Porous tissue grafts sandwiched with multilayered mesenchymal stromal cell sheets induce tissue regeneration for cardiac repair

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Aims To provide the basis for uniform cardiac tissue regeneration, a spatially uniform distribution of adhered cells within a scaffold is a prerequisite. To achieve this goal, a bioengineered tissue graft consisting of a porous tissue scaffold sandwiched with multilayered sheets of mesenchymal stromal cells was developed.

Methods and results This tissue graft (sandwiched patch) was used to replace the infarcted wall in a syngeneic Lewis rat model with an experimentally chronic myocardial infarction (MI). There were four treatment groups (n = 10): sham, MI, empty patch, and sandwiched patch. After a 7 day culture of the sandwiched patch, a tissue graft with relatively uniform cell concentrations was obtained. The cells were viable and tightly adhered to the tissue scaffold, as the endogenous extracellular matrix inherent with multilayered cell sheets can act as an adhesive agent for cell attachment and retention. At retrieval, the area of the empty patch was relatively enlarged, suggesting reduced structural support, while that of the sandwiched patch remained about the same (P = 0.56). In the immunofluorescent staining, host cells together with neo-microvessels were clearly observed in the empty patch; however, there were still a large number of unfilled pores within the patch. In the sandwiched patch, besides host cells, originally seeded cells were populated within the entire patch. No apparent evidence of apoptotic cell death was found in both studied patches. Thus, the sandwiched-patch-treated hearts demonstrated a better heart function to the empty-patch-treated hearts (P < 0.05).

Conclusion The results demonstrated that this novel bioengineered tissue graft can serve as a useful cardiac patch to restore the dilated left ventricle and stabilize heart functions after MI.

KEYWORDS
Myocardial infarction; Angiogenesis; Tissue regeneration; Scaffold; Cell-sheet engineering

1. Introduction

Myocardial infarction (MI) often leads to left ventricular (LV) dilation, thus impairing cardiac functions.1 Restoring the dilated LV is therefore desirable for the treatment of MI.2 To restore the dilated LV, a possible strategy is to replace the infarcted myocardium with bioengineered tissue grafts.3,4 The goal of tissue engineering is to repair or replace the damaged organ or tissues by delivering functional cells on supporting scaffolds to areas in need.5 However, several potential drawbacks accompanying scaffold tissue constructs have been reported in the literature.6–8

Cell-sheet engineering has been developed by Okano’s group as an alternative approach for tissue engineering.9,10 In our recent study, a method, using a thermo-responsive hydrogel [methylcellulose blended with phosphate-buffered saline (PBS)] coated on tissue culture polystyrene (TCPs) dishes, was developed for harvesting living cell sheets.11 We demonstrated that the use of cell sheets can serve as a cell-delivery vehicle by providing a favourable extracellular matrix (ECM) environment to retain cells at the sites of intramuscular injection.12,13 Additionally, it was reported that monolayered mesenchymal stem cells attached onto the anterior wall of the scarred myocardium reversed its wall thinning in infarcted rat hearts, with minimal cell loss.10

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However, the harvested cell sheets lack the mechanical strength to replace the infarcted myocardium for the reconstruction of dilated LV.

In this study, a novel bioengineered tissue graft, a porous acellular bovine pericardium sandwiched with multilayered sheets of mesenchymal stromal cells (MSCs), was developed for the treatment of MI. It was previously shown by our group that the acellular bovine pericardium fixed with genipin can provide a natural microenvironment for host cell migration and may be used as a tissue-engineering scaffold. We hypothesized that this newly developed tissue graft can provide the required mechanical strength to support the sandwiched, multilayered sheets of MSCs for tissue regeneration to restore the dilated LV and improve cardiac functions in a syngeneic rat model.

2. Methods

2.1 Production of multilayered cell sheets

Detailed methods used in the preparation of the cell-sheet culture system can be found in our previous publication and in the online data supplement. Bone marrow MSCs were isolated from femora and tibia of Lewis rats. Procedural details and characteristics of isolated cells (see Supplementary material online, Figure S1) are described in the online-only data supplement. The DNA-demethylating agent 5-azacytidine (5-Aza, Sigma-Aldrich, St Louis, MO, USA) was added on the third day and incubated with MSCs for 24 h. Subsequently, the induced MSCs were labelled for later analysis by adding 100 μg/mL of 5-bromo-2′-deoxyuridine (BrDU, Sigma-Aldrich) containing media to 50% confluent cultures for 24 h. The labelled MSCs were then seeded evenly on the cell-sheet culture system at a density of 5 × 10^5 cells/cm², and a monolayered cell sheet was formed 7 days later. When placed at room temperature (~20 °C), the cell sheet detached gradually from the surface of the thermo-responsive hydrogel spontaneously, without treating with any enzymes. The harvested cell sheet was folded into a five-layer sheet and was cultured for additional 1 day to form an integrated multilayered cell sheet (Figure 1).

The obtained cell sheets were fixed in 4% paraformaldehyde and then stained with a monoclonal antibody against BrdU (BD, Bedford, MA, USA) containing media to 50% confluent cultures for 24 h. Subsequently, the induced MSCs were labelled for later analysis by adding 100 μg/mL of 5-bromo-2′-deoxyuridine (BrDU, Sigma-Aldrich) containing media to 50% confluent cultures for 24 h. The labelled MSCs were then seeded evenly on the cell-sheet culture system at a density of 5 × 10^5 cells/cm², and a monolayered cell sheet was formed 7 days later. When placed at room temperature (~20 °C), the cell sheet detached gradually from the surface of the thermo-responsive hydrogel spontaneously, without treating with any enzymes. The harvested cell sheet was folded into a five-layer sheet and was cultured for additional 1 day to form an integrated multilayered cell sheet (Figure 1).

The obtained cell sheets were fixed in 4% paraformaldehyde and then stained with a monoclonal antibody against BrdU (BD, Bedford, MA, USA), collagen type I (clone 1-B95, MP Biomedical, Solon, OH, USA), collagen type III (clone 3G4, Chemicon, Temecula, CA, USA), fibronectin (clone 7G8, Abcam, Cambridge, UK), or laminin (clone ZEB, Chemicon). A Cy-5-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was then used as the secondary antibody. Cell sheets were co-stained to visualize F-actins and nuclei by phalloidin (Oregon Green® 514 phalloidin, Molecular Probes, Invitrogen, Karlsruhe, Germany) and propidium iodide (PI, P4864, Sigma-Aldrich), respectively. The stained cell sheets were examined using an inverted confocal laser scanning microscope (CLSM, TCS SL, Leica, Wetzlar, Germany) with excitations at 488, 543 and 633 nm, respectively. Three-dimensional images were reconstructed using an LCS software (Leica).

2.2 Preparation of porous tissue scaffolds

Procedures used to prepare and characterize porous acellular bovine pericardia were described previously. Details can be found in the Supplementary data online. Porous acellular tissues were fixed in a 0.05% genipin (Challenge Bioproducts, Taichung, Taiwan) aqueous solution. The pore size of fixed porous acellular tissues (scaffold), stained with hematoxylin and eosin, was determined using a microscope and their porosity was measured by helium pycnometry (n = 10). Mechanical testing of the porous tissue scaffold was conducted by an Instron material testing machine (n = 10, Mini 44, Canton, MA, USA). The porous tissue scaffold was frozen and then sliced into three layers using a cryostat microtome (Leica CM3050S, Nussloch, Germany, ~0.3 mm thickness for each layer).

2.3 Porous tissue grafts sandwiched with multilayered cell sheets

The above-mentioned multilayered cell sheets of MSCs were sandwiched into the sliced tissue scaffold under aseptic conditions and then cultured in TCP5 dishes for 7 days (Figure 1). Ultrastructures of porous tissue scaffolds fixed by genipin, before and after cell seeding, were examined using an inverted CLSM. The fluorogenic characteristics of genipin render it a promising candidate as a visualization reagent.

2.4 Animal study

The investigation conducted with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). MI was created by the ligation of the left coronary artery (LCA) in male syngeneic Lewis rats weighing 300–350 g. Four weeks later, animals with infarcts >25% of the LV free wall were randomly divided into all studied groups. The rat heart was exposed through a median sternotomy. A purse-string suture (5–6 mm in diameter) was placed on the edge of the infarcted wall of LV. Each end of the suture was passed through a 24-gauge plastic vascular cannula, which was used as a tourniquet. The tourniquet was tightened and the bulging part of the infarcted wall inside the purse-string stitch was lopped off.

Subsequently, the above-mentioned sliced tissue scaffold without cell seeding (empty patch, 5 mm diameter, 1.2 ± 0.1 mm thickness, preserved in PBS prior to implantation) or that sandwiched with multilayered sheets of MSCs (sandwiched patch, with 1.5 × 10^6 cells) was sutured along the margin of the purse-string stitch with 7–0 polypropylene to cover the defect created in the LV. After surgery, rats were removed from ventilation and recovered under a warming lamp. Control groups consisted of rats without the LCA ligation (sham group) and those subjected to the LCA ligation but without further treatments (MI group). Animals were coded, so that all measurements were made without knowledge of treatment groups. The study was continued until at least 10 rats survived for at least 3 months in each of the four coded groups.

2.5 Left ventricular function assessment

Echocardiography was performed before patch implantation and at 4 and 12 weeks after patch implantation. Dimension data were presented as the average of measurement of 10 consecutive beats. The fractional shortening (FS) of LV was calculated as follows:

\[
\text{LVFS} = \frac{\text{LVESD} - \text{LVEDD}}{\text{LVEDD}} \times 100\%
\]

where LVEDD and LVESD represent LV dimensions in end-diastole and end-systole, respectively, and LVFS refers to left ventricular fractional shortening. Pressure measurements were performed at 12 weeks postoperatively. The aforementioned measurements were conducted by investigators blinded to the experimental conditions.

2.6 Histological examinations

Specimens of each studied group were retrieved at 12 weeks after patch implantation and were used for gross and histological examinations. The patch areas before implantation (pre-PA) and at retrieval (post-PA) were measured, using a computer-based image analysis system, and graft expansion expressed as post-PA/pre-PA × 100%. Specimens used for light microscopy were fixed in 10% phosphate-buffered formalin, embedded in paraffin and stained with Masson’s trichrome. Measurements of thickness were taken from the middle of each implant with the above-mentioned image analysis system. For immunofluorescent staining, after rehydration and microwave antigen retrieval with 0.1 mol/L sodium citrate, sections were
washed and incubated at 4°C for 12 h with the anti-BrdU antibody resuspended in the dilution buffer. Sections were then double-stained with antibodies against factor VIII (DAKO, Carpinteria, CA), keratin pan (clone C-11, Chemicon), α-smooth muscle actin (α-SMA, clone 1A4, DAKO), smooth muscle myosin heavy chain (SMMHC, clone 1G12, Abcam), cleaved caspase-3 (clone SA1, Cell Signaling Technology, Beverly, MA, USA), or α-actinin (clone EA 53, Sigma-Aldrich). Different Alexa Fluor secondary antibodies (Invitrogen) were used to obtain fluorescent colours. The stained sections were counterstained to visualize nuclei by Sytox blue (Invitrogen) or PI, and examined with excitations at 458, 488, 543 and 633 nm, respectively, using CLSM.

Angiogenesis in each studied patch was examined and quantified by immunofluorescent staining with factor VIII to visualize capillaries and with α-SMA to detect arterioles. The numbers of capillaries and arterioles were counted in a blinded fashion and compared between groups. In addition to the labelling of smooth muscle cells (SMCs) in arterioles, α-SMA was used to identify myofibroblasts, which were characterized by elongated cell morphology without visible lumen organization. The number of apoptotic cells per field, immunostained for cleaved caspase-3, was counted and expressed as a percentage of total cells.

2.7 Statistical analysis

Comparison between the two groups was analysed by the one-tailed Student’s t-test, and multiple group comparison was performed by one-way ANOVA followed by Fisher’s LSD using statistical software (SPSS, Chicago, IL, USA). Data are presented as mean ± SEM. A difference of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Characteristics of the sandwiched patch

Extracellular matrix molecules [collagen type I (data not shown) and collagen type III (Figure 2A)] and integrative adhesive agents [fibronectin (Figure 2B) and laminin (data not shown)] were clearly identified in the monolayered sheet of MSCs (82.2 ± 2.5% BrdU-positive). When folded and subsequently recultured, the folded layers of cell sheet adhered together via their endogenous ECM and became an integrated multilayered sheet of MSCs (Figure 2C).

When viewed under CLSM, the genipin-fixed tissue scaffold was clearly identified by the appearance of distinguishing bright white (Figure 2D). As shown, the tissue scaffold revealed a porous structure with an apparent interconnectivity; and its pore size and porosity were 127.5 ± 28.5 μm and 90.8 ± 1.6%, respectively. The ultimate tensile strengths of the tissue scaffold before (2.7 ± 0.3 MPa) and after slicing into three layers (2.6 ± 0.2 MPa, Figure 2E) were comparable ($P = 0.89$).

The obtained multilayered sheets of MSCs were sandwiched in the sliced tissue scaffold. After a 7 day culture, the three layers of the tissue scaffold were stuck together and seeded cells were populated within the entire scaffold (Figure 2F–H, 760 ± 34 cells/mm²). Immunofluorescence analyses indicated that cells were tightly adhered to a robust fibronectin meshwork within the tissue scaffold, and there was little detectable cleaved caspase-3 (<0.4%). Additionally, ECM molecules (collagen type I and collagen type III, data not shown) were found in the scaffold.

3.2 Animal study

The overall surgical mortality rate, defined as animal death within 4 weeks after LCA ligation, was 5.2% (three of 58 rats), and the late mortality rate (death within 12 weeks after patch implantation) was 7.3% [four of 55 rats (MI group, $n = 2$; empty-patch group, $n = 1$; sandwiched-patch group, $n = 1$)]. The numbers of rats survived until retrieval (at 12 weeks after patch implantation) were: sham group, $n = 10$; MI group, $n = 13$; empty-patch group, $n = 14$; sandwiched-patch group, $n = 14$.

3.3 Left ventricular function assessment

Results of echocardiography (Table 1) and catheterization (Figure 3) revealed a superior heart function in the
empty- and sandwiched-patch groups to the MI group. The empty- and sandwiched-patch groups showed a significantly higher LVFS than the baseline (before patch implantation) and the MI group. Additionally, echocardiographic data showed that the thickness of the sandwiched patch was thicker than that of the empty patch (2.0 ± 0.1 vs. 1.4 ± 0.1 mm, \(P = 0.025\)) at 12 weeks after patch implantation. In pressure measurements, the sandwiched-patch group demonstrated significant improvements in LV end-systolic and end-diastolic pressures (LVEDP) when compared with the empty-patch and MI groups.

### 3.4 Gross examination

At retrieval, the area of the empty patch (graft expansion, 107.6 ± 2.1%) was significantly larger than that of the sandwiched patch (102.1 ± 1.2%, \(P = 0.041\)). On the epicardial surface, 10 out of the 13 studied animals in the MI group had a moderate thoracic adhesion (required sharp dissection, see Supplementary material online, Figure S2). In contrast, minimal thoracic adhesions (readily separated with a forceps) were observed in the empty- and sandwiched-patch groups. On the endocardial surface,
Capillaries in the patches were identified as a single layer of factor VIII-positive cells with a flattened morphology, whereas arterioles were recognized as staining positive for α-SMA and as having a visible lumen. The densities of capillaries and arterioles observed in the sandwiched patch (525 ± 46 and 18 ± 1 vessels/mm²) were significantly greater than those in the empty-patch (250 ± 56 and 10 ± 1 vessels/mm², P = 0.0019 and P = 0.0038, respectively) and MI (120 ± 23 and 5 ± 1 vessels/mm², P = 0.00046 and P = 0.00028, respectively) groups.

In the sandwiched patch, a large number of BrdU-labelled cells were further stained positively for α-SMA (Figure 5P) or SMA-HE (Figure 5Q), indicating that a substantial portion of the implanted MSCs had been differentiated into myofibroblasts or SMCs. Some capillary (or arteriole) walls composed of BrdU-labelled endothelial cells (or SMCs, Figure 5L–N) were recognized. However, no mature cardiomyocytes (α-actin-positive cells) were identified. Factor VIII-positive endothelial cells were visible on the endocardial surfaces of the empty and sandwiched patches, whereas mesothelial cells, positively stained with keratin pan, were observed on their epicardial surfaces (Figure 5S–V).

4. Discussion

One of the major difficulties in cardiac tissue engineering is how to grow 3-D cell/scaffold constructs that contain more than a few layers of seeded cells. To overcome this difficulty, researchers have designed bioreactors, which portray different patterns of fluid dynamics and vessel geometry. These bioreactors may not, however, be the optimal cultivation vessels for seeded cells. The turbulent fluid flow at the surface of constructs is usually characterized by eddies that destroy seeded cells. Moreover, after transplantation, cell washout from the surface of the scaffold, due to an intense shear rate of blood flow exerted on the weakly attached cells, remains a critical issue.

In this study, we successfully established a technique for seeding cells uniformly in a porous tissue scaffold. The application of this technique resulted in tightly attached cells, coupled with high cell viability and relatively uniform cell distribution within the scaffold, factors that are critical in creating a useful implant for cardiac repair.

Cell sheets harvested from the culture system preserved the endogenous ECM with integrative adhesive agents (Figure 2A and B). It is known that ECM provides not only a physical support but also outside-in signals that regulate many cellular functions such as adhesion, migration, proliferation, and differentiation and must be maintained for optimal cellular benefits. The porous tissue scaffold used in the study was made from decellularized bovine pericardia (Figure 2D and E). These decellularized biological tissues are composed of native ECM proteins that can serve as scaffolds for cell attachment, migration, and proliferation. This can be a large advantage over synthetic materials. The tissue scaffold was sliced into three layers and then sandwiched with multilayered sheets of MSCs.

After reculture, a relatively uniform cell distribution within the tissue scaffold was observed (Figure 2F–H). Additionally, we observed that cells were tightly adhered to the scaffold. This may be attributed to the fact that the endogenous ECM fibronectin inherent with cell sheets can act as an adhesive agent for cell attachment. There

3.5 Histological findings

Masson’s trichrome staining demonstrated a severe degree in LV dilation and myocardial fibrosis in the MI group, while dilation of the LV cavity was beneficially reduced in the empty- and sandwiched-patch groups (Figure 4A). Neoconnective tissue fibrils were observed in filling all pores in the sandwiched patch, while there were still a large number of unfilled pores in the empty patch (Figure 4B). The thickness of the sandwiched patch (2.2 ± 0.2 mm) was significantly greater than their counterparts in the empty-patch (1.6 ± 0.2 mm, P = 0.025) and MI (0.7 ± 0.3 mm, P = 0.0012) groups.

In the immunofluorescent staining, host cells (Figure 5A–H) together with neo-microvessels (Figure 5I–N) were clearly observed in the empty patch. In the sandwiched patch, besides host cells, BrdU-positive cells (originally seeded cells) were found within the entire patch (Figure 5F–H, 1460 ± 114 cells/mm²). No apparent evidence of apoptotic cell death was found in both studied patches, as indicated by cleaved caspase-3 staining (<0.2%, Figure 5O).

intimal thickening was observed for both studied patch groups; however, no thrombus formation was found.

Figure 3 The sandwiched-patch group demonstrated significant improvements in the LV function compared with the empty-patch group. Results obtained by catheterization at 12 weeks after patch implantation: (A) LVESP, left ventricular end-systolic pressure and (B) LVESD, left ventricular end-diastolic pressure.

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are reports of fibronectin’s role in regulating anchorage-dependent growth, which was linked to the ability of fibronectin to promote cell adhesion. It was shown that fibronectin was able to induce cell growth on a variety of ECM substrates.

At retrieval, in the sandwiched-patch group, the thickness of the patch increased significantly, while its patch area remained almost the same. This indicated that the mechanical strength of the sandwiched patch was strong enough to tolerate the LV pressure and reduced dilation of the LV cavity.
its extent of dilation. In contrast, although the thickness of the empty patch increased slightly, it was relatively enlarged, suggesting reduced structural support.

An intimal thickening covered with endothelial cells was found on the endocardial surfaces in both the empty- and sandwiched-patch groups (Figure 5S and U). It is speculated that host endocardial endothelial cells or endothelial progenitor cells were involved in the endothelialization of the inner surface of each implanted patch and prevented it from thrombus formation.31 Interestingly, some BrdU-positive cells were identified in the endothelialized inner surface of the sandwiched patch (Figure SU). Endothelialization is one of the most promising mechanisms to reduce thrombogenicity of any cardiovascular implants.32

Host cells together with neo-connective tissue fibrils and neo-microvessels were clearly observed in the outer layers of the empty and sandwiched patches (Figure 5K and N), indicating that the epicardial layers of both studied groups became well integrated with their host tissues. Additionally, an intact layer of neo-mesothelial cells, resting on the regenerated tissues, was recognized by the keratin pan stain (Figure 5T and V [including some BrdU-labelled cells]). It was reported that multipotential cells present within the connective matrix can differentiate into mesothelial cells and contribute to surface reepithelialization.33 Remesothelialization on the outer surface of each implanted patch is assumed to play an important role in the prevention of postsurgical adhesions (see Supplementary material online, Figure S2). Whittaker et al.34 reported that mesothelial cells prevent adhesions.

Tissue regeneration (host cells, neo-connective tissue fibrils and neo-microvessels) was observed in both studied patches. It is known that SMCs (host cells or differentiated from seeded MSCs) permit formation of a muscular tissue in addition to collagen formation.35 The extent of tissue regeneration observed in the sandwiched patch was much more prominent than in the empty patch, particularly in the middle layer of the patch (Figure 4B). This may be attributed to the fact that the density of neo-microvessels observed in the sandwiched patch was significantly greater than in the empty patch.

We found that MSCs (BrdU-labelled cells) originally seeded in the sandwiched patch remained viable and the amount of MSCs increased significantly when compared with that before implantation, implying that cell damage and washout from the implanted patch was minimal. Some of seeded MSCs were differentiated into capillaries and arterioles (Figure 5L–N). Evidences of MSCs incorporation into neovascularules have been reported in the literature.36 Additionally, MSCs have been shown to express angiogenic growth factors in a paracrine way to stimulate neovascularization at the sites of the cell graft.37,38 These facts may explain why there was a significantly greater vascular density observed in the sandwiched patch than in the empty patch (Figure 5I–N). To promote cardiac tissue regeneration, sufficient numbers of cells need to be delivered and maintained within the patch, and the new tissue must be vascularized.38

The sandwiched-patch treated hearts demonstrated reduced graft expansion and stabilized heart functions when compared with the empty-patch treated hearts (Figure 3). However, no mature cardiomyocytes were found in the sandwiched patch, a significant limitation of this study. It was suggested that true cardiac regeneration requires restoring sufficient cardiomyocytes and ensuring their synchronous contraction via electromechanical junctions with host myocardium.39

5. Conclusions

We demonstrated the possibility of using a porous tissue scaffold sandwiched with multilayered sheets of MSCs to overcome the long-standing problem associated with uniform cell distribution within the scaffold and the limitation of cell washout from the scaffold surface when implanted in vivo. The results showed that this novel bioengineered tissue graft can serve as an effective cardiac patch to restore the dilated LV and improve heart functions in a syngeneic rat model with an experimentally chronic MI.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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Bioengineered tissue grafts


