardiogenic shock. The indications for the use of ECLS have yet to be established, however, and its use for elderly patients is still controversial. We retrospectively evaluated the impact of ECLS on the survival of patients with cardiogenic shock to determine the validity of using ECLS in elderly patients (≥75 years of age).

Methods. Between 2000 and 2004, 91 patients were emergently placed on percutaneous ECMO. The patients were divided into two groups by age (group 1, n = 79: less than 75 years; group 2, n = 12: 75 years or older), which were compared for clinical outcome. Logistic regression analysis of the variables was performed to identify predictors of ability to be weaned from ECLS.

Results. Weaning from ECLS was achieved in 50 patients in group 1 (63.3%) and 6 patients in group 2 (50%; $p = 0.37$). Thirty-five patients in group 1 (44.3%) and 5 patients in group 2 (41.7%) were discharged from the hospital ($p = 0.86$). Logistic regression analysis revealed that patients with a body surface area of more than 1.50 m^2, patients with cardiomyopathy, and patients who underwent interventions under ECMO support were more likely to be successfully supported by ECMO.

Conclusions. Extracorporeal life support using percutaneous ECMO systems provides excellent cardiac support. It is also effective in resuscitating elderly patients, yielding hospital survival similar to that for younger patients.

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The discouraging survival rate after prolonged cardiopulmonary resuscitation with conventional methods such as external compression of the chest has prompted the development of more aggressive methods for saving the lives of patients with refractory cardiogenic shock. Since the advantages of peripherally applied cardiopulmonary bypass for the resuscitation of cardiogenic shock patients were first demonstrated [1–3], this technology has made consistent progress, with the introduction of miniaturized pumps and circuit biocompatibility. Extracorporeal life support (ECLS) using percutaneous extracorporeal membranous oxygenation (ECMO) is now considered one of the important means of resuscitating patients suffering from refractory cardiogenic shock. With developments in ECMO systems and improvement of resuscitation teams, many patients previously considered impossible to resuscitate are now being rescued. Indications for the use of ECLS have yet to be established, however, and the use of ECLS for elderly patients is still controversial. Some previous reports have included age 75 years or older as a contraindication for ECLS [4, 5].

We retrospectively evaluated the impact of ECLS on the survival of elderly patients (≥75 years of age) with profound cardiogenic shock and examined the validity of using ECLS.

Patients and Methods

Between 2000 and 2004, 91 patients were emergently placed on ECLS using percutaneous ECMO at the National Cardiovascular Center (NCVC), Osaka, Japan. The hospital records and ECLS records were retrospectively reviewed. The Ethics Committee of NCVC approved this study, and individual consent was waived because individual patients were not identified in this study.

Patients were selected by the resuscitating physician or surgeon for emergent ECLS owing to cardiac arrest refractory to advanced cardiac life support or for intractable cardiogenic shock with imminent cardiac arrest. In cases of postcardiotomy cardiogenic shock, all patients who could not be weaned from cardiopulmonary bypass in the operating room were placed on ECMO. Extracorporeal life support was contraindicated in patients with previous irreversible brain damage or patients with ob-

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Table 1. Extracorporeal Membrane Oxygenation Systems

	CICU	Capiox EBS	NCVC Unit
Centrifugal pump	Dura Flow (HPM-15)	Capiox (CX-SP45)	Jostra Rota Flow (RF32(F))
Oxygenator	Alpha Cube 4000	Capiox (CX-SX18)	MERA Excelung Prime (HPO-20WH-C)
Priming volume (mL)	280	480	550
Time for setup (min)	5	5	20
Durability	24 hours	1–3 days	3–7 days
Flow (L/min)	4	7	7
Heat exchanger	—	+	+
Heparin coating	—	+	+

Capiox EBS = Capiox emergent bypass system (Terumo, Tokyo, Japan); CICU = compact integrated cardiopulmonary bypass unit (Edwards-DIC, Tokyo, Japan); NCVC = National Cardiovascular Center.

vious pupil dilation and no light reflex that did not recover with external cardiac massage. Patients who underwent elective percutaneous ECMO-supported angioplasty or coronary artery bypass grafting were excluded. In cases of cardiomyopathy, patients who did not require right heart support were placed on the left ventricular assist system (LVAS) instead of ECMO if their condition permitted their transfer to the operating room, and these cases were also excluded from this study. The mean age of the patients was 51.5 ± 20.5 years (range, 9 to 88), and 59 (64.8%) were male.

In this series, three different ECMO systems were used based on patient requirements, and are summarized in Table 1. The compact integrated cardiopulmonary bypass unit (CICU; Edwards-DIC, Tokyo, Japan) [6] and Capiox emergent bypass system (Capiox EBS; Terumo, Tokyo, Japan) are designed for rapid induction. Their priming volumes are 280 mL and 480 mL, respectively, and each can be set up in 5 minutes. The CICU circuit is not heparin-coated and does not include a heat exchanger. Maximal flow is about 4 L/min, and it can be used for only about 24 hours. The CICU is the easiest to set up, however, and has been used in our institution by cardiologists when clinical engineers or cardiac surgeons familiar with the set-up and establishment of other units are not readily available. The Capiox EBS circuit is heparin-coated and has a heat exchanger, but can be used for only about 24 to 72 hours. When mechanical support was required for a longer duration of time, these systems were replaced by a NCVC unit consisting of a centrifugal pump (Jostra Rota Flow; Jostra Medizintechnik AG, Hirrlingen, Germany) and a heparin-coated hollow-fiber membrane oxygenator with an integral heat exchanger (MERA Excelung Prime; MERA, Tokyo, Japan). Because the priming volume of the NCVC unit is 550 mL and set-up time is about 20 minutes, it is not suitable to support patients with shock when mechanical support is needed without delay. However, its circuit is heparin-coated and the system includes a heat exchanger, and maximal flow is about 7 L/min. It can be used for about 3 to 7 days.

Cannulation was achieved peripherally by either percutaneous or cut-down placement of cannulas in the femoral artery and vein. In patients with lower extremity ischemia or for whom prolonged perfusion was required, the peripheral femoral artery was also cut down and cannulated.

During ECMO assistance, continuous injection of heparin was used to maintain an activated clotting time of 180 to 220 s.

The 91 patients were divided into two groups by age (group 1, n = 79: less than 75 years; group 2, n = 12: 75 years or more). The groups were compared for indications for the use of ECMO, ECMO management, and clinical outcomes.

To compare outcomes between the two groups, statistical analysis was performed using the unpaired Student's *t* test, with *p* values less than 0.05 considered significant. Predictors for weaning from ECMO or successful bridging to LVAS were analyzed with respect to sex, age, body surface area, diagnosis, incidence of cardiac massage or intra-aortic balloon pump use or incapability of weaning from cardiopulmonary bypass before ECMO, place of ECMO initiation, incidence of interventions during ECMO, and incidence of complications related to ECMO. Univariate logistic regression analysis of these variables was performed. For variables with a *p* value less than 0.3, multivariate logistic regression analysis was performed, with *p* value less than 0.05 considered significant.

Results

Indications and ECMO Management

Patient background and the reasons for ECLS assistance are summarized in Tables 2 and 3, respectively. Fifty-one

Table 2. Patient Background

	Demographic Value
Male	59/91 (64.8%)
Mean age (years)	51.5 ± 20.5
Mean body surface area (m^2)	1.6 ± 0.2
Place of ECMO initiation	
Operating room	36
Intensive care unit	14
Coronary care unit	31
Emergency room	7
Others	3

ECMO = extracorporeal membrane oxygenation.

Table 3. Patient Indications for ECMO Support

Indication	No. of Patients (No. of Group 2 Patients)				
	Total	Cardiac Massage Before ECMO	IABP Before ECMO	Incapable of Weaning From CPB	Interventions on ECMO
Primary cardiac failure	51 (9)	33 (8)	23 (6)	—	14 (5)
Diagnosis					
Acute myocardial infarction	18 (7)	18 (7)	12 (5)	—	10 (5)
Without free wall rupture	12 (4)	12 (4)	9 (3)		6 (2)
With free wall rupture	6 (3)	6 (3)	3 (2)		4 (3)
Myocarditis	15 (2)	8 (1)	6 (1)	—	0 (0)
Cardiomyopathy	14 (0)	3 (0)	4 (0)	—	1 (0)
Arrhythmia	2 (0)	2 (0)	0 (0)	—	1 (0)
Others	2 (0)	2 (0)	1 (0)	—	2 (0)
Postcardiotomy cardiogenic shock	37 (3)	5 (1)	15 (2)	30 (2)	1 (0)
Preceding surgery					
Coronary surgery	6 (0)	3 (0)	5 (0)	1 (0)	1 (0)
Valve surgery	8 (1)	2 (1)	2 (0)	6 (0)	0 (0)
Aortic surgery	11 (1)	0 (0)	5 (1)	11 (1)	0 (0)
Pulmonary endoarterectomy	7 (0)	0 (0)	0 (0)	7 (0)	0 (0)
Others	5 (1)	0 (0)	3 (1)	5 (1)	0 (0)
Pulmonary embolism	2 (0)	2 (0)	0 (0)	—	2 (0)
Anaphylactic shock	1 (0)	1 (0)	0 (0)	—	0 (0)

CPB = cardiopulmonary bypass; ECMO = extracorporeal membrane oxygenation; IABP = intra-aortic balloon pump.

patients needed ECLS for primary cardiac failure. Thirty-three (64.7%) required external cardiac massage before ECLS, and 23 (45.1%) required intra-aortic balloon pump before ECLS. All 18 patients with acute myocardial infarction (AMI) needed external cardiac massage before ECLS, and 6 were complicated with free wall rupture. Ten of 18 patients with AMI underwent interventions such as percutaneous coronary intervention, coronary artery bypass grafting, and release of cardiac tamponade. Thirty-seven patients needed ECLS for postcardiotomy

Table 4. Outcome

	No. of Patients (No. of Group 2 Patients)		
	Bridged to LVAS	Weaned From ECMO	Hospital Discharge
Primary cardiac failure	12 (0)	24 (6)	27 (5)
Diagnosis			
Acute myocardial infarction	0 (0)	10 (4)	7 (4)
Without free wall rupture	0 (0)	7 (2)	5 (2)
With free wall rupture	0 (0)	3 (2)	2 (2)
Myocarditis	0 (0)	11 (2)	10 (1)
Cardiomyopathy	12 (0)	1 (0)	8[a] (0)
Arrhythmia	0 (0)	2 (0)	2 (0)
Others	0 (0)	0 (0)	0 (0)
Postcardiotomy cardiogenic shock	1 (0)	17 (0)	10 (0)
Preceding surgery			
Coronary surgery	1 (0)	4 (0)	2 (0)
Valve surgery	0 (0)	5 (0)	2 (0)
Aortic surgery	0 (0)	2 (0)	0 (0)
Pulmonary endoarterectomy	0 (0)	4 (0)	4 (0)
Others	0 (0)	2 (0)	2 (0)
Pulmonary embolism	0 (0)	2 (0)	2 (0)
Anaphylactic shock	0 (0)	1 (0)	1 (0)

[a] Including 5 transplantations.

ECMO = extracorporeal membrane oxygenation; LVAS = left ventricular assist system.

Table 5. Complications Related to Extracorporeal Membrane Oxygenation

Complications	Group 1	Group 2	Total
Leg ischemia	5	0	5
Bleeding from cannulation site	5	2	7
Bleeding from surgical site	9	1	10
Airway bleeding	5	0	5
Cerebral infarction	1	0	1
Total	25 (31.6%)	3 (25.0%)	28 (30.8%)

cardiogenic shock. In 30 of them, ECLS was needed because they could not be weaned from cardiopulmonary bypass. Fifteen patients also required intra-aortic balloon pump. The other reasons for ECLS were pulmonary embolism in 2 patients and anaphylactic shock in 1 patient. In group 2, 9 of 12 patients required ECLS for primary cardiogenic shock, and in 7 of these 9, the diagnosis was AMI. In group 1, there was no such tendency, and 14% were AMI patients.

Mean ECMO assist flow was 2.9 ± 0.9 L/min in group 1 and 2.7 ± 0.8 L/min in group 2 ($p = 0.62$).

The CICU was replaced by a NCVC unit after 24-hour support, and the Capiox EBS by a NCVC unit after 24 to 72 hours of support. For cases in which ECLS was established in the operating room, or when the patient's condition permitted waiting until the NCVC unit could be set up, the NCVC unit was used from the beginning. System exchange was also required when oxygenator function diminished, severe hemolysis was observed, thrombus formation was observed within the circuit, or massive serum leakage occurred. The mean frequency of system exchange was 0.78 times (range, 0 to 5) per patient in group 1, and 0.67 times (range, 0 to 5) per patient in group 2 ($p = 0.77$).

Clinical Outcomes

The mean length of ECMO assistance was 103 ± 129 hours (range, 0.5 to 528) in group 1 and 84 ± 100 hours (range, 2 to 360) in group 2 ($p = 0.61$). Weaning from ECLS or bridging to LVAS was achieved in 50 patients (63.3%) in group 1 and 6 patients (50%) in group 2 ($p = 0.37$; Table 4). Thirty-five patients in group 1 (44.3%) and 5 patients in group 2 (41.7%) were discharged from the hospital ($p = 0.86$). The oldest patient who survived to hospital discharge was 84 years of age. Mean duration from weaning from ECLS to hospital discharge was 101 ± 83 days in group 1 and 147 ± 108 days in group 2 ($p = 0.38$).

Complications related to ECLS occurred in 28 patients (30.8%; Table 5). The frequencies of complications in the two groups were similar. The most frequent complication was bleeding due to anticoagulation therapy, with bleeding events accounting for 78.6% of complications (22 of 28). Arterial thromboembolism occurred in only 1 patient in group 1, who suffered cerebral infarction. Five patients in group 1 had lower extremity ischemia, which was controlled by adding distal perfusion.

In the 34 patients who could not be weaned from ECMO, causes of death were multiple organ failure in 30, septic shock in 3, and airway bleeding in 1.

Predictors of Successful ECMO Support

Predictors of weaning from ECMO or successful bridging to LVAS were analyzed using univariate and multivariate logistic regression analysis. Among the variables used for univariate logistic analysis, the p value was less than 0.3 in males, body surface area greater than 1.50 m^2, cardio-

Table 6. Univariate Logistic Regression of Variables Associated With Weaning From ECMO

Variables	Parameter Estimate	Standard Error	χ^2	p Value	Odds Ratio	95% Confidence Intervals (Lower, Upper)
Male	−0.700	0.473	2.193	0.1386	0.497	0.197, 1.254
Age <75 years	0.545	0.623	0.875	0.3817	1.724	0.509, 5.844
Body surface area >1.50 m^2	0.629	0.452	1.935	0.1642	1.875	0.773, 4.546
Diagnosis						
Postcardiotomy cardiogenic shock	−0.799	0.925	0.745	0.3880	0.450	0.073, 2.758
Acute myocardial infarction	−0.470	0.987	0.227	0.6341	0.625	0.090, 4.330
Cardiomyopathy	1.872	1.352	1.918	0.1661	6.500	0.460, 91.948
Myocarditis	0.318	1.044	0.093	0.7604	1.375	0.177, 10.652
Cardiac massage before ECMO	0.145	0.434	0.111	0.7391	1.156	0.493, 2.706
Intra-aortic balloon pump before ECMO	−0.116	0.434	0.071	0.7894	0.891	0.381, 2.084
Incapable of weaning from ECMO	−1.139	0.463	6.047	0.0139	0.320	0.129, 0.794
Place of ECMO initiation						
Operating room	−0.693	1.269	0.298	0.5850	0.500	0.042, 6.019
Intensive care unit	−0.105	1.346	0.006	0.9376	0.900	0.064, 12.586
Coronary care unit	0.539	1.298	0.172	0.6779	1.714	0.135, 21.825
Emergency room	−0.981	1.443	0.462	0.4968	0.375	0.022, 6.350
Interventions on ECMO	1.705	0.791	4.646	0.0311	5.500	1.167, 25.921
Without complications related to ECMO	0.479	0.462	1.077	0.2997	1.165	0.653, 3.995

CPB = cardiopulmonary bypass; ECMO = extracorporeal membrane oxygenation.

Table 7. Multivariate Logistic Regression of Variables Associated With Weaning From ECMO

Variables	Parameter Estimate	Standard Error	χ^2	*p* Value	Odds Ratio	95% Confidence Intervals (Lower, Upper)
Male	−0.812	0.588	1.909	0.1670	0.444	0.140, 1.405
Body surface area >1.50 m^2	1.531	0.611	6.271	0.0123	4.622	1.395, 15.320
Cardiomyopathy	2.364	1.140	4.303	0.0380	10.633	1.139, 99.255
Incapable of weaning from ECMO	−0.376	0.547	0.472	0.4920	0.687	0.235, 2.006
Interventions on ECMO	2.130	0.902	5.575	0.0182	8.415	1.436, 49.319
Without complications related to ECMO	0.354	0.534	0.438	0.5079	1.424	0.500, 4.061

ECMO = extracorporeal membrane oxygenation.

myopathy, incapability of weaning from cardiopulmonary bypass, interventions during ECMO, and complications related to ECMO (Table 6). Multivariate logistic regression analysis was performed using these variables, and the *p* value was less than 0.05 in body surface area greater than 1.50 m^2, cardiomyopathy, and interventions during ECMO (Table 7).

Comment

Extracorporeal life support is an essential technology for cardiopulmonary resuscitation in patients with refractory cardiogenic shock, and ECMO is widely used for ECLS because of its portability, rapid priming, and ease of handling. In ECLS, percutaneous access through the femoral artery and vein is preferable because these vessels are easily and rapidly approached, with less invasion than that associated with the open-chest approach. These methodologies for ECLS are now well established, and published reports have demonstrated overall survival rates between 20% and 60% [7–12]. In our study, a satisfactorily high percentage of patients were resuscitated by ECLS and survived to hospital discharge: weaning from ECLS was achieved in 56 patients (61.5%) with hospital discharge for 40 patients (44.0%).

In this study, the most frequent complications related to ECLS were bleeding events. That was clearly due to our use of anticoagulation therapy during ECMO support. We used continuous injection of heparin and maintained activated clotting time of 180 to 220 s. Arterial thromboembolism occurred in only 1 patient. Our findings thus differed from those in the studies of Magovern and coworkers [9] and Muehrcke and coworkers [12], in which continuous systemic heparinization was not routinely performed. Intracardiac clot formation and arterial thromboembolism are the most serious complications of ECLS and may potentially limit the use of ECLS. Although it may increase the frequency of bleeding events, we believe that systemic heparinization is essential for ECMO support to avoid thromboembolic events.

Although methodologies of ECLS are now well established, the indications for its use have yet to be clearly determined. Although guidelines have been proposed in previous reports to avoid futile efforts at resuscitation [9–13], the use of ECLS for elderly patients is still controversial. It is apparent that elderly age is an important risk factor for mortality in patients with cardiogenic shock [14], and in some reports being 75 years old or older is considered a contraindication for ECLS [4, 5].

In this study, patients required ECLS for a variety of reasons. In the elderly group, however, the indication for ECLS was primary cardiac failure in 9 of 12 patients, and AMI had been diagnosed in 7 of these 9 patients. The onset of circulatory failure is often acute in AMI patients, with rapid progression. Patients are often already in cardiopulmonary arrest on arrival at the hospital. In such cases, when conventional methods of resuscitation fail, the resuscitating physician or surgeon must decide whether ECLS should be used for the patient, despite insufficient time and information on the patient's background and risk factors. For elderly patients in particular, decision-making can be very difficult, because it has been believed that the likelihood of survival is quite small for such patients. This study demonstrated that ECLS using percutaneous ECMO is as effective in saving the lives of elderly patients as for younger patients. We used the same ECMO devices for elderly as for younger patients, and there were no differences in ECMO management between the two groups. Weaning from ECLS was achieved at a satisfactorily high rate not only in younger but also in elderly patients, with no difference in survival rate between the two groups.

The only drawback to the use of ECLS in elderly patients is that a long period of recovery is required after resuscitation by ECLS and after weaning from it. In this study, although the difference between groups was not significant because of the relatively small number of patients in group 2, the mean duration from weaning from ECLS to hospital discharge was 46 days longer in group 2 than in group 1. However, this is insufficient reason to avoid resuscitation of elderly patients with refractory cardiogenic shock, given the satisfactory rate of survival of such patients.

In logistic regression analysis, it was again confirmed that age is not a predictor of successful ECMO support. Patients with body surface area more than 1.50 m^2 were more likely to be weaned. Patients with a small body size are sometimes associated with malnutrition, which increases the risk of mortality. Another predictor of successful ECMO support was cardiomyopathy. This result is obviously due to the effective use of LVAS. In this study, 12 of 14 patients were bridged to LVAS and 1 was weaned from ECMO. We also found that interventions during ECMO were predictors of successful ECMO sup-

port. The ability to correct the underlying pathology seemed to play a role in successful weaning from ECMO. These are the same findings as other authors [2, 3, 10].

In conclusion, we have demonstrated that ECLS using percutaneous ECMO systems provides excellent cardiac support. It is effective in resuscitating elderly patients more than 75 years of age, yielding hospital survival similar to that for younger patients. Although rehabilitation often requires a longer period of time after weaning from ECLS in elderly patients, ECLS resuscitation should be considered even for elderly patients with refractory cardiogenic shock.

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