Myocardial Layer-Specific Effect of Myoblast Cell-Sheet Implantation Evaluated by Tissue Strain Imaging

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Background: The implantation of skeletal myoblast (SMB) cell-sheets over the damaged area of a myocardial infarction (MI) has been shown to improve global left ventricular (LV) function through a paracrine effect. However, the regeneration process has not been fully evaluated. We hypothesized that the use of tissue Doppler strain M-mode imaging to assess myocardial layer-specific strain might enable detailed visual evaluation of the regenerative ability of SMBs.

Methods and Results: SMBs were cultured on temperature-responsive culture dishes to generate cell-sheets. At 4 weeks after inducing anterior MI, the animals were divided into 2 groups: SMB cell-sheet implantation and sham operation (n=6 in each). A total of 30 cell-sheets (1.5×10^7 cells/sheet) were placed on the epicardium, covering the infarct and border regions. Subendocardial and subepicardial strain values were measured in the infarct, border, and remote regions by tissue Doppler strain analysis. SMB cell-sheet implantation produced the following major effects: progression of LV remodeling was prevented and global LV ejection fraction increased; the subendocardial strain was significantly greater than the subepicardial strain in the treated border region; vascular density in the subendocardium was significantly higher than in the subepicardium in the treated region; the expression of vascular endothelial growth factor was significantly increased.

Conclusions: Tissue Doppler strain analysis allows precise evaluation of the effect of cell-sheet implantation on layer-specific myocardial function. (Circ J 2013; 77: 1063–1072)

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Measure myocardial layer-specific strain values, based on the transmural myocardial strain profile (TMSP). Within the myocardium, the specific characteristics of each myocardial layer confer a different ability to improve regional myocardial performance. We hypothesized that the myocardial layer-specific strain values might enable an assessment of regional functional improvement, based on the paracrine effects of cytokines following cell-sheet implantation. To investigate our hypothesis, we assessed the TMSP in a porcine model of myocardial infarction (MI).

Methods

Ethics

All studies were performed with the approval of the Ethics Committee of Osaka University. Humane animal care was used in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Animal Resources and published by the National Institutes of Health (Publication No 85-23, revised 1996). All authors had full access to the data and take full responsibility for its integrity. All authors have read and agreed to the manuscript as written. All procedures and evaluations, including the assessment of cardiac parameters, were carried out in a blinded manner.

Animal Models and Study Protocol (Figure 1)

We used 20 female mini-pigs (8–10 months old, 20–25 kg; Japan Farm Co Ltd, Kagoshima, Japan). They were anesthetized with intravenous ketamine (6 mg/kg) and sodium pentobarbital (10 mg/kg) for endotracheal intubation and then maintained with inhaled sevoflurane (1–2%). The pericardial space was exposed by left thoracotomy through the 4th intercostal space. The distal portion of the left anterior descending coronary artery (LAD) was directly ligated, followed by placement of an ameroid constrictor around the LAD just distal of the branching of the left circumflex coronary artery (LCX) to prevent sudden cardiac death from lethal ventricular arrhythmia and intolerance of ischemia. This technique produces an MI model that has clinical relevance and can be used for appropriate preclinical studies with minimal procedure-related mortality (6 (30%) of the 20 mini-pigs died within 48 h of surgery primarily from acute cardiac failure).

Computer-generated random allocation generated 2 randomized study groups at 1 week after the induction of MI, and autologous cells were then isolated and grown in culture for 3 weeks for implantation. At 4 weeks after MI induction, the mini-pigs were again placed under general anesthesia for echocardiography followed by either cell-sheet implantation or sham operation. Two mini-pigs in which the LV ejection fraction (LVEF) was >40%, measured by transthoracic echocardiography using the Simpson’s method before the treatment, were excluded from the study. At 4 and 8 weeks after either cell-sheet implantation or sham operation, the mini-pigs were again placed under general anesthesia for echocardiography examination. The mini-pigs were killed humanely following the 8-week echocardiography study for histological and biochemical analysis of the heart tissue.

Preparing and Grafting Skeletal Myoblast Cell Sheets

Autologous skeletal muscle weighing approximately 10–15 g was removed from the quadriceps femoris muscle, and purified autologous SMB cells were cultured for 3 weeks in preparation for implantation as described previously. The cells were incubated in 60-mm temperature-responsive culture dishes (UpCell®; Cellseed, Tokyo, Japan) at 37°C for 24 h, with the cell numbers adjusted to 1.5×10⁷ cells/dish. The dishes were then transferred to another incubator set at 20°C for 1 h to release the cultured cells as intact cell-sheets. SMB spontaneously detached to generate free-floating monolayer cell-sheets.

At 4 weeks after MI induction, the mini-pigs were randomly divided into the 2 treatment groups (n=6 in each): SMB cell-sheet implantation (Sheet group) or sham operation (Sham group). In the Sheet group, 30 cell-sheets (1.5×10⁶ cells/sheet) with the total cell number being 4.5×10⁸ were implanted on the epicardium of the ischemic area (LAD region) via median sternotomy approach under general anesthesia. Cell sheets were attached and fixed to the epicardial surface by stitching around the edge of the sheet.

Conventional Echocardiography

Global cardiac function was assessed using a commercially available echocardiograph machine with a 4.0-MHz transducer (Aplio; Toshiba, Otawara, Japan) before, and 4 and 8 weeks after cell-sheet implantation. Echocardiographic measurements included LV end-diastolic and end-systolic volumes (LVEDV and LVESV), left ventricular ejection fraction (LVEF), and LV mass index (LVMI). These echocardiographic parameters were measured by 2 readers, who were blinded to the treatment groups.

Figure 1. Study protocol for the assessment of cardiac function and histological analysis. TDI, tissue Doppler imaging; STE, speckle tracking echocardiography; MCE, myocardial contrast echocardiography.
and LVESV, respectively), and LVEF, calculated as:

$$\text{EF} (\%) = 100 \times \frac{\text{LVEDV} - \text{LVESV}}{\text{LVEDV}}.$$  

**Myocardial Layer-Specific Strain Using Tissue Doppler Strain M-Mode Imaging**

Tissue strain M-mode imaging (frame rate, 82–118 frames/s) based on the tissue Doppler technique and the corresponding analysis software (TDI-Q, Toshiba, Otawara, Japan) were used to assess myocardial layer-specific strain. Parasternal short-axis images were recorded at the level of base, mid-ventricle, and apex by tissue Doppler imaging (Figure 2A). To obtain a strain image, TDI-Q first calculates the myocardial displacement of all pixels of tissue by integrating myocardial velocity over a certain period. Next, strain is obtained by evaluating the change in the distance between pairs of points defined on all pixels of the image by utilizing the displacement values. The initial time frame is set at end-diastole to evaluate myocardial deformation occurring in systole. To measure local strain accurately, it is essential to accurately obtain local velocity. Therefore, the present imaging system used tissue Doppler tracking and angle-correction techniques. Tissue Doppler tracking is an automatic motion tracking technique based on tissue Doppler information. By integrating the velocity of an index point on the ventricular wall, identified from tissue Doppler imaging, we could obtain myocardial displacement and predict where the index point would move next. By repeating this procedure, the system can automatically track the motion of the index point (Figure 2B). With this technique, the influence of myocardial translation can be ignored. The angle-correction technique enables Doppler incident angle dependency to be partially overcome. To correct the Doppler incident angle, a contraction center is set at the center of the LV cavity at end-systole in the short-axis view. The software automatically calculates the tissue velocity toward the contraction center (V motion) by dividing the velocity toward a transducer (V beam) by the cosine of the angle (θ) between the Doppler beam and the direction to the contraction center as follows:

$$\text{V motion} = \frac{\text{V beam}}{\cos \theta}$$

Using these 2 techniques, the software TDI-Q automatically cancelled the effect of myocardial translation and angle dependency, accurately assessing myocardial velocity, displacement, and strain. In previously described experiments, the displacement data obtained by this method correlated with true displacement. Myocardial radial strain distribution over the myocardium is obtained as M-mode color-coded images and the profile of distribution (TMSP) at end-systole is shown as in Figure 2C. We divided the myocardium into subendocardial and subepicardial half-layers by the mid-point of the myocardium at end-systole. Mean strain values in the subendocardial half-layer and in the subepicardial half-layer were calculated by averaging the strain values over each layer in the infarcted (center of segment 13), border (edge of segment 7), and remote regions (center of segment 10). In this study, the “infarcted” region was assigned predominantly to territories of the LAD, and the “remote” region was assigned to the LCX or right coronary artery.

**Histological and Immunohistochemical Analyses**

At 8 weeks after the treatment, the hearts were dissected and embedded in optimum cutting temperature compound, snap-frozen in liquid nitrogen, and cut into sections. The 5-μm-thick, paraffin-embedded sections fixed in 4% paraformaldehyde were stained with hematoxylin-eosin (HE) or Masson’s trichrome. Using Image J software, the infarcted area was expressed as a percentage calculated as the positively stained LV area/total LV area in sections stained with Masson’s trichrome. The 5-μm thick cryosections fixed in 4% paraformaldehyde were immunofluorolabeled with anti-von Willebrand factor.
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The average copy number of gene transcripts for each sample was normalized to that for GAPDH.

**Statistical Analysis**
SPSS software (version 11.0, Chicago, IL, USA) was used for statistical analyses. Continuous values are expressed as the mean (standard deviation). The significance of differences was determined using a 2-tailed multiple t-test with Bonferroni correction following repeated measures analysis of variance for individual differences. P<0.05 was considered statistically significant.

**Results**
Gradual Recovery of Global Systolic LV Function
Serial changes in global systolic and diastolic LV function after cell-sheet implantation were assessed by conventional echocardiogram. LVEDV increased significantly at 8 weeks in the Sham group compared with baseline, and remained unchanged in the Sheet group. LVESV significantly decreased in the Sheet group at 4 and 8 weeks after implantation, showing a significantly lower value than that in the Sham group. LVEF significantly increased in the Sheet group at 4 and 8 weeks after implantation, showing a significantly higher value than that in the Sham group. n=6 in each group; *P<0.05. LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVEF, left ventricular ejection fraction.

**Figure 3.** Echocardiographic analysis. LVEDV increased significantly at 8 weeks in the Sham group compared with baseline, and remained unchanged in the Sheet group. LVESV significantly decreased in the Sheet group at 4 and 8 weeks after implantation, showing a significantly lower value than that in the Sham group. LVEF significantly increased in the Sheet group at 4 and 8 weeks after implantation, showing a significantly higher value than that in the Sham group. n=6 in each group; *P<0.05. LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVEF, left ventricular ejection fraction.
Figure 4. Myocardial layer strain value. In the treated border region, subendocardial strain significantly increased at 4 and 8 weeks after cell-sheet implantation, showing a significant increase in comparison with subepicardial strain. In the infarcted region, both subendocardial and subepicardial strain values were greater in the Sheet group than in the Sham group. In the remote region, no significant changes were observed. n=6 in each group; *P<0.05.
cardiography. Following sham operation, LVEDV and LVESV tended to increase till 8 weeks, while LVEF did not change significantly. In contrast, following SMB cell-sheet implantation, LVEDV did not change significantly, but LVESV significantly decreased and LVEF increased significantly at 4 and 8 weeks after SMB cell-sheet implantation compared with before the implantation. At 4 weeks after the treatment, LVESV was significantly smaller and LVEF was significantly greater in the Sheet group than in the Sham group, but there was no significant difference in LVEDV between them. At 8 weeks

Figure 5. Histological findings. (A) Macroscopic (×40) view of the heart (HE). (B) Macroscopic (×40) views of the heart (Masson’s trichrome). (C) The size of the infarcted area of the heart was significantly reduced in the Sheet group as compared with the Sham group. (D) Microscopic (×200) views of sections of the subendocardium and subepicardium in the treated border zone area stained with anti-von Willebrand factor (vWF) antibody (factor VIII) (bar=20μm). (E) A greater number of vWF-positive blood vessels in the subendocardium compared with the subepicardium of the border zone area in the Sheet group. No significant changes are seen in the infarcted and remote areas. *P<0.05.
Tissue Strain Analysis of Cell-Sheet Implantation

Cardiac and subepicardial strain values tended to be greater in the Sheet group than in the Sham group. In the remote region, no significant changes were observed.

Modulation of Myocardial Structure
Myocardial structure, including fibrosis and vascularity, was assessed by HE staining, Masson’s trichrome staining and immunohistochemistry for vWF at 8 weeks after the treatment (Figures 5A, B). The LV cavity was enlarged after the sham operation compared with after sheet implantation, and myocardial structure was well maintained post-sheet implantation compared with post-sham operation, as assessed by HE staining. Collagen had densely accumulated in the infarct area and was globally distributed in the remote area post-sham operation, whereas less collagen accumulated in either the infarct or remote area post-cell-sheet implantation compared with post-sham operation, as assessed by Masson’s trichrome staining.

After the treatment, both LVEDV and LVESV were significantly smaller and LVEF still greater in the Sheet group than in the Sham operation group (Figure 3, Table).

Myocardial Layer-Specific Recovery
Regional LV function in the infarct, border and remote areas was also examined in a myocardial-layer specific manner to assess the regional effects of SMB cell-sheet implantation in more detail by using the TMSP at 4 and 8 weeks post cell-sheet implantation (Figure 4, Table). Before the treatment, myocardial strain values of both the subendocardium and subepicardium were significantly smaller in the infarct and border areas compared with the remote area. After the sham operation, myocardial strain in both the subendocardium and subepicardium showed similar values for the infarct, border, and remote areas for 8 weeks. In contrast, subendocardial strain significantly increased at 4 and 8 weeks after cell-sheet implantation and was significantly larger than subepicardial strain in the treated border region. In the infarcted region, both subendo-
assessed by computer-based planimetry of Masson’s trichrome-stained heart tissue, was significantly smaller in the Sheet group than in the Sham group (Figure 5C).

Vascular density, assessed by immunohistochemistry for vWF, in both the endocardium and epicardium, tended to be greater in the infarct and border areas than in the remote area of sham-operated hearts. In addition, vascular density in the endocardium was significantly greater than that in the epicardium in the border area post-sham operation, whereas vascular density did not differ significantly between the subendocardium and subepicardium in either the infarct or remote area of the sham-operated hearts. After cell-sheet implantation, vascular density did not differ between the subendocardium and subepicardium in either the infarct or remote area, but it was significantly greater in the subendocardium than in the subepicardium in the border region in the Sheet group. Only vascular density in the subendocardium of the border zone showed a significant difference between the sheet-implanted and sham-operated hearts (Figures 5D,E).

Profiles of Expression of Reverse LV Remodeling-Related Molecules

A variety of molecules that are expressed intramyocardial and potentially related to reverse LV remodeling were assessed by real-time PCR. Relative expression of VEGF was significantly increased in the Sheet group compared with the Sham group, whereas other factors, such as TNF-α, IL-6, bFGF and IGF-1, did not show any significant differences (Figure 6). Relative expression of BNP was significantly smaller post-cell-sheet implantation than post-sham operation.

Discussion

In the present study, SMB cell-sheet implantation produced the following major effects: (1) progression of LV remodeling was prevented and global LVEF decreased; (2) subendocardial strain was significantly greater than subepicardial strain in the treated border region; (3) vascular density in the subendocardium was significantly higher than in the subepicardium of the treated region; and (4) the expression of VEGF was significantly increased. Our data therefore suggest that SMB cell-sheet implantation enhanced the paracrine effect (eg, VEGF), inducing angiogenesis and thus improving regional myocardial performance in the targeted area, and these effects were more significant in the subendocardium than in the subepicardium of the border lesion.

The mechanism of restoration of damaged myocardium by SMB cell-sheet implantation is complex and many pathways are involved in the recovery of treated myocardium. Recent reports have described the beneficial results of SMB cell-sheet implantation in several animal experimental models and patients with heart failure, which were primarily attributed to the following factors: the secretion of cytokines from the implanted cell-sheets (ie, paracrine effect), including angiogenic growth factors, the formation of capillary networks, and finally, mechanical inhibition of LV dilatation by implantation of cell-sheets. Previous studies supported this and have shown that SMB and bone marrow-derived mesenchymal stem cell sheets secrete growth factors (eg, VEGF) into the myocardium, and that these factors accelerate neovascularization in the damaged area. Among the many complex molecular and cellular mechanisms, the role of VEGF and its signaling pathway has been intensively investigated in vivo. Toyota et al reported that the expression of VEGF is critical to the growth of coronary collateral vessels. In the present study, VEGF expression was significantly increased in the Sheet group compared with the Sham group, suggesting that SMB cell-sheet implantation induced an angiogenic response via VEGF. Although many studies have proved that released cytokines from implanted cells play a major role in generating therapeutic effects on ischemic myocardium, there is currently no modality to precisely evaluate the section of damaged myocardium affected by released cytokines.

For tissue engineering as cardiac therapy, the creation of mature and functional vessels as neo-vascularization is essential. It has been reported that capillary formation occurs via 2 basic vessel-constructing processes: angiogenesis (ie, the formation of new capillaries via sprouting or intussusception from preexisting vessels), and vasculogenesis. It has been also reported that angiogenesis requires dynamic temporal and spatial regulated interaction among endothelial cells, pericytes, and angiogenic factors. Together with the morphology of vessels forming within myocardial tissues, including the diameter and stability of the vessel walls, we propose another possible mechanism that vessel maturation may occur under pathological stimuli such as increased blood perfusion in the in vivo environment.

To separately elucidate the effects of SMB cell-sheet implantation on LV regional function in the treated infarcted and border areas, we used tissue Doppler derived strain and the corresponding analysis software. SMB cell-sheet implantation therapy induced an improvement in regional myocardial performance in the treated border area, but not the treated infarcted area. Moreover, we speculate that regional functional recovery may correlate well with our data for the upregulation of VEGF gene expression and significant angiogenesis in the border region of the ischemic/infarcted myocardium. In addition, on the basis of the results of an improvement in the strain value as determined by tissue Doppler derived strain, the model used in the present study can be considered as the hibernating state, especially in the border region, instead of as a model of chronic MI. Taken together, the results suggest that SMB cell-sheet therapy may rescue potentially salvageable myocardium partially by reperfusion, thus improving myocardial performance. Together with the paracrine effects of the implanted SMB cell-sheet, humoral substances might have a beneficial effect on native cardiomyocytes and viable surrounding muscle cells, leading to the prevention of global myocardial remodeling. Our results may support the concept of a molecular mechanism of paracrine effect associated with cardioprotective factors released following SMB cell-sheet implantation.

The TMSP showed that SMB cell-sheet implantation induced a more significant regional recovery in the subendocardium than in the subepicardium, despite the SMB cell-sheet being implanted on the epicardium. To understand this mechanism in more detail, we performed tissue strain imaging and the results reflect the fundamental differences in functional properties within the LV myocardium. Ischemic injury did not occur in a uniform manner throughout the LV myocardium. Regional differences in metabolism and energy requirements render the endocardium more vulnerable to injury. Myocardial injury and stunning therefore usually originate in the endocardium and, with time, progress to include the epicardium. In general, VEGF expression is activated under hypoxic conditions, a reasonable mechanism for holding oxygen tension constant. Some previous investigators suggested that a soluble VEGF receptor (ie, sVEGFR1) increases in response to hypoxia. It seems reasonable to assume that paracrine signaling between VEGF and sVEGFR1 might be evoked predominantly
in the ischemic region to regulate angiogenesis, and improve regional myocardial performance, in the face of hypoxia. Thus, the conceptual approach of SMB cell-sheet implantation is the eliciting of a cardiac protective response (eg, angiogenesis and microcirculation) during ischemia and prevention of the progression of ischemic injury and tissue necrosis. A possible mechanism to explain our results is that SMB cell-sheet implantation induces the release of cytokines and enhances the development of microvasculature (ie, microcirculation) that might be particularly vulnerable to injury during ischemia, and upon reperfusion, enhances the recovery of myocardial performance. There is currently an emerging theory that the microcirculation could be the primary target for the amelioration of the potentially devastating consequences of ischemic injury. Nevertheless, it remains to be determined whether the primary benefits of SMB cell-sheet implantation are a consequence of (1) a cardioprotective effect by contributing directly to cardiomyocyte regeneration, (2) paracrine effects emanating from the SMB cell-sheet, or (3) a combination of these effects. Also, it is unclear whether the source of the therapeutic cytokines (eg, VEGF) is the implanted cells or native cardiac cells, such as ischemic cardiomyocytes, endothelial cells, or resident macrophages.

**Study Limitations**

Considerable caution must be exercised in extrapolating the present results. We did not validate myocardial strain values using other methods (eg, sonomicrometry). However, sonomicrometry is not always suitable for the assessment of transmural distribution of myocardial strain. We believe that our measurements were accurate because the displacement data obtained by our method were shown to be accurate. TDI is generally recognized as a 1D method and can measure myocardial deformation along the beam direction only. TDI-based strain estimation suffers from decorrelation caused by both axial motion and motion transverse to the beam direction. 2D speckle tracking strain imaging was introduced to overcome these limitations to myocardial imaging by estimating the 2D in-plane displacements with moderate frame rates. These 2 methods are very different in principle and detail, directly affecting estimation accuracy, even of the same parameters. These differences must be noted when parameters from either method are applied clinically to myocardial contractility characterization. Moreover, the operator must avoid myocardium with large transverse motion to minimize the effect of transverse motion on TDI measurements.

Several investigators have suggested that a zone of dysfunctional myocardium caused by coronary artery occlusion might exist at the border of an infarct, with graded hyperperfusion extending out from the central region of infarction. Subsequent reports demonstrated that coronary microvessels function essentially as end vessels with sharp boundaries between adjacent vascular beds, but that intermediate levels of mean blood flow can exist as a result of admixture of peninsulas of ischemic tissue intermingled with regions of normally perfused myocardium. Although there is tremendous variability in the coronary artery blood supply to myocardial segments, it was believed to be appropriate to assign individual segments to specific coronary artery territories.

**Conclusions**

In conclusion, assessment of the TMSP enabled precise evaluation of the effect of cell-sheet implantation on layer-specific myocardial function. Autologous SMB cell-sheet implantation enhanced the paracrine effect, induced angiogenesis, and increased blood perfusion, thus improving regional myocardial performance more effectively in the subendocardium as compared with the subepicardium of the treated border zone area.

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**Disclosure**

There is no conflict of interest related to this article.

**References**


