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ORIGINAL ARTICLE

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Fibroblast sheets co-cultured with endothelial progenitor cells improve cardiac function of infarcted hearts

Abstract We have already confirmed that cell sheet transplantation can improve damaged heart function via continuous cytokine secretion. In this study, we hypothesized that cytokine-secreting cell sheets co-cultured with an endothelial cell source may be more effective for repairing ischemic myocardium. Confluent rat fibroblasts cultured on temperature-responsive culture dishes were harvested as contiguous cell sheets by temperature reduction. Green fluorescent protein (GFP)-positive endothelial progenitor cells (EPCs) were seeded on fibroblast sheets to create co-cultured cell sheets, and sandwich-like constructs were engineered by stacking of the co-cultured cell sheets. These constructs were transplanted into rat myocardial infarction models. Cardiac function and histology were assessed in four groups: the sham operation (C) group, the isolated EPC injection (E) group, the transplantation of triple-layer fibroblast sheets (F) group, and the transplantation of triple-layer sandwich-like constructs (E + F) group. Echocardiography showed significant improvement of the fractional shortening in the E + F group in comparison with the C group (0.25 ± 0.05 vs. 0.16 ± 0.02). On histological examination, significantly less connective tissue formation was observed in the E, F, and E + F groups when compared to the C group (C, E, F, and E + F groups: 53 ± 2%, 41 ± 4%, 40 ± 4%, and 32 ± 7%, respectively). Additionally, increased blood vessel formation was detected in the E, F, and E + F groups compared with the C group (C, E, F, and E + F groups: 1.9% ± 0.6%, 6.7% ± 0.6%, 7.8% ± 0.9%, and 10.2% ± 2.4%, respectively). Furthermore, GFP-staining demonstrated that the newly formed blood vessels were composed of the co-cultured EPCs. Transplantation of cell sheets co-cultured with an endothelial cell source may be a new therapeutic strategy for myocardial tissue regeneration.

Key words Tissue engineering · Cell sheet · Endothelial progenitor cells · Heart

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Introduction

Recently, several approaches involving regenerative medicine have been investigated as potential treatments for the improvement of cardiac function after myocardial injury.[1–3] The most popular approach has been the direct injection of isolated cells into damaged hearts, and studies using animal models have revealed improvement of heart function after the transplantation of various cell types. Myoblasts or bone marrow cells have already been transplanted into patients suffering from severe ischemic heart diseases and significant efficacies of these treatments have been reported.[4,5]

In the direct transplantation of dissociated cells, it is difficult to control the shape, size, and location of the transplanted grafts. Moreover, cell loss due to various factors such as physical strain, hypoxia, or cell washout through blood vessels remains problematic.[6–8] To overcome these obstacles, implantation of tissue-engineered myocardial patches has been investigated.[9,10] In contrast to conventional approaches using three-dimensional (3-D) biodegradable scaffolds, we have previously developed a novel scaffold-free tissue engineering technology to harvest and layer cultured cell sheets for 3-D myocardial patch fabrication.[11] Cell sheets are obtained by using unique culture dishes whose surfaces are covalently grafted with a temperature-responsive polymer.[12,13] The polymer surface is hydrophobic

above 32°C, but changes to become hydrophilic below 32°C. By simple temperature modifications, these surface changes allow cultured cells to be spontaneously detached as contiguous cell sheets from these surfaces. With this technique, cells can be more effectively delivered as thin, but large-area constructs without cell loss when compared to isolated cell transplantation.[14] Using this approach, we previously confirmed restoration of impaired heart functions by cell sheet transplantation in myocardial infarction models.[15–17] Cardiomyocyte, skeletal myoblast, and adipose mesenchymal stem cell sheets were able to improve damaged heart function.

Regarding the mechanism for repairing damaged myocardium, various cytokines secreted from transplanted cells, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), accelerate new blood vessel formation and limit fibrosis in the ischemic areas.[16–19] Other studies have also revealed that the transplanted cells can become incorporated into the reconstructed vascular structures, suggesting that transplantation of vascular-composing cells may be an effective methodology.[18,20,21] We therefore hypothesized that cytokine-secreting cell sheets co-cultured with an endothelial cell source may be more effective for repairing ischemic heart tissue. In this study, upon consideration of less-invasive cell isolation in clinical settings, we sandwiched peripheral blood endothelial progenitor cells (EPCs) between subcutaneous tissue-derived fibroblast sheets and transplanted the constructs onto rat ischemic hearts. The efficacy of this combination therapy was evaluated in comparison with three groups: rats received a sham operation involving thoracotomy and cardiac exposure but without transplantation, transplantation of fibroblast sheets alone, and isolated-EPC injection.

Materials and methods

All procedures using animals in this study were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health, USA.

Rat myocardial infarction model

Male athymic rats (8–10 weeks old, Charles River, Yokohama, Japan) were used. Myocardial infarction was produced by means of ligation of the left anterior descending coronary artery (LAD), as previously described.[22] Briefly, rats were anesthetized by intraperitoneal injection of ketamine (40 mg/kg) and xylazine (4 mg/kg). The heart was exposed via a left thoracotomy and the left anterior descending coronary artery was ligated at a position 2–3 mm from its origin between the pulmonary conus and left atrium.

Isolation and culture of EPCs

Rat EPCs were isolated according to a previously described methodology.[23,24] Peripheral blood mononuclear cells were isolated from 5- to 6-week-old Green fluorescent protein (GFP)-expressing Sprague-Dawley rats (SD-Tg[Act-EGFP] CZ-004, SLC, Hamamatsu, Japan) by density gradient centrifugation with Hisotopaque 1083 (Sigma, Saint Louis, MO, USA). A total of 1×10^7 isolated cells were plated on 35-mm culture dishes coated with vitronectin (Sigma) and cultured in endothelial cell basal medium-2 (Cambrex, East Rutherford, NJ, USA). After 4 days, nonadherent cells were removed by washing and the cultures were maintained. After a further 2 days, adherent EPCs were harvested by trypsinization for needle injection or seeding onto fibroblast sheets.

Fibroblast culture

Primary dermal fibroblasts were prepared by explant culture.[25] Fibroblasts from male Wistar rats (5 weeks old, Nisseizai, Tokyo, Japan) were cultivated from abdominal area skin samples (about 2×2 cm). Skin samples were cut into 0.5×0.5-cm portions and placed into culture dishes containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Approximately 1 week later, the explanted rat fibroblasts were harvested by trypsinization and used for cell sheet fabrication.

Preparation of co-cultured cell sheets

Fibroblast cells were seeded at a density of 5×10^5 cells/dish onto 35-mm-diameter temperature-responsive culture dishes (provided by CellSeed, Tokyo, Japan).[12,13] After 1 week of culture at 37°C, confluent fibroblasts on the temperature-responsive dishes were transferred to another CO_2 incubator set at 20°C for about 30 min, upon which the fibroblast sheets detached spontaneously. To create co-cultured cell sheets, 1×10^5 isolated EPCs were seeded over the 6-day-cultured confluent fibroblasts on the temperature-responsive culture surfaces. After 1 additional day in culture, confluent fibroblasts topped with EPCs were harvested as co-cultured cell sheets by the low-temperature treatment. The harvested cell sheets were stacked by pipetting according to the previously described procedures.[14]

Transplantation protocol

In this study, all rats received a second operation 1 week after LAD ligation and were randomly divided into four experimental groups. In the first group, as a control, rats received a sham operation involving thoracotomy and cardiac exposure but without transplantation (C group, n = 5). In the second group, 2×10^5 isolated EPCs were injected into three sites around the infarcted area using a 29-gauge needle (E group, n = 5). In the third group, triple-layer fibroblast sheets were transplanted onto infarcted hearts (F group, n = 5). In the fourth group, sandwich-like triple-layer constructs were fabricated by layering two co-cultured cell sheets and one fibroblast sheet. The sandwich-like constructs were transplanted onto the infarcted hearts (E + F

group, $n = 5$). In this group, the total number of the sandwiched EPCs was equivalent to the EPC injection group (2×10^5 EPCs). In both the F group and E + F group, the sheet covered about 70% of the myocardial infarction area.

Measurement of rat cardiac function

Echocardiographies were performed just before transplantation (baseline) and at 1, 2, 3, and 4 weeks after the procedure. M-mode tracings were obtained at the papillary muscle level using a commercially available echocardiograph with a 12-MHz phased-array transducer (Nemio 30, Toshiba, Tokyo, Japan). The left ventricular (LV) dimension at end-diastole (LVDd), the LV dimension at end-systole (LVDs), and the LV anterior wall thickness (AWTh) were measured according to the leading edge method of the American Society of Echocardiography.[26] Fractional shortening (FS) was calculated as a measure of systolic function: FS = (LVDd – LVDs)/LVDd.

Histological analyses

Animals were killed by intraperitoneal injection of an overdose of ketamine and xylazine and the hearts were excised 4 weeks after the transplantation. The samples were fixed with 4% paraformaldehyde, embedded in paraffin, and sagittally sectioned into 10-μm-thick tissue sections. Azan staining was performed by conventional methods. To determine the quantity of connective tissue, blue areas from azan-stained tissues were measured by NIH-Image software (National Institutes of Health, Bethesda, MD, USA). Randomly selected fields from each cross section were analyzed at a magnification of ×200 at the LV papillary muscle level. For endothelial cell staining, fixed tissue sections were incubated with a 1:200 dilution of Alexa Fluor-568 conjugated isolectin B4 (Molecular Probes, Eugene, OR, USA) for 2 h at room temperature and examined using fluorescence microscopy (LSM510-V3.2, Carl Zeiss, Oberkochen, Germany). Capillary densities of the cross-sections at the LV papillary muscle level were evaluated morphometrically as the percentage of areas containing stained blood vessels, using NIH-Image software. Ten randomly selected fields were analyzed at a magnification of ×400 for each cross section at the LV papillary muscle level. To enhance GFP-expression, samples were incubated with a 1:200 dilution of anti-GFP rabbit polyclonal antibody (Molecular Probes) for 2 h at room temperature and subsequently treated with a 1:500 dilution of tetramethylrhodamine isothiocyanate (TRITC)-labeled anti-rabbit IgG antibody (Sigma) for 2 h at room temperature. The double-stained images with isolectin B4 and anti-GFP antibody were obtained with the same equipment as described above.

Statistical analyses

All data are expressed as mean ± SD and were analyzed by multiple analysis of variance (ANOVA) using StatView 5.0 software (Abacus Concepts, Berkeley, CA, USA). Echocardiographic data were first analyzed by two-way repeated measures ANOVA for differences across the whole time course, and one-way ANOVA with the Tukey-Kramer posthoc test was used to verify specific comparisons at each time point. Other numerical data were analyzed by one-way ANOVA with the Tukey-Kramer posthoc test. A P-value of less than 0.05 was considered significant.

Results

Cell sheet transplantation onto the infarcted myocardium

When the culture temperature was decreased from 37°C to 20°C, confluently cultured fibroblast or fibroblasts co-cultured with EPCs were detached as contiguous cell sheets from temperature-responsive culture surfaces and could be stacked into triple-layer constructs (Fig. 1a). For cell sheet transplantation, the constructs were placed onto polypropylene supporting sheets (2 × 1.5 cm) and transplanted over the anterior wall including the infarcted area (Fig. 1b). After transplantation, the constructs formed a tight attachment to the heart surface and covered the infarcted areas (Fig. 1c).

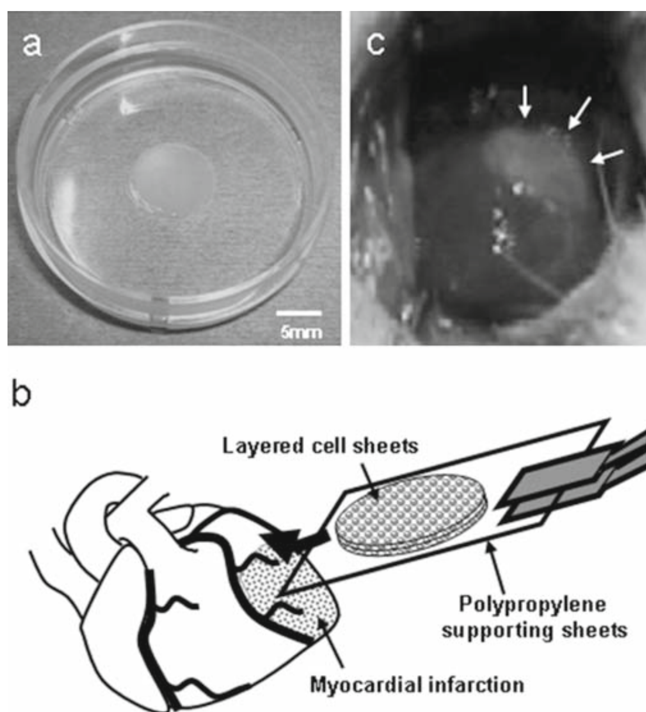

Fig. 1a–c. Stacked cell sheets and transplantation to infarcted rat hearts. Three cell sheets harvested from temperature-responsive culture surfaces were successfully stacked, resulting in a triple-layer construct (**a**). Schematic illustration of cell sheet transplantation (**b**). The tissue grafts were transplanted over the anterior wall of the infarcted heart and formed a stable attachment to the heart surface (*arrows*) (**c**)

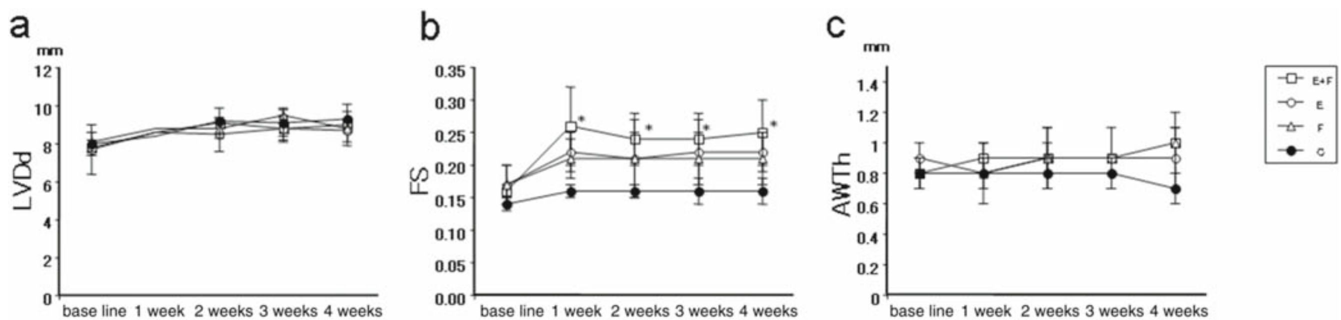

Fig. 2a–c. Echocardiographic analyses. Baseline represents 1 week after left anterior descending coronary artery ligation. The left ventricular (LV) dimension at end-diastole (*LVDd*), the fractional shortening (*FS*), and the LV anterior wall thickness (*AWTh*) at baseline were not significantly different among the four groups. No significant differences in LVDd were observed between the four groups (**a**). The FS in the transplantation group with fibroblast sheets co-cultured with endothelial progenitor cells (EPCs) (*E* + *F*) was significantly improved at all time points after transplantation, in comparison with the control group (*C*). The EPC injection group (*E*) and the fibroblast sheet transplantation group (*F*) showed only a tendency for FS recovery (**b**). The E, F, and E + F groups also showed tendencies for AWTh increases when compared to the C group (**c**). $^*P < 0.05$ versus control group, $n = 5$

Functional recovery of the infarcted myocardium

One week after LAD ligation, all animals exhibited deterioration of LV function and thinning of the infarct wall. The LVDd, FS, and AWTh measurements at baseline were not significantly different among the four groups. Additionally, no significant differences in LVDd were observed between the four groups throughout the observation period (C, E, F, and E + F groups, 4 weeks: 9.3 ± 0.4, 8.7 ± 0.6, 8.8 ± 0.9, and 9.0 ± 1.1 mm, respectively, $n = 5$) (Fig. 2a). However, FS in the E + F group was significantly improved 1 week after the transplantation compared with the C group, and this functional improvement was preserved at 2, 3, and 4 weeks after transplantation (C vs. E + F, 4 weeks: 0.16 ± 0.02 vs. 0.25 ± 0.05, $P < 0.05$, $n = 5$) (Fig. 2b). In contrast, the E and F groups showed only a tendency for recovery of FS (E and F, 4 weeks: 0.22 ± 0.03 and 0.21 ± 0.04, respectively, $n = 5$) (Fig. 2b). In addition, tendencies of increasing AWTh were observed in the E, F, and E + F groups compared with the C group (C, E, F, and E + F, 4 weeks: 0.7 ± 0.1 mm, 0.9 ± 0.2 mm, 1.0 ± 0.2 mm, and 1.0 ± 0.1 mm) (Fig. 2c).

Histological findings

Azan staining of heart cross sections revealed LV anterior wall thinning, LV dilatation, and fibrotic tissue formation in all groups. To determine the quantity of connective tissue, blue areas from azan-stained tissues were measured by NIH-Image software. It was found that the ratio of connective tissue in the infarcted area was significantly reduced in the E group, the F group, and the E + F group compared with the C group (C, E, F, and E + F group: 53% ± 2%, 41% ± 4%, 40% ± 4%, and 32% ± 7%, respectively, $P < 0.05$, $n = 5$) (Fig. 3). In addition, the connective tissue ratio of the E + F group was significantly less than that of the E group.

Regarding the vascularization in the infarcted wall, the percentage of areas composed of blood vessels in the E group, the F group, and the E + F group were significantly greater than in the C group (C, E, F, and E + F group: 1.9% ± 0.6%, 6.7% ± 0.6%, 7.8% ± 0.9%, and 10.2% ± 2.4%, respectively, $P < 0.05$, $n = 5$) (Fig. 4a–e). Vessel formation in the E + F group was also significantly enhanced in comparison to the E group. In the E + F group, both GFP and isolectin B4-positive mature endothelial cells were identified in the infarcted region, indicating that the newly formed blood vessels were composed of cells derived from the EPC-fibroblast sandwich co-cultures (Fig. 4f).

Discussion

The present study demonstrated that the transplantation of fibroblast sheets co-cultured with EPCs could improve damaged heart function with the inhibition of fibrotic tissue formation and acceleration of neovascularization in the infarcted myocardium. Furthermore, co-cultured EPCs participated in new blood vessel formation, indicating that co-cultured cell sheet transplantation was more effective than either cell sheet transplantation alone or EPC injection.

In cell transplantation therapies for damaged hearts, the survival of transplanted cells is crucial for improved efficacy of cardiac function. With injected cell suspensions however, most of the cells are washed-out into blood vessels and can die due to hypoxia. Previous studies have revealed that approximately 90% of cells injected into myocardium were lost within a few days.[7,8] In contrast to isolated cells, cell sheets maintain cell-to-cell junctions and can be transplanted over myocardium as thin, but large-area tissues without cell loss. While cells harvested by enzymatic proteolysis lack adhesive proteins, cell sheets preserve the deposited extracellular matrix proteins on the basal surface of the cultured cells.[27] These adhesive proteins play an important role in enhancing the attachment between stacked cell sheets and also between cell sheets and the myocardial surface, thereby allowing for stable fixation of the cell sheet constructs to the target tissues. We previously confirmed that significantly more cells survived via cell sheet delivery

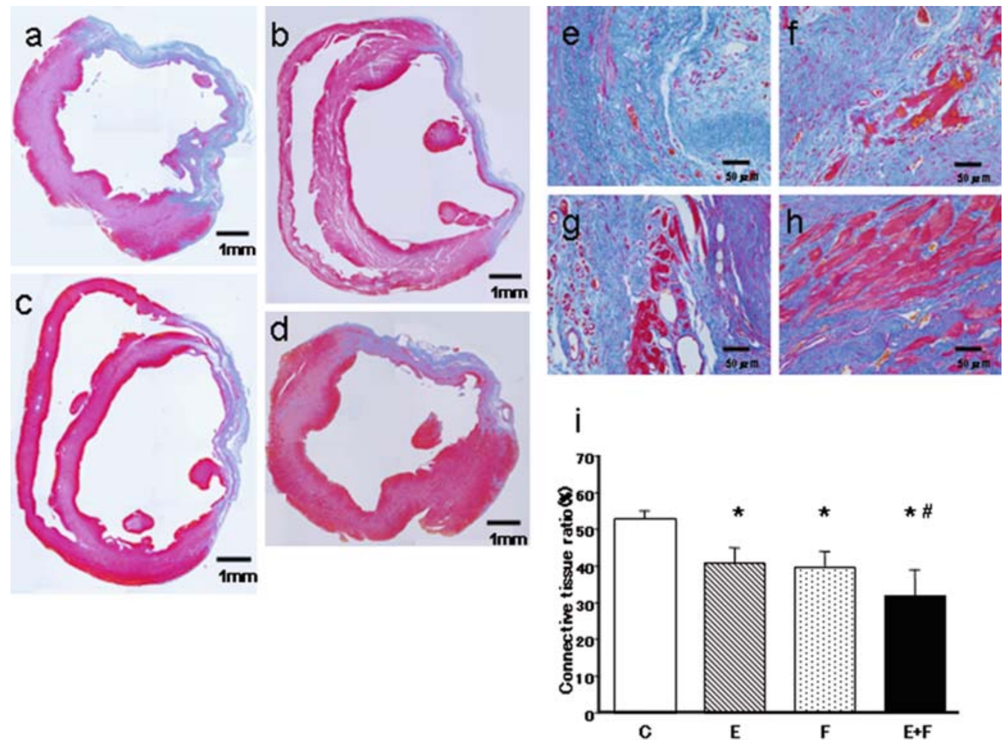

Fig. 3a–i. Azan staining of heart cross sections and inhibition of fibrotic tissue formation. Azan staining of heart cross sections at the left ventricular papillary muscle level are shown in lower magnification (× 20) (**a–d**) and in higher magnification (× 200) (**e–h**). In contrast with the C group (**a, e**), fibrotic tissue formation was diminished in the E group (**b, f**), the F group (**c, g**), and the E + F group (**d, h**). Quantitative analysis of connective tissue formation demonstrated significant inhibition of fibrosis in the E group, the F group, and the E + F group compared with the C group. The connective tissue ratio of the E + F group was significantly less than that of the E group (**i**). $*P < 0.05$ versus the control group, $n = 5$; $^{\#}P < 0.05$ versus the E group, $n = 5$

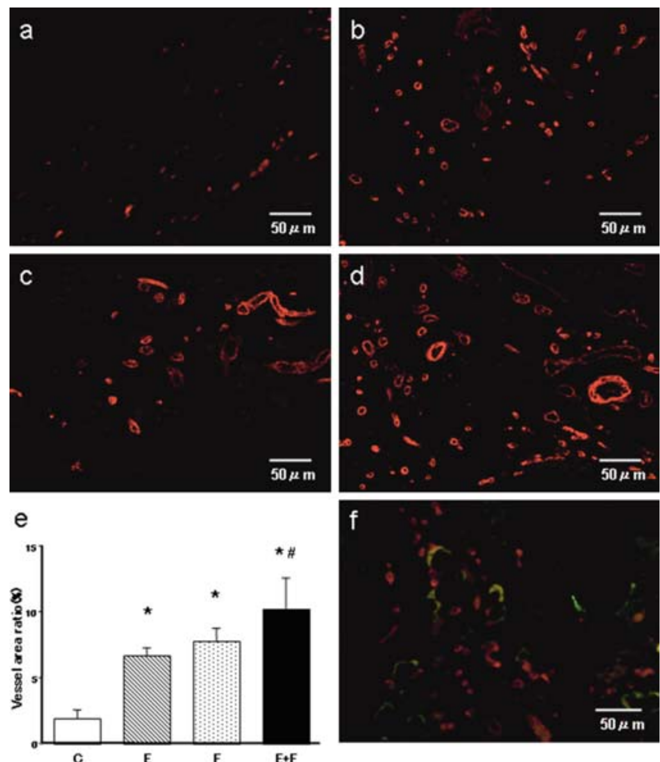

Fig. 4a–f. Vascularization in the infarcted wall. Isolectin B4 staining of the infarcted areas at the left ventricular papillary muscle level are shown (**a**, the C group; **b**, the E group; **c**, the F group; **d**, the E + F group). Quantitative analysis of blood vessel formation demonstrated significant increases in blood vessel numbers in the E, F, and E + F groups, in contrast to the C group (**e**). Vessel formation in the E + F group was also significantly larger than in the E group. Double-staining with anti-Green fluorescent protein (GFP) antibody and isolectin B4 showed that the newly formed vessels were composed of GFP-positive EPCs transplanted with the fibroblast sheets (**f**). $*P < 0.05$ versus the control group, $n = 5$; $^{\#}P < 0.05$ versus the EPC injection group, $n = 5$

than via cell injection delivery. Cell sheet transplantation also improved damaged heart function more effectively than injection of suspended cells.[14,16,28] Therefore, cell sheet transplantation may provide a useful alternative as a new methodology for cell delivery.

In the present study, we proposed an approach that utilizes the transplantation of endothelial cell sources in combination with growth factor secreting cell sheets to accelerate neovascularization and repair of the infarcted myocardium. Regarding endothelial cell sources, it has been previously

reported that bone marrow-derived cells and peripheral blood EPCs possess the ability to differentiate into mature endothelial cells and can participate in blood vessel formation within ischemic hearts.[20,21] We have also recently reported that endothelial cells present within cardiac tissue sheets could induce vascular network formation when transplanted in vivo.[29] In this study, EPCs inserted between fibroblast sheets also survived and contributed to new blood vessel formation. These data confirmed the effectiveness of cotransplantation with an endothelial cell source to treat ischemic hearts.

Previous studies have demonstrated that skeletal myoblast and mesenchymal stem cell sheets have the ability to secrete growth factors including VEGF and HGF, and that these factors can accelerate neovascularization and inhibit fibrotic tissue formation and thus improve damaged heart function.[16,17] In the present study, dermal fibroblasts were used as a cell source because the fibroblasts can be easily isolated and expanded ex vivo. Additionally, dermal fibroblasts have also been shown to have the potential to produce a heterogeneous population of cytokines and growth factors including VEGF, HGF, transforming growth factor-β_1 and angiopoietin-1.[30,31] The present study revealed that fibroblast sheet transplantation alone could increase vascular density and diminish connective tissue formation, but was not able to significantly improve cardiac function. Previous studies have also shown a lack of significant improvement as a result of fibroblast sheet transplantation when performed as control comparison experiments.[15,17] From these data, it appears that the secretion of angiogenic factors by fibroblast sheets may be insufficient for functional myocardial recovery. In contrast, when fibroblast sheets were cocultured with EPCs, the function of damaged hearts could be significantly improved, indicating the additive effects of the combination therapy. EPCs inserted between fibroblast sheets were fixed on the myocardial surface without the cell loss that was observed during isolated cell injection. In clinical settings, large amounts of peripheral blood are generally required to obtain sufficient numbers of EPCs for effective cell injection therapies. Therefore this technique for inserting isolated cells between cell sheets with minimal cell loss may reduce the number of EPCs required. An additional advantage of the use of a combination of fibroblast sheets with EPCs is that the secretion of growth factors from the fibroblast sheets may have stimulatory effects on not only host endothelial cells but may also induce proliferation and differentiation of the inserted EPCs. This combination of beneficial effects may improve functional repair of ischemic hearts.

Regarding the timing of cell sheet transplantation, we transplanted allogeneic cell sheets onto athymic rat hearts 1 week after infarction was induced. For clinical application, it will take about 1 week to expand EPCs and about 2 weeks to expand skin fibroblasts. Therefore, autologous cocultured cell sheet transplantation may be possible at least 2 weeks after the onset of myocardial infarction. For future clinical application, further studies using models with larger animals will be needed to optimize the culturing period and transplantation timing.

If fibrotic tissue formation can be inhibited, it is expected that cardiac remodeling such as LV dilatation and wall thinning will be suppressed. However, in this study, the LVDd and AWTh were not significantly improved despite significant fibrosis reduction in E, F, and E + F groups in comparison with C group and only a tendency of increasing AWTh was observed. This discrepancy may be due to the small sample size.

Several methods have been previously reported regarding bioengineered myocardial patches,[10] with classical tissue engineering approaches utilizing 3-D biodegradable scaffolds.[32–34] However, scaffold-based myocardial patches can present some disadvantages, such as insufficient cell migration into prefabricated scaffolds and inflammatory reactions due to polymer biodegradation. Zimmermann's group have developed a principally different approach by entrapping cardiomyocytes in liquid biological matrix and have successfully engineered functional heart tissues.[35,36] With our technology, cell sheets are layered directly without the use of potentially immunogenic or pathogenic biodegradable scaffolds. Therefore, cell sheet-based bioengineered myocardial patches which are composed solely of living cells and deposited adhesive proteins may be advantageous for future clinical applications.

Conclusion

We have demonstrated the efficacy of fibroblast sheets cocultured with EPCs for myocardial tissue repair. Using this method, cell sheets can be delivered without cell loss and provide a simultaneous supply of vascular-composing cells and angiogenic factors. Thus, transplantation of cell sheets co-cultured with endothelial cell sources may present a new therapeutic strategy for myocardial tissue regeneration.

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