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The similar effect of transplantation of marrow-derived mesenchymal stem cells with or without prior differentiation induction in experimental myocardial infarction

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Abstract

Marrow-derived mesenchymal stem cells (MSCs) have been heralded as a source of great promise for the regeneration of the infarcted heart. There is no clear data indicating whether or not *in vitro* differentiation of MSCs into major myocardial cells can increase the beneficial effects of MSCs. The aim of this study is to address this issue. To induce MSCs to transdifferentiate into cardiomyocyte-like and endothelial-like cells, 5-azacytidine and vascular endothelial growth factor (VEGF) were used, respectively. Myocardial infarction in rabbits was generated by ligating the left anterior descending coronary artery. Animals were divided into three experimental groups: I, control group; II, undifferentiated mesenchymal stem cell transplantation group; III, differentiated mesenchymal stem cell transplantation group; which respectively received peri-infarct injections of culture media, autologous undifferentiated MSCs and autologous differentiated MSCs. General pathology, immunohistochemistry, electron microscopy and echocardiography were performed in order to search for myocardial regeneration and improvement of cardiac function. In Groups II and III, implanted cells trans-differentiate into myocardial cells within 28 days post injection in a similar manner, and well-developed ultra structures formed within transplanted cells. Improvements in left ventricular function and reductions in infarcted area were observed in both cell-transplanted groups to the same degree. Vascular density was similar in Groups II and III and significantly higher in these groups compared with the control group. There is no need for prior differentiation induction of marrow-derived MSCs before transplantation and peri-infarct implantation of MSCs can efficiently regenerate the infarcted myocardium and improve cardiac function.

Introduction

The remodeling process following myocardial infarction (MI) will eventually lead to impairment of left ventricular function [1, 2]. The use of bone marrow-derived mesenchymal stem cells (MSCs) is an auspicious method which prevents deleterious remodeling and improves left ventricular (LV) function [3, 4].

5-Azacytidine which is a DNA demethylating agent, and vascular endothelial growth factor (VEGF) can induce MSCs to transdifferentiate into cardiomyocytes and endothelial cells, respectively

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[5, 6]. Some authors used a mesenchymal stem cell pretreatment with 5-azacytidine to increase the probability that these cells will be directed toward a cardiomyocyte-differentiation pathway [7–9], but the significance of this approach is not yet clear [10]. On the other hand, it has been suggested that an immature, more plastic cell may be more effective than an ex vivo pre-differentiated, committed cell [11].

However, there is no published data from a systematic study which might indicate whether or not *in vitro* differentiation induction of MSCs have more beneficial effects for the regeneration of the infarcted myocardium and improvement of LV function rather than undifferentiated MSCs. The goal of this study is to address this issue with an extensive investigation. In this study, we chose a rabbit model whose heart has been suggested as a good experimental model for infarction-related researches [12].

Methods

This study was performed in accordance with guidelines published in Guide for the Care and Use of Laboratory Animals (NIH publication 8523, revised 1996).

Cell isolation and expansion

Male New Zealand White rabbits were anesthetized with ketamin (50 mg/kg i.m.) and xylazine (5 mg/kg i.m.). Then 5–8 ml of marrow was aspirated from the iliac crest with an 18-gauge needle connected to a syringe containing 3000 units of heparin. Bone marrow-derived mononuclear cells (BM-MNCs) were isolated by centrifugation on a Ficoll gradient. Then, BM-MNCs were washed twice with phosphate-buffered saline (PBS), pelleted by centrifugation and resuspended in culture medium DMEM (Sigma) supplemented with 20% fetal bovine serum (FBS). Cells were then introduced into 25 cm^2 flasks and incubated with 95% air and 5% CO_2 at 37 °C. Medium was changed subsequently twice per week. Non-adherent mononuclear and red blood cells were removed during the first few medium changes. The attached cells grew and developed colonies in approximately 5–7 days. Mesenchymal stem cells (MSCs) were passaged prior to confluency by detachment using 0.25% trypsin/EDTA (Gibco). Flow cytometry analysis showed that marrow-derived MSCs were CD29 and CD166 positive, but were negative for both CD34 and CD45 (data not shown).

Cardiomyocyte-like and endothelial-like cell differentiation of MSCs

The differentiation of cardiomyocyte-like cells from MSCs was performed as described previously [13, 14]. The second passage of MSCs was seeded into flasks at a concentration of 20000 cells/cm^2. On the second day, the medium was changed, then cells were treated for 24 h with medium containing 6 μM/l 5-azacytidine. Thereafter, cells were washed and incubated with DMEM containing 10% FBS. The medium was changed every 3 days. Differentiation was confirmed by immunostaining and ultrastructural analysis. For fluorescent immunostaining, cells were washed with PBS and fixed by incubating in 4% paraformaldehyde for 20 min, then washed three times with PBS, permeabilized with 0.1% triton ×-100 for 15 min and rinsed three times in PBS and proteins blocked by incubating the cells in 1% bovine serum albumin (BSA). Then the cells were incubated overnight at 4 °C with primary antibodies including anti-sarcomeric α-actinin (1:200; Sigma), anti-sarcomeric myosin, MF20 (1:20; Developmental studies, Hybridoma Bank), anti-desmin (1:200; Sigma), anti-pan-cadherin (1:300; Sigma) and anti-troponin T (1:300; Sigma). Afterwards, cells were washed three times with PBS and incubated with the 1:100 diluted fluorescence isothiocyanate (FITC)-conjugated anti-mouse antibody (Sigma) for 3 h at room temperature. After rinsing cells in PBS, the cells were analyzed using a fluorescence microscope (Olympus). Non-induced MSCs were stained as negative control.

For ultrastructural analysis, the cells were mechanically detached using a cell scraper. After washing with PBS, the cells were fixed in 3% glutaraldehyde in 0.1 mol/l PBS at 4 °C for 1 h and post-fixed in 1% osmium tetroxide for 1 h. The samples were then dehydrated through a graded ethanol series and embedded in epoxy resin. The thin section was prepared and double-stained with uranylacetate for 10 min and lead citrate for 4 min, then ultrastructurally scoped using an electron microscope (Philips).

To induce MSCs to differentiate into endothelial-like cells, the second passage of MSCs were treated for 2 weeks with medium containing 20 ng/ml VEGF. The medium was changed every 3 days. To confirm the differentiation, fluorescent immunostaining was carried out for VEGF receptor-2 (KDR) and von Willebrand factor (vWF) with the following primary antibodies: anti-VEGF receptor-2 (KDR) (1:100; Sigma) and anti vWF (1:100; Sigma). Phycoerythrin (PE)-conjugated anti-mouse antibody (1:100; Sigma) was used as secondary antibody. Finally, the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). As negative control, non-induced MSCs were stained through a similar method.

Myocardial infarction (MI) generation and experimental groups

After anesthesia, rabbits were mechanically ventilated and their hearts were exposed through left thoracotomy. The left anterior descending coronary artery was ligated with a 6-0 polypropylene, just below the tip of the left auricle. The regional pallor of the anterior cardiac surface confirmed myocardial ischemia.

Animals were divided into the following three experimental groups: I, control group (n = 6); II, undifferentiated mesenchymal stem cell (UMSC) transplantation group (n = 7); III, differentiated mesenchmal stem cell (DMSC) transplantation group (n = 7). They were respectively injected with 200 μl of culture media, 10^6 undifferentiated MSCs and 5×10^5 cardiomyocyte-like plus 5×10^5 endothelial-like cells differentiated from marrow MSCs, at four sites bordering the infarcted area 14 days post-MI.

Labeling of cells for transplantation

To identify the transplanted cells in the recipient heart, the cells were labeled with bromodeoxyuridine (BrdU, Sigma) 24 h before implantation. For injection, the cells were washed with PBS, trypsinized, washed with PBS, counted and resuspended in serum free culture medium.

Cardiac function assessment

Trans-thoracic echocardiography was performed using an echocardiographic system (Toshiba SSA-380A) provided with a 7.5 MHz linear transducer, before MI, 14 days post-MI before injection and 28 days post-injection. LV anterior wall thickness (AWT, mm), posterior wall thickness (PWT, mm), body weight-corrected LV end-diastolic dimension (EDD/BW), LV ejection fraction (EF, %) and fractional shortening (FS, %) were measured by two blind examiners.

General pathology

Twenty-eight days after injection, each of the animals was heparinized (500 U/kg), then sacrificed by an overdose of pentobarbital, after which, body weight and LV weight were measured. LV was then fixed in 10% buffered formalin and cut into six transverse slices from base to apex. The slices were embedded in paraffin and two 7 μm tick serial sections were cut from each slice. These two sections were stained with hematoxylin-eosin and Masson's trichrome and scanned. For each transversely sliced preparation with infarction, the LV wall area as well as the infarcted area was calculated as described previously [15].

Immunohistochemistry

For immunohistochemical staining with standard labeled streptavidin biotin method, the following monoclonal antibodies were used: mouse ant-BrdU (1:100; Sigma), mouse anti-human smooth muscle myosin heavy chain (1:100; DAKO), mouse anti-human CD31 (1:100; DAKO) and mouse anti-human desmin (1:200; DAKO). Biotinylated goat anti-mouse was used as secondary antibody. The sections were colored with streptABComplex/HRP kit (DAKO) according to the company's instructions. For BrdU immunostaining, 3,3-diaminobenzidine (DAB) was used as chromagen to produce a brown color. For other markers, 3-amino-9-ethylcarbazole (AEC) was used to produce a red color. The sections were counterstained with Mayer's hematoxylin solution, and the numbers of small arteries positive for smooth muscle-specific myosin heavy chain and capillaries positive for CD31were counted as described previously [15, 16].

Electron microscopy

Immediately after sacrifice of two random animals from each group, very small proportions of the

infarcted anterior LV wall and non-infarcted posterior LV wall of each heart were removed and fixed in 3% glutaraldehyde in 0.1 mol/l PBS at 4 °C for 4 h then, post-fixed in 1% osmium tetroxide for 2 h, and thin sections prepared as described above.

Statistical analysis

All values are expressed as mean ± SD. The differences in echocardiographic data between three groups were determined by two-way repeated-measures analysis of variance (ANOVA), followed with a post hoc Tukey-Kramer's test. After evaluation of the homogeneity of variance and normal distribution of post-sacrifice data, statistical comparison of post-sacrifice data was performed using one-way ANOVA followed by Tukey-Kramer multiple comparison test. Values of $p < 0.05$ were considered statistically significant.

Results

In vitro differentiation of MSCs into cardiomyocyte-like and endothelial-like cells

Within 2 weeks after 5-azacytidine treatment, fluorescent immunostaining of the differentiated cells revealed that cardiomyocyte markers including sarcomeric myosin, sarcomeric α-actinin, desmin, pan-cadherin and troponin T were expressed in the cells (Figure 1a–h). MSCs treated with VEGF expressed KDR and vWF (Figure 1i–n). Non-induced undifferentiated MSCs were negative for these antigens.

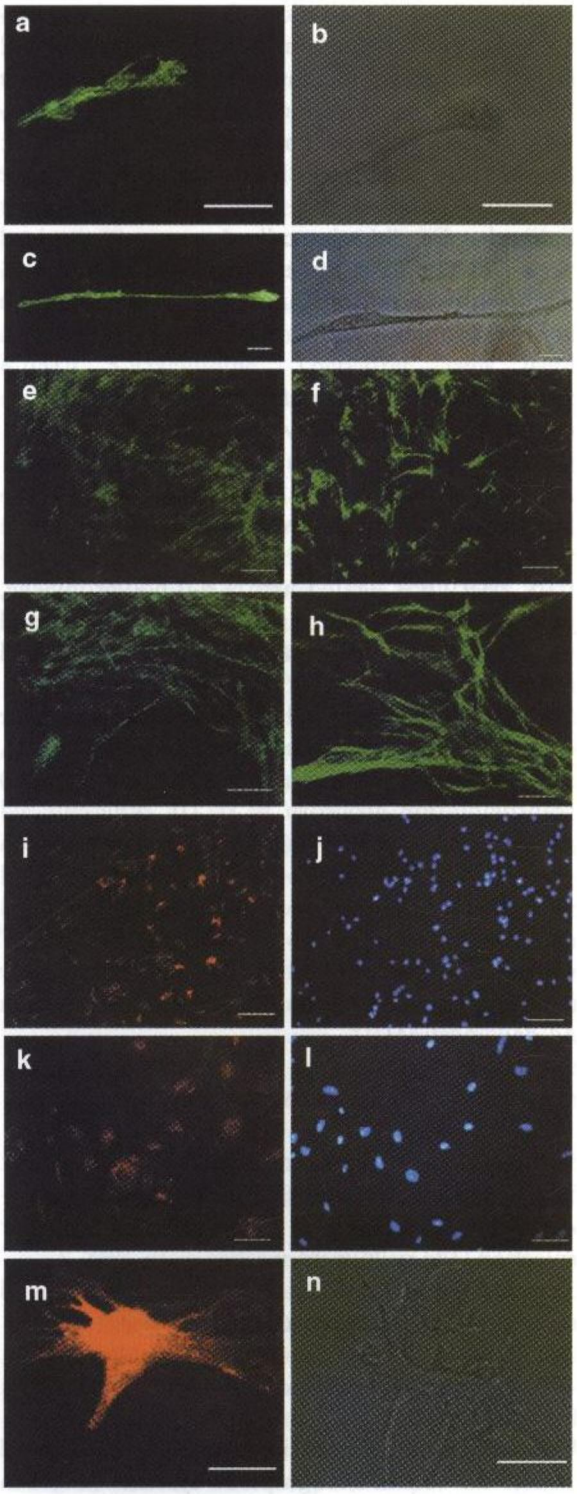

Figure 1. Fluorescent immunostaining of cardiomyocyte-like and endothelial-like cells differentiated from marrow-derived MSCs, 2 weeks after induction with 5-azacytidine and VEGF, respectively. (a, c, e-h) FITC immunostaining for sarcomeric α-actinin (a), sarcomeric myosin (MF20) (c) and (e), desmin (f), pan-cadherin (g) and troponin T (h) revealed that these cardiac markers were strongly expressed in 5-aza treated cells. (b and d) Phase contrast photographs, which are related to (a) and (c), respectively. (i and k) PE immunostaining for KDR (i) and vWF (k) showed significant presence of these markers in VEGF treated cells. (j and l) DAPI-stained nuclei related to panels (i) and (k), respectively. (m) The photograph shows a single endothelial-like cell with intense staining for KDR. (n) Phase contrast photograph, which is related to (m). Non-induced marrow-derived MSCs were negative for these antigens (data not shown). Scale bars: 50 μm in panels (a), (b), (c), (d), (k) and (l); 100 μm in panels (e), (f), (g), (h), (i) and (j); 10 μm in panels (m) and (n).

Ultrastructural analysis of the cardiomyocyte-like differentiated MSCs showed that 2 weeks after induction with 5-azacytidine, myofilaments were seen within cytoplasms of the induced cells (Figure 5a). We did not find Z disc-like structures in the cytoplasms of the cells harvested for electron microscopy two weeks after induction.

Echocardiography

Before MI and 2 weeks post-MI, echocardiographic parameters between Groups I, II and III were not statistically different. Twenty-eight days post-injection, a significant increase in LV anterior wall thickness, EF, FS, and a significant decrease in EDD/BW were observed in the cell-transplanted groups compared with those of the control group (Figure 2a, c–e). However, there was no significant difference in these parameters between Groups II and III. Posterior wall thickness was similar between Groups I, II and III at all times (Figure 2b).

General pathology

Twenty-eight days post-injection, we observed scar tissue consisting of collagen and fatty tissue, in the infarcted zone of all groups. The infarct sizes in Groups I, II and III were 16.8 ± 3.0%, 5.9 ± 2.0% and 5.5 ± 1.7%, respectively. A significant reduction in the infarcted area occurred in cell-transplanted groups versus control group ($p < 0.0001$) (Figure 3d). However, statistical analysis of the infarcted area of two transplanted groups (II and III) did not show a significant difference ($p = 0.951$) (Figure 3d).

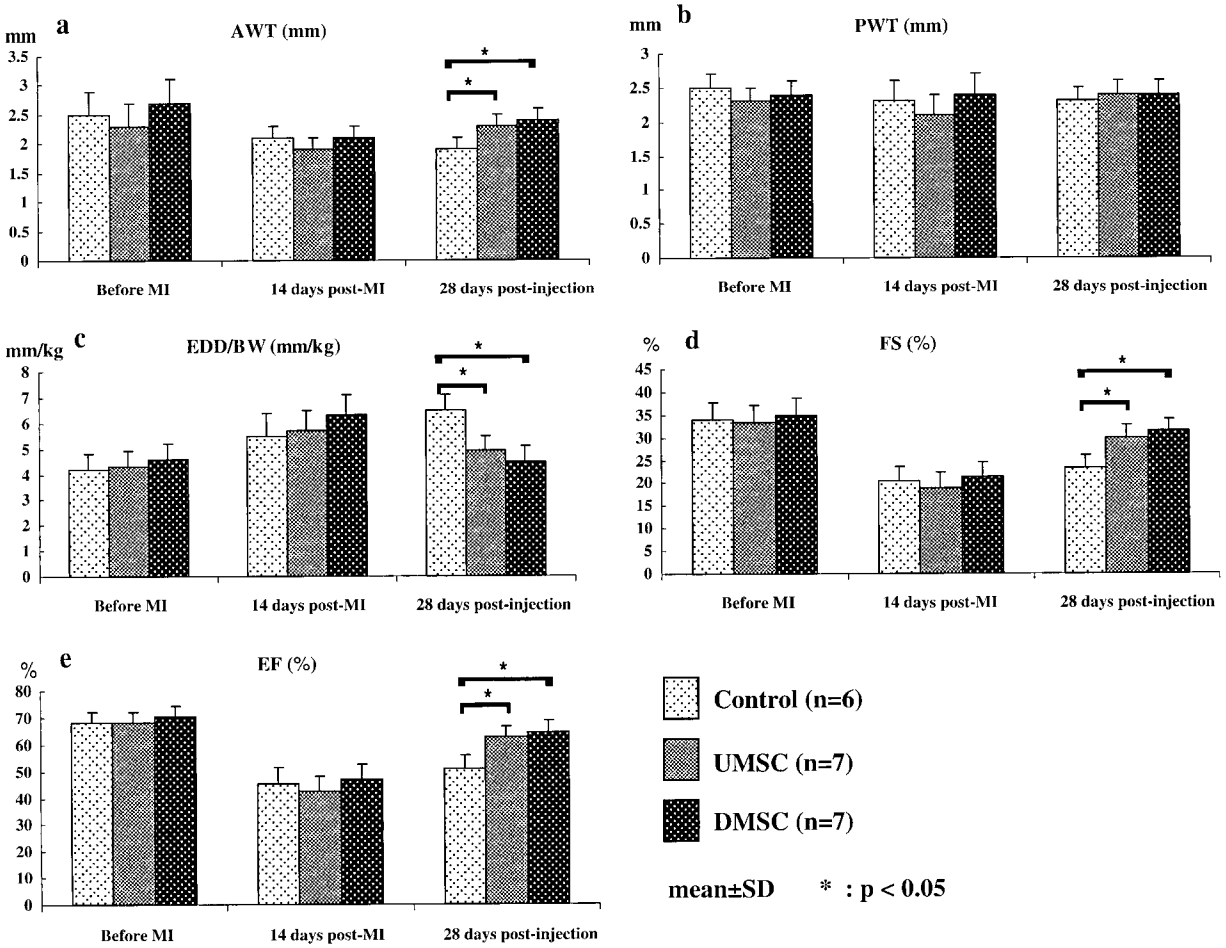

Figure 2. Improvement in cardiac function after cell transplantation. AWTs, PWT, FEs, FSs and EDD/BW ratios obtained before MI, 14 days post-MI and 28 days post-injection.

No significant differences were observed in LV weight and LV wall area between the three groups (Figure 3b, c). Respective transverse diameters of the cardiomyocytes in the left ventricular anterior wall and left ventricular posterior wall of the three groups were similar, but anterior wall cardiomyocyte diameter was significantly greater than that of the posterior wall cardiomyocytes (Figure 3a).

Immunohistochemical analysis

In the cell-transplanted groups, accumulations of BrdU-positive cardiomyocytes were observed in some portions of the anterior wall (Figure 4a, b). In Groups II and III BrdU-labeled cells were also observed in the structure of vessels and colocalized with endothelial and smooth muscle cells (Figure 4c–f). However, a few BrdU positive cells were found scattered in the remote non-infarcted regions.

Immunohistochemical staining using monoclonal antibodies against CD31 and smooth muscle myosin heavy chain for respective detection of capillaries and small arteries revealed a significant increase in neovascularization in Groups II and III compared with control group (Figure 3e, f). However, vascular density was similar between Groups II and III.

Electron microscopy

Twenty-eight days after injection of differentiated as well as undifferentiated MSCs, we observed these cells in the infarcted anterior wall. Close ultrastructural scrutiny of these cells revealed an excellent transdifferentiation into mature cardiomyocytes. Well-developed myofilaments with clear Z lines formed parallel striated arrangements within the cytoplasm (Figure 5c, d). Highly-developed intercalated discs with fascia adherence had been created between adjacent cells, and distinct desmosomes and gap junctions were also present in the intercalated discs (Figure 5e, e_1 and g). The same cells were not present in control hearts, which did not receive cell therapy.

An interesting ultrastructural finding was that the plasma membrane of the injected cells developed tubular invaginations in the vicinity of the Z lines in order to from transverse tubules (T-tubules) (Figure 5f, f1). The structure and location of these T-tubules were similar to those specifically existing in the ventricular cardiomyocytes.

Discussion

The significant decrease of scar tissue in cell-transplanted groups compared to control group, can be attributed to reduction in collagen synthesis and/or formation of new cardiomyocytes. Since the total LV area and weight as well as the transverse size of cardiomyocytes were similar in all experimental groups, hypertrophy of preexisting myocytes could not be responsible for the reduction of infarcted areas in cell-transplanted hearts compared to control hearts. Recently, it has been demonstrated that in bone marrow-derived mononuclear cell-treated hearts as opposed to saline-treated hearts, the expression of repair-related cytokines such as transforming growth factor (TGF)-β, a mediator stimulating collagen synthesis, were down-regulated and this is likely to contribute to the improved LV function as well as reduction in scar tissue [15]. However, the improvement in cardiac function may be ascribed to the self-renewal ability of MSCs and para-secretion of growth factors, in addition to prevention of apoptosis in ischemic myocardium [17–19].

Our ultrastructural observation indicates that marrow-derived MSCs have an excellent capability for transdifferentiation into mature cardiomyocytes within one month after injection into an infarcted rabbit heart model. This situation was similar in both UMSC and DMSC groups. Strong evidence suggest that the regeneration of the infarcted myocardium following cell transplantation is due to differentiation of transplanted cells into myocardial tissue cells and is independent of cell fusion [20–22]. In our study, new cardiomyocytes formed independent clusters in the anterior infarcted wall although it seems that they have a great talent for creation a syncytium with the spared myocardium.

Transplanted cells formed almost all structures existing normally in mature cardiomyocytes. Myofilaments and intercalated discs were well-developed and cell membrane developed T-tubular system. In mammalian skeletal muscle, T-tubules pass transversely across the fiber and lie specifically in the plane of the junction of the A (dark bands) and I bands (light bands). Unlike in skeletal muscle, the T-tubules of cardiac muscle cells are located in the vicinity of the Z lines [23], as we observed in transplanted cells. Moreover, ventricular myocytes from embryonic and newborn

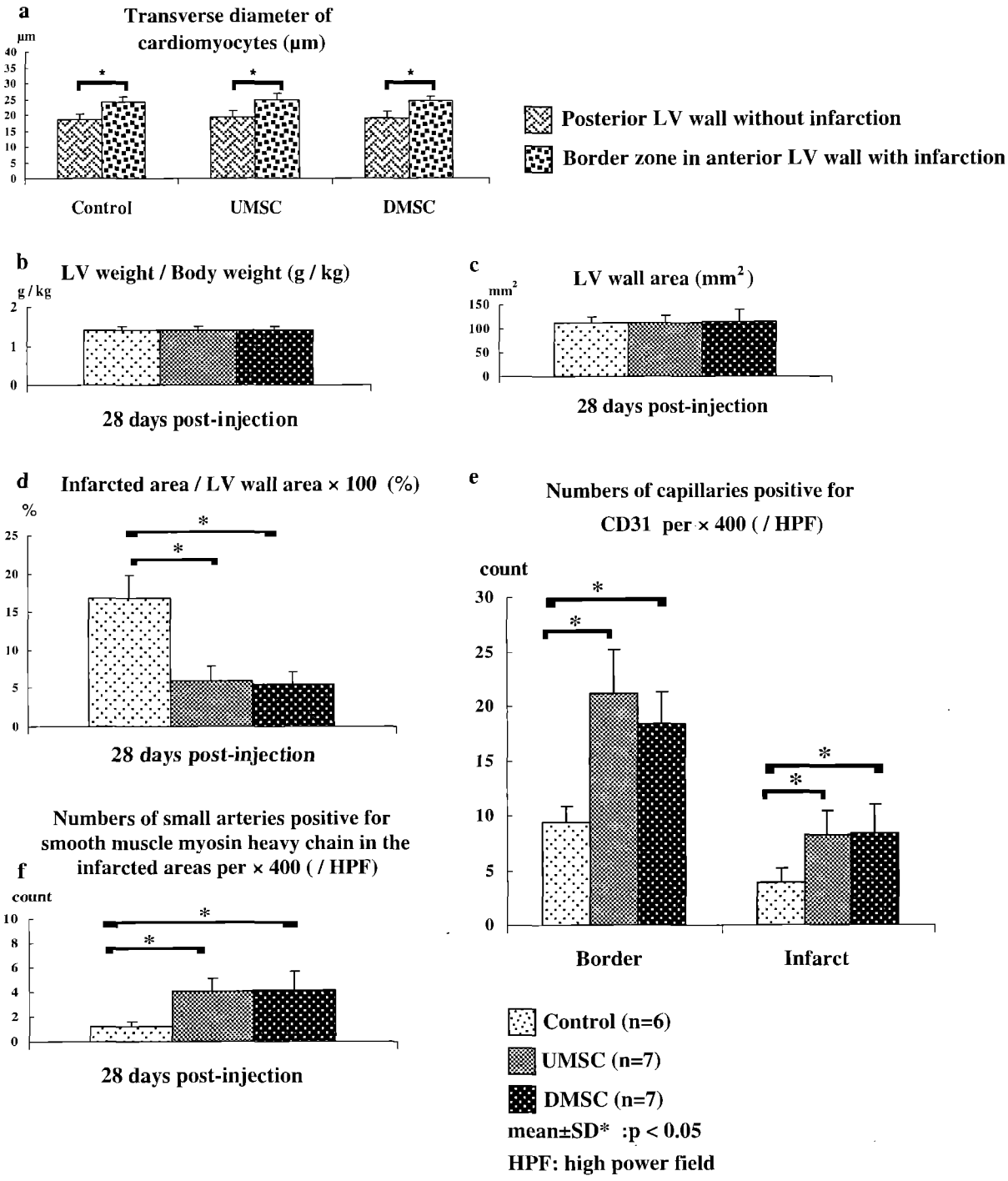

Figure 3. Changes in infarct size and vascular density in the cell-transplanted groups. Cardiomyocyte diameter, LV weight/body weight ratio, LV wall area, infarct size and the numbers of CD31-positive capillaries and smooth muscle myosin heavy chain-positive small arteries observed 28 days after transplantation. Cells were counted in each of 15 serial fields per rabbit. The border zone was defined as surviving myocardial tissue areas within 1 mm of infarcted areas.

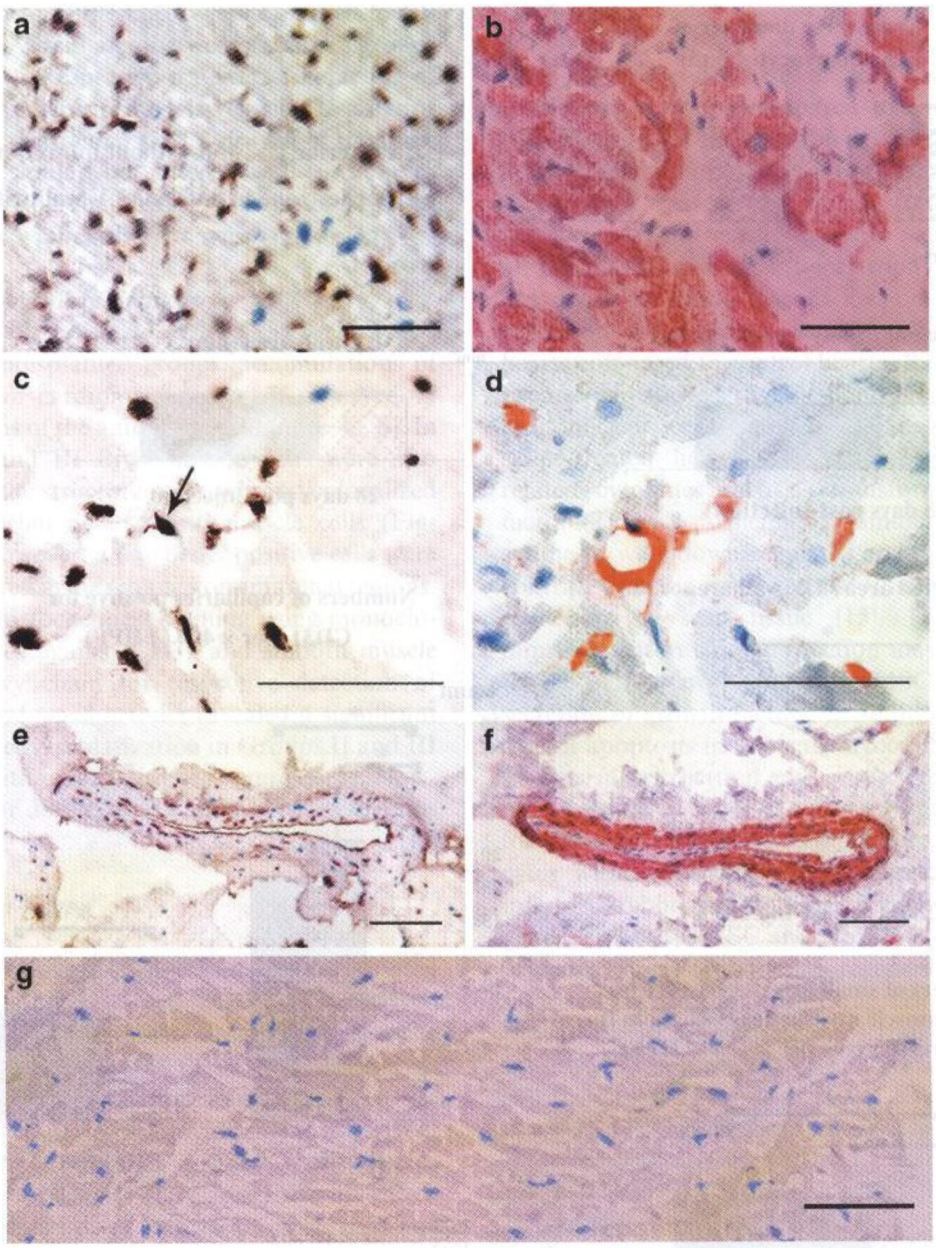

Figure 4. Immunohistochemical analysis. (a and b) Accumulations of BrdU-positive cells (a) which are also positive for desmin (b) in the border zone. (c and d) Colocalization of a BrdU-labeled cell (c) and a CD31-positive endothelial cell (d). (e and f) BrdU-labeled cells (e) in the structure of a small artery which is positive for smooth muscle-specific MHC (f). (g) As negative control, immunohistochemical staining of culture media-injected (control) hearts with anti-BrdU revealed no DAB signal. Scale bars: 20 μm.

animals, atrial cells, and Purkinje cells do not have a T-tubular system or have only a very sparse system [24, 25]. These all confirm that marrow-derived MSCs directly transdifferentiate into mature ventricular myocytes following transplantation into the infarcted myocardium. However, future studies remains to be done in investigating whether stem cell-derived T-tubular system has normal physiologic functions.

BrdU-positive endothelial cells, observed in the cell-transplanted groups, must be derived from marrow stem cells. BrdU-positive cells also incorporated in the structure of large vessels and colocalized with endothelial and smooth muscle

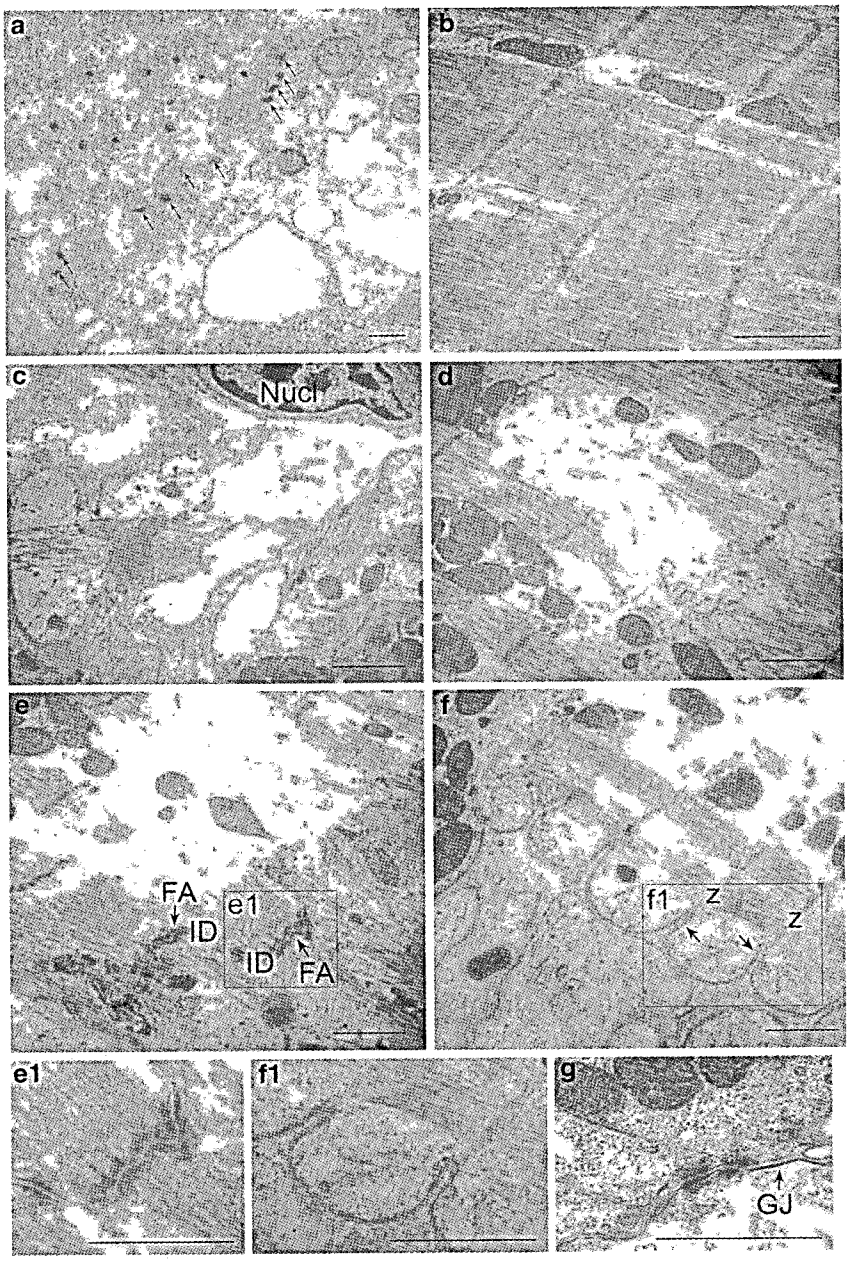

Figure 5. Electron microscopic findings. (a) Formation of thin filaments within the cytoplasm of marrow MSCs, 2 weeks after induction with 5-azacytidine. (b) Normal adult cardiomyocytes showing normal striated configuration. (c and d) Formation of well-developed myofilaments with clear Z lines within the cytoplasms of transplanted cells, 4 weeks after injection of differentiated as well as undifferentiated MSCs. (e) Highly developed intercalated discs (ID) with fascia adherence (FA) between adjacent transplanted cells, 4 weeks after injection. (e_1) Highly magnified image of the rectangular part of panel (e). (f) Tubular invagination of the plasma membrane in the vicinity of the Z lines. (f_1) Highly magnified photograph of the rectangular portion of panel (f). (h) A well-developed gap junction (GJ) has formed in region where cells are lying side by side. Scale bars: 0.1 μm in panel (a) and 1 μm in the other panels.

cells. These evidence may be explained by the hypothesis that the neovascularization induced by MSCs involves paracrine factors secreted by MSCs to facilitate vessel sprouting and growth and incorporation of MSCs into newly forming blood vessels by differentiation of MSCs *in situ*

[19, 26–29]. Increase in blood supply in infarcted area, as a result of neovascularization, would prevent further necrosis or apoptosis of hypertrophied but otherwise viable myocardium, and increase viability of implanted cells, thus improving ventricular function [30, 31].

Bittira et al. reported that pretreatment of marrow stromal stem cell with 5-aza-2′ deoxycytidine, which is an active metabolite of 5-azacytidine, had better phenotypic outcome compared with untreated stromal cells when they were injected directly into the scar created by cryoinjury [32]. They concluded that when MSCs are implanted directly within the scar, because of the nonmyogenic milieu of the scar, pretreating MSCs with 5-aza may enhance their myogenic differentiation [32]. However, they used only routine pathologic methods in their study and they did not use more sophisticated methods such as electron microscopy or at least, measurement of the infarcted area or vascular density in their experimental groups.

Our aim in this study was to direct marrow-derived MSCs toward cardiomyocyte-like and endothelial-like cells through pretreatment of cells with previously proven cardiomyogenic and angiogenic inducers (5-azacytidine and VEGF) two weeks before injection. Immunocytochemical analysis showed that our treatments successfully directed MSCs toward cardiomyocyte-like and endothelial-like cells. In the present study we have focused on comparison between treated and untreated marrow-derived MSCs for myocardial regeneration and improvement of cardiac function after experimental myocardial infarction. Our findings demonstrated that cells without differentiation induction were as effective as cells with prior differentiation induction. These effects can be due to strong signals from the peri-infarct microenvironment that efficiently guides the *in vivo* differentiation of marrow MSCs [33].

In conclusion, directing MSCs toward cardiac cells before transplantation is not needed. Peri-infarct injection of marrow-derived MSCs can effectively regenerate infarcted myocardium and improve cardiac function.

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