

FIGURE 4. Evaluation of cardiac performance. (a) The left ventricular end-diastolic dimension (LVDD) and (b) left ventricular end-systolic dimension (LVDs) were determined by echocardiography 8 weeks after cell transplantation. (c) The left ventricular ejection fraction (LVEF) was calculated as $LVEF(\%) = (LVDD^3 - LVDs^3) / LVDD^3 \times 100$. The positive control, group S, showed a lower LVDs and higher LVEF compared with group C, whereas group A did not show any differences from group C. Filled columns, group C; checked columns, group S; and open columns, group A. **P* less than 0.05.

transplantation might improve the therapeutic effect by minimizing the immune response.

We observed a strong immune reaction and low donor integration in group A, which were associated with a loss of therapeutic benefit. These findings have implications for the mechanisms involved in SMB transplantation therapy for MI. In MSC transplantation therapy for AMI, paracrine factors

may function as the main regulators, and donor cell survival might not be so important (21). In addition, some reports have suggested the contribution of soluble factors in SMB transplantation (22–26). Farahmand et al. (24) reported that the beneficial effect of SMB transplantation is because of the inhibition of matrix remodeling in noninfarcted tissue, which is probably mediated by a paracrine effect. However, it seems unlikely that the therapeutic gain in SMB transplantation is entirely due to a paracrine effect. In this study of SMB transplantation, the donor cells were much more abundant and survived for a longer period (Fig. 3), compared with MSC transplantation. We previously evaluated the number of MSC in infarct myocardium using the same technique (8). The number of syngenic MSC in infarct myocardium decreased notably within the first 24 hr and then continued to decrease slowly, although it was effective. Conversely, even in allogenic SMB transplantation, this number was higher compared with syngenic MSC (day 1: $1.71 \times 10^5 \pm 84617$ vs. $4.52 \times 10^4 \pm 13061$). In SMB transplantation therapy, it is possible that the grafted SMB themselves contribute directly to reduce wall stress, increase scar elasticity, and buttress infarcted LV walls, which would limit LV remodeling (9). Although controversy remains, it was also reported that SMBs in infarcted myocardium differentiate into a myogenic lineage and compensate for lost contractile ability (27). The low level and short-term existence of allogenic SMBs in the myocardium may thus relate to the absence of a therapeutic effect.

The number of donor SMBs was increased on day 4 after transplantation (Fig. 3). Some reports have noted that grafted SMBs proliferate in both infarcted and noninfarcted myocardium (28, 29). Furthermore, we previously reported that MSCs do not proliferate (8). These findings may indicate differences in the skeletal muscle cell properties, which potentially have a greater tolerance than other cell types to hypoxic conditions (29). The donor SMBs began to decline on day 7 (Fig. 3), which correlated with the activation of the immune response (Fig. 2). In addition, the donor cells disappeared in group A, in which a stronger and more rapid immune response occurred than in group S, strongly suggesting that the disappearance of donor cells is influenced by their immunogenicity.

In summary, in a rat model of infarcted myocardium, MHC-mismatched allogenic SMB transplantation induced an immune response with CD4- and CD8-positive T-cell activation; this response was associated with accelerated donor cell clearance and loss of therapeutic effect. Furthermore, our findings suggest that donor cell survival and inflammation are important aspects of the therapeutic mechanism of SMB transplantation for AMI, although additional investigation is needed.

MATERIALS AND METHODS

Humane animal care was performed in compliance with the “Principals 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 Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 85-23, revised 1996).

SMB Harvest and Culture

SMBs were harvested from the tibialis anterior muscle tissues of 3-week-old ACI (ACI/NJcl: RT-1^{av}, CLEA Japan, Inc., Tokyo, Japan) and LEW

TABLE 1. Primers and probes used in this study

	Forward primer	Reverse primer	Probe
IL-2	GCCTTGTGTGTTATAAGTAGGAGGC	AGTGCCAATTCGATGATGAGC	TCTCCTCAGAAATTCACCACAGTTGCTG
SRY	GCCTCAGGACATATTAATCTCTGGAG	GCTGATCTCTGAATTCTGCATGC	AGGCGCAAGTTGGCTCAACAGAATCC
GAPDH	CCATCACTGCCACTCAGAAGAC	TCATACTTGGCAGGTTTCTCCA	CGTGTTCCTACCCCAATGTATCCGT
IL-2R	CCCTCAGGTGTTTCTTGAGCTT	CTTTCCCAGAGAGTGAGGCTTC	TGGCCACTGCTACCTGATACTCCTTTGTGA
IFN- γ	ATCGAATCGACCTGATCACTA	TTCTTATTGGCACACTCTTACCC	AACAACCCACAGATCCAGCACAAGC

IL-2R, interleukin-2 receptor; IFN, interferon.

(LEW/Sea: RT-1¹, Kyudo, Kumamoto, Japan) rats, as described previously (30). Briefly, cells from the muscle mass were obtained by enzymatic dissociation, by adding 0.2% collagenase type II (Life Technologies, CA) and shaking vigorously for 1 hr at 37°C. The extracts of muscle cell were then preplated in collagen-coated flasks (BD, NJ). Twenty-four hours after isolation, the nonadherent cell suspension was collected and seeded on Matrigel (0.5 mg/mL; BD)-coated flasks and allowed to attach for the next 48 hr. SMBs were grown in Dulbecco's modified Eagle's medium (D-MEM; Life Technologies) containing 20% heat-inactivated fetal bovine serum (lot selected for promoting rapid SMB expansion; MP Biochemicals, LLC, CA), 2 mM L-glutamine (Life Technologies), and penicillin streptomycin (Life Technologies) at 37°C and 5% CO₂ for 7 days. To identify implanted cells in vivo, SMBs were labeled using a PKH26 red fluorescent linker kit (Sigma-Aldrich Corp., MO) at the end of culture, following the manufacturer's instructions. To enhance SMB immunogenicity, SMBs were pretreated with 10 ng/mL recombinant rat IFN- γ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 4 days before use (31).

Animal Experiments

LEW rats were prepared as a model of acute heart failure and placed randomly into three treatment groups: those that underwent transplantation of LEW SMBs (syngenic SMB transplantation: group S), those that underwent transplantation of ACI SMBs (allogenic SMB transplantation: group A), and those that underwent cell-free buffer injection, as the no-treatment control (control: group C). Twenty-five rats in each group were used for DNA and RNA preparations at 0 (15 min), 1 (24 hr), 4, 7, and 28 days after transplantation (five rats at each time point). Six rats in each group were used for histologic analysis at 4 and 7 days after transplantation (three at each time point), whereas 10 rats in each group were examined by echocardiography 8 weeks after transplantation.

Eight-week-old female LEW rats weighing 150 to 180 g were used as SMB recipients. MI was produced by ligation of the left anterior descending coronary artery, as described previously (32), then subjected to cell transplantation 15 min later. A suspension of SMBs (5.0×10^6 in 200 μ L of Hank's buffered salt solution; Sigma-Aldrich) obtained from a male ACI or LEW rat, or 200 μ L of cell-free Hank's buffered salt solution, was injected into the LV anterior wall of heart of each recipient female rat at five points, using a 26-gauge needle. Injection was performed under direct observation with a surgical microscope to ensure there was no leakage of the cell suspension. If leakage was suspected, the sample was excluded from the study. The rats were allowed to recover under care.

The recipient rats were killed at 0 (15 min), 1 (24 hr), 4, 7, and 28 days after surgery by intravenous injection of pentobarbital (200 mg/kg body weight; DS Pharma Biomedical Co., Ltd, Osaka, Japan) and potassium chloride (30 mM; Wako Pure Chemical Industries) to cause cardiac arrest in diastole under terminal anesthesia, and the heart was excised. Hearts used for *sry* gene and RNA analyses were dissected to remove the right ventricular free wall and soaked in RNA Later (Qiagen, Hilden, Germany). Hearts used for histologic examinations were cut into three segments, embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), and snap-frozen in liquid nitrogen.

Measurement of Donor Cell Number

The procedure used to determine the number of donor cells in the recipient hearts was described previously (8, 20). The number of donor SMBs was determined at 0 (15 min), 1 (24 hr), 4, 7, and 28 days after transplantation. Quantitative PCR assays for *sry* and *IL-2* were performed with the primers and probes listed in Table 1. The probes were labeled with a 5' fluorogenic probe (6-carboxyfluorescein [6FAM]) and a 3' quencher (5/6-carboxy-tetramethyl-rhodamine [TAMRA]).

Histologic Analysis

Histologic analyses were performed at 4 and 7 days after transplantation. The hearts were cut transversely from the apex to the base into three equal slice samples and frozen in liquid nitrogen. These frozen samples were cut into 5- μ m cryosections. To evaluate T-cell infiltration into the border zone, the sections from day 7 were stained with antibodies for CD4 and CD8 (1:100 dilutions, Millipore, MA). The second antibody was Alexa488-conjugated goat anti-mouse antibody (1 μ g/mL, Life Technologies). The samples were then counterstained with 6-diamidino-2-phenylindole (1 μ g/mL, Dojindo Laboratories, Kumamoto, Japan). To identify implanted cells, which had been labeled with fluorescent dye, the sections from day 4 were stained with 6-diamidino-2-phenylindole or hematoxylin-eosin and examined by fluorescence microscopy (Keyence Corp., Osaka, Japan) and confocal microscopy (Bio-Rad Laboratories Inc., CA).

Statistical Analysis

All values are expressed as the mean \pm standard error of the mean. To assess the significance of differences between individual groups, statistical evaluations were conducted using one-way analysis of variance with Fisher's protected least significant difference (PLSD). *P* less than 0.05 was considered significant.

An expanded Materials and Methods section is available as **Supplemental Digital Content 2** (<http://links.lww.com/TP/A338>).

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Biventricular support using implantable continuous-flow ventricular assist devices

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KEYWORDS:

biventricular assist device;
implantable BiVAD;
continuous-flow;
centrifugal pump;
axial-flow pump;
bridge to transplantation

A 34-year-old woman with fulminant myocarditis underwent emergent implant with the Toyobo (Nipro, Osaka, Japan) paracorporeal biventricular assist device (BiVAD). The patient had been stable for 6 months, until she started to develop heart failure symptoms due to severe pulmonary insufficiency. Pulmonary valve closure and BiVAD conversion to implantable rotary pumps was performed. A DuraHeart centrifugal pump (Terumo Heart Inc, Ann Arbor, MI) was used for left ventricular assist, and a Jarvik 2000 axial-flow pump (Jarvik Heart Inc, New York, NY) was used for right ventricular assist. Although strict management was required to balance the flow rates of the 2 different types of devices, her postoperative course was uneventful and she was discharged home.

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Despite excellent long-term results of implantable continuous-flow left ventricular assist devices (LVAD),¹⁻³ extracorporeal devices are often used for patients who require biventricular support as a bridge to transplantation.^{4,5} This is problematic, especially in Japan, where the average waiting time for transplantation with device support exceeds 2 years. We report a case of successful implantation of continuous-flow VADs for biventricular support.

Case report

A 34-year-old woman with cardiogenic shock due to fulminant myocarditis was referred to our institute under extracorporeal life support. Emergent biventricular assist device (BiVAD) implantation with the Toyobo (Toyobo-Nipro, Osaka, Japan) paracorporeal device was performed.

Intraoperatively, neither ventricle had visible contractions and no significant electrical activity, even with epicardial pacing. Histopathologic study of the myocardium

showed very few viable cardiomyocytes due to massive inflammatory cell infiltration.

Two ischemic strokes occurred postoperatively; fortunately, the patient recovered from both events without any significant neurologic sequela. Owing to the lack of recovery of cardiac function, she was listed as a potential heart transplant recipient. Even though her native cardiac function had been completely absent, the patient was stable with BiVAD support for 6 months, until further heart failure symptoms developed secondary to severe pulmonary insufficiency.

Because of the long waiting times expected for heart donation, pulmonary valve closure and BiVAD conversion to implantable rotary pumps was performed. We selected the DuraHeart (Terumo Heart Inc, Ann Arbor, MI) as the LVAD and the Jarvik 2000 (Jarvik Heart Inc, New York, NY) as the right VAD (RVAD).

Cardiopulmonary bypass (CPB) was established and the Toyobo BiVAD was explanted. When the inflow cannula of the Toyobo LVAD was removed, a fresh wedge of thrombus was found around the inflow cannula. This was completely removed to prevent further thromboembolism. The main pulmonary artery was opened, and the pulmonary valve was directly closed using 5-0 Prolene (Ethicon, Somerville, NJ) continuous suture. Because the DuraHeart LVAD has the same size inflow cannula as the Toyobo LVAD, the previous

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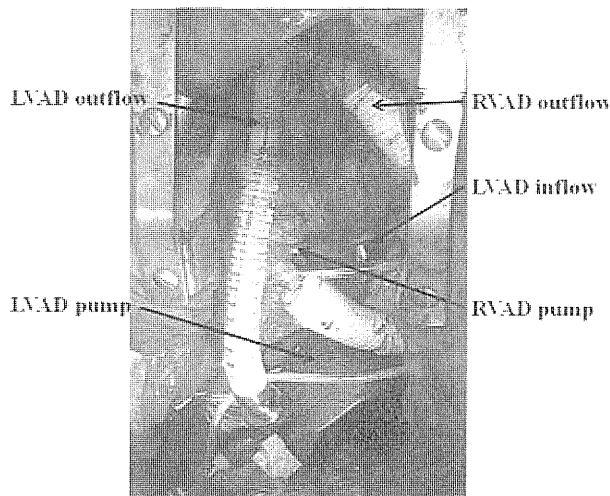


Figure 1 The inflow cannula of DuraHeart left ventricular assist device (LVAD) is inserted in the LV apex and the outflow graft is anastomosed to the ascending aorta. The Jarvik 2000 right ventricular assist device (RVAD) is within the RV, and the outflow graft is anastomosed to the pulmonary trunk. The alignment of the 2 devices was excellent and there was no difficulty in closing the chest.

Toyobo LV apical cuff was left in place, and the DuraHeart inflow cannula was connected. The inflow cuff of Toyobo RVAD, which was put on the RV free wall, was replaced with a new Jarvik 2000 inflow cuff. The anterior leaflet of the tricuspid valve was sutured to the anterior wall of the RV to avoid RVAD inflow obstruction. The outflow graft was routed through the left pleural space and anastomosed to the main pulmonary artery in end-to-side fashion. The alignment of the 2 devices is shown in Figure 1.

Weaning from CPB was somewhat difficult because it required a delicate adjustment of the BiVAD flow. Even though the Jarvik 2000 RVAD flow rate was set at the minimum (dial 1: 8,000 rpm), the RV was sucked down, and an adequate forward flow could not be obtained initially. Transesophageal echocardiography (TEE) showed that the ventricular septum was markedly shifted towards the RV (Figure 2). With adequate volume resuscitation and careful flow adjustment by

monitoring both LV and RV volume with TEE, the patient was successfully weaned from CPB, and BiVAD flow finally reached approximately 4.5 liters/min.

At the time of the second operation, the contraction of the both ventricles was still completely absent. The myocardial biopsy specimen revealed that the myocardium had been replaced with fibrous tissue.

The patient's postoperative course was uneventful. She was extubated on the fifth post-operative day; however, severe pulmonary distress developed and the patient was reintubated 6 hours later. Chest X-ray imaging showed severe pulmonary congestion (Figure 3A), which was immediately resolved by reintubation and increasing the LVAD flow by 10% (Figure 3B). This BiVAD flow imbalance seemed to be caused by a sudden reduction of the pulmonary vascular resistance after extubation. She was successfully extubated 5 days later by increasing the flow rate of DuraHeart LVAD (Figure 3C) and keeping her in room air to control the pulmonary circulation. Pulmonary congestion has not recurred, and the LVAD flow is stable (Figure 3C). Her ventricles have now been standstill for 17 months, and 11 months have passed after the BiVAD conversion. She is living an almost normal daily life as a housewife and is waiting at home for heart transplantation.

Discussion

Various types of implantable LVADs have been used successfully for patients with end-stage heart failure as a bridge to transplantation, bridge to recovery, or destination therapy. Although recently developed small, implantable, continuous-flow pumps have demonstrated excellent results,¹⁻³ extracorporeal devices are still the first choice in patients who require biventricular support for their easy management.^{4,5} Extracorporeal devices have several limitations, including poor blood compatibility, high infection rates, high cerebrovascular event rates, and the need for a prolonged hospital stay with a reduced quality of life.^{5,6} Their use is limited to short-term to medium-term support. This makes it very difficult to bridge a

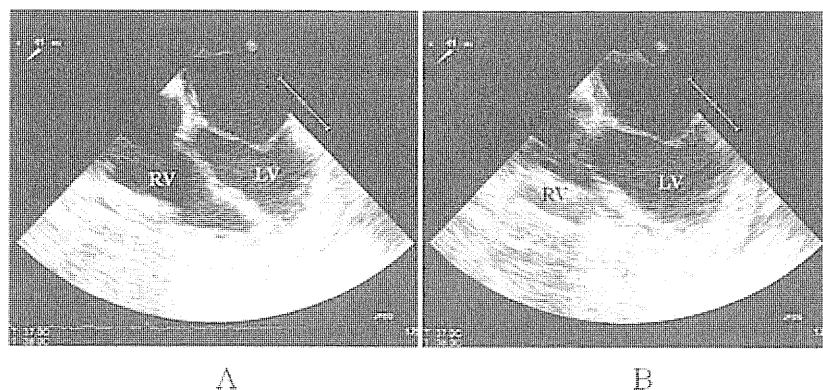


Figure 2 Intraoperative transesophageal echocardiographic study (A) while the patient was on cardiopulmonary bypass and before biventricular assist device (BiVAD) support was started and (B) on BiVAD support (Jarvik 2000 RVAD: dial 1, DuraHeart LVAD: 1600 rpm). On starting the BiVAD, the interventricular septum shifted immediately to the right side and sucking of the right ventricular (RV) wall by the Jarvik 2000 was observed. LV, left ventricle.

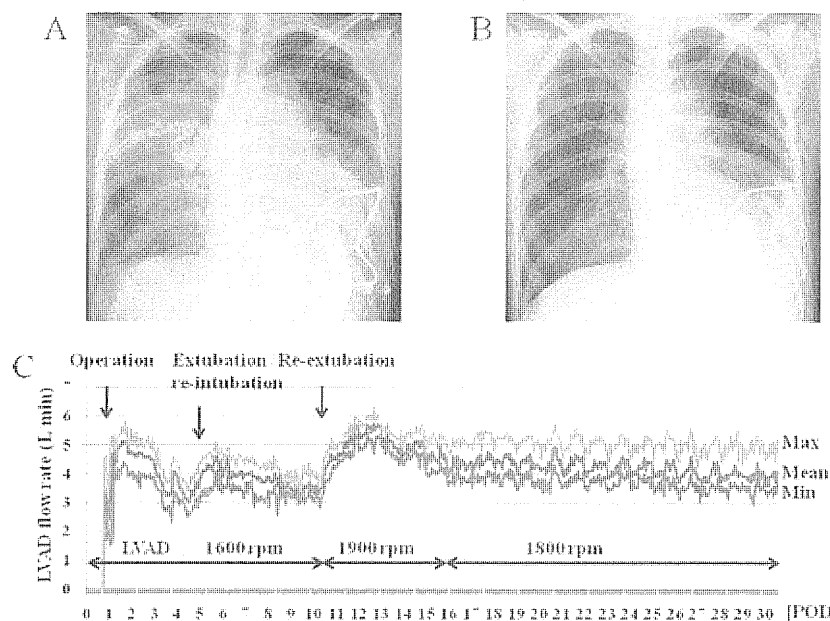


Figure 3 (A) Severe pulmonary congestion was observed after extubation. (B) The pulmonary congestion was improved immediately after reintubation. (C) Left ventricular assist device (LVAD) flow increased after extubation. Although dynamic flow change occurred in the acute phase, the flow rate was stabilized in the chronic phase. POD, postoperative days.

patient with the Toyobo paracorporeal BiVAD to transplantation in Japan, where the average waiting time for heart transplantation exceeds 2 years.

We selected the DuraHeart for LVAD and the Jarvik 2000 for RVAD because the size of the inflow cannula and the outflow graft of DuraHeart are the same as those of the Toyobo pump. Subsequently, the inflow cuff did not need to be exchanged, and the aortic side clamp was not needed because the outflow graft could be directly anastomosed to that used with the Toyobo pump. The Jarvik 2000 is not considered suitable as an LVAD for patients in whom LV contraction is totally absent.⁷ The Jarvik 2000 is operated in an intermittent low-speed mode⁸ to allow aortic valve opening, but this function would only adversely affect in patients whose aortic valve never opens and whose cardiac output is completely dependent on the LVAD flow. On the other hand, the Jarvik 2000 is small and does not require a pump pocket, and was considered suitable as an RVAD to be implanted together with an implantable LVAD, even in small Japanese patients (Figure 1).

Although we expected that the same device for both sides would be easier to control, the dual DuraHeart BiVAD was impossible to implant in the patient because it requires a large pump pocket. The dual Jarvik 2000 BiVAD would have been one of the choices if the patient's LV function had been better. However, in fact, the handling of the unrelated right and left devices, although their output and function are not automatically coordinated, was not too difficult. Because we used the Jarvik 2000 as the RVAD, all we had to do was to set the RVAD at the minimal pump speed and control the LVAD speed.

The importance of balancing the right and left device flow was first learned with the original Jarvik total artificial heart (TAH).⁹ Even a very small excess in the output of the right-sided device compared with that of the left-sided de-

vice results in immediate onset of pulmonary edema. Rousset et al¹⁰ reported that pulmonary edema was one of the important adverse events resulting in respiratory dysfunction in patients receiving implantation of Jarvik TAH.¹⁰

A leading factor that has prevented the use of continuous-flow devices in BiVADs or TAHs is the lack of pre-load sensitivity. Pulsatile devices are generally pre-load-sensitive and efficient at adapting to the patient's physiologic condition with a Frank-Starling-like response to venous return. However, continuous-flow pumps are generally much more after-load-sensitive, making it more difficult to respond to varying patient pre-load. A group at Cleveland Clinic is now developing a TAH that could passively self-regulate the flow balance between left and right artificial hearts in response to atrial pressure balance.^{11,12} Clinical applications of such devices would overcome the drawbacks of the continuous-flow BiVAD/TAH.

In the present case, the optimal flow balance between the 2 pumps was initially difficult to achieve. As soon as CPB was converted to the BiVAD support, severe sucking of the RV by the Jarvik 2000 RVAD occurred, even with the minimal setting of the device (Figure 2A and B). Post-operatively, an acute decrease in pulmonary vascular resistance by termination of positive pressure ventilation resulted in severe pulmonary congestion (Figure 3A). Once the optimal flow balance between the right and left pump was achieved, the flow imbalance never occurred again, even with changes in the patient's activity (Figure 3C). Interestingly, it appears that the patient's body regulates the balance itself in the chronic phase. We speculate that the patient's remaining ventricles, although they totally lack contractility, act as buffer chambers and prevent the acute flow mismatch between the LVAD and the RVAD. In that sense, it would be more difficult to control the balance

between systemic and pulmonary flow with a TAH because it needs to remove both ventricles.

In conclusion, we report a patient with fulminant myocarditis with completely absent cardiac function, in whom the Toyobo paracorporeal BiVAD was successfully converted to the DuraHeart LVAD and Jarvik 2000 RVAD. Thus, we report the simultaneous implantation of centrifugal-flow and axial-flow implantable VADs for LV and RV assist, respectively.

Disclosure statement

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None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclose.

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Tissue-Engineered Cardiac Constructs for Cardiac Repair

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Several recent basic research studies have described surgical methods for cardiac repair using tissue cardiomyoplasty. This review summarizes recent advances in cardiac repair using bioengineered tissue from the viewpoint of the cardiac surgeon. We conclude that the results of many basic and preclinical studies indicate that bioengineered tissue can be adapted to conventional surgical techniques. However, no clinical studies have yet

proved bioengineered tissue is effective as a treatment for human heart failure. Today's cardiac surgeons can look forward to the advent of new techniques to benefit patients who respond poorly to existing treatment for heart failure.

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Heart failure, which is mainly caused by ischemic or dilated cardiomyopathy (DCM), is a life-threatening disorder worldwide. Many excellent surgical techniques for treating cardiac diseases have been developed, and current cardiac surgical treatments are stable and prolong human life. Surgical treatments for end-stage heart failure were unexplored until innovative cardiac surgeons and researchers introduced left ventricular assist device (LVAD) implantation [1] and heart transplantation [2] to this field.

Owing to the untiring efforts of many doctors and researchers, these strategies have made tremendous contributions to the treatment of heart failure for several decades. These procedures have some limitations, however, because of the limited durability of the LVAD [3] and the shortage of heart donors [4]. Thus, cardiac surgeons have considered alternative strategies for treating end-stage heart failure. Recently, there have been many reports on the use of the LVAD to induce myocardial regeneration [5] in addition to its powerful LV support [6]. This innovative treatment is called the bridge to recovery, but its efficacy has only been shown in selected patients [7].

Recently, remarkable progress has been made in myocardial regeneration therapy using cellular cardiomyoplasty, a technique that involves injecting skeletal myoblasts [8] or bone marrow mononuclear cells [9] into the myocardium. This technique has been introduced in clinical trials for treating heart failure and was shown to be feasible and safe, although its efficacy was insufficient to repair the severely damaged myocardium. Thus, a

next-generation strategy for myocardial regeneration therapy, tissue-engineered cardiomyoplasty, which uses cell sheet and cell-based scaffold implantation techniques, is being developed in the laboratory and in the clinic. In addition, to induce angiogenesis to treat ischemic heart diseases, injectable scaffolds and new drug delivery methods have been developed. Other attractive techniques for cardiovascular surgery include tissue-engineered valves [10] and a right ventricular outflow patch that grows in situ [11, 12].

In this environment, these recent advances suggest that cardiovascular surgeons will soon be able to use these advanced techniques to treat heart failure patients whose disease is currently intractable. Moreover, these techniques have the potential to enhance the effectiveness of conventional surgical procedures when used in combination with them. The aim of this review is to analyze recent advances in myocardial regeneration that make use of bioengineered cardiac tissue and to depict their potential application and possible drawbacks from the viewpoint of the cardiac surgeon.

Literature Search

PubMed was searched for terms including, "cell," "sheet," "heart failure," "scaffold," "clinical trial," "myocardial infarction," "temperature responsive cell culture surface," "tissue engineering," "biomaterial," and "cardiomyocyte." We reviewed the abstracts of the obtained articles and chose to analyze critically only those that addressed cell sheets and cell scaffolds. During the review of these articles, if we found a reference that was not captured in the initial literature search but appeared relevant to the topic, it was retrieved and included.

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Recent Advancements in Bioengineered Myocardial Grafts

In recent studies using animals, instead of implanting scattered cells, artificial cardiac tissue was created *ex vivo* and implanted into the distressed heart. This cardiac tissue can be implanted into the failed heart only by a surgical procedure, so cardiac surgeons hold an unchallenged position to introduce this technique in the clinic. This method can provide viable muscle tissue (myogenesis) to the severely damaged myocardium that has few myocytes and massive fibrosis and can also induce angiogenesis. These distinctive features of bioengineered cardiac tissue are attractive to clinicians who seek to regenerate the severely damaged myocardium.

Bioengineered Myocardial Grafts Without a Scaffold

Development of Engineered Cell Sheets

In 1999 a 3-dimensional cardiac-like tissue with spontaneous contractile activity was developed using the self-assembling properties of neonatal cardiomyocytes [13]. Kelm and colleagues [14] created a 3-dimensional micro-tissue using the same approach, and Baar and colleagues [15] prepared a cylindrical construct that spontaneously contracted and produced force. Another encouraging approach for creating myocardial tissue without a scaffold is the cell sheet technique, which was developed by Okano and colleagues [16] and has been applied to several diseased organs, including the heart [17], eye [18], and kidney [19], in the laboratory or clinic, or both.

The cell sheets are generated on and removed from special dishes that are coated with a temperature-responsive polymer, poly[N-isopropylacrylamide], that changes from hydrophobic to hydrophilic when the temperature is lowered without destroying the cell-cell or cell-extracellular matrix (ECM) adhesions in the cell sheet (Fig 1). The greatest advantage of this technique is that the sheet is made only of cells, and the cells produce the ECM without requiring an artificial scaffold [20]. The

cell sheet has a high ability to integrate with native tissues because of the preservation of the adhesion molecules on its surface [21].

Cardiomyocyte Sheets

Shimizu and colleagues [22] developed a contractile chick cardiomyocyte sheet in special dish, without enzymatic or ethylenediaminetetraacetic acid treatment, that had a recognizable heart-tissue-like structure and showed electrical pulsatile amplitude [22]. They layered one-cell sheets to make bilayer cell sheets (the electrically communicative 3-dimensional cardiac construct) that showed spontaneous and synchronous pulsation and showed that the cell sheets adhered together rapidly, as indicated by the presence of desmosomes and intercalated disks [23].

A 4-layered neonatal rat cardiomyocyte sheet was also developed that had electrical communication between the sheets by connexin43. This pulsatile cardiac tissue, when implanted subcutaneously, could survive for up to 1 year and showed spontaneous beating, heart tissue-like structure, and neovascularization, as well as increasing in size, conduction velocity, and contractile force in proportion to the host's growth [24, 25].

Cardiomyocyte sheets are flexible and can change their shape. Myocardial tubes have also been created that can produce pressure and follow Starling's law [26]. Sekine and colleagues [27] wrapped a myocardial tube around the rat thoracic aorta and showed that it could produce pressure *in vivo*.

Interestingly, the electrical coupling between 2 sheets starts approximately 34 minutes after they are brought into contact and is completed by about 46 minutes, as determined by a multiple-electrode extracellular recording system; histologic examination revealed the presence of connexin43 within 30 minutes [28]. These data predict that the electrical coupling between a cardiomyocyte sheet and the host myocardium should occur within 1 hour of implantation.

Miyagawa and colleagues [17] demonstrated that a neonatal cardiomyocyte sheet could survive in infarcted

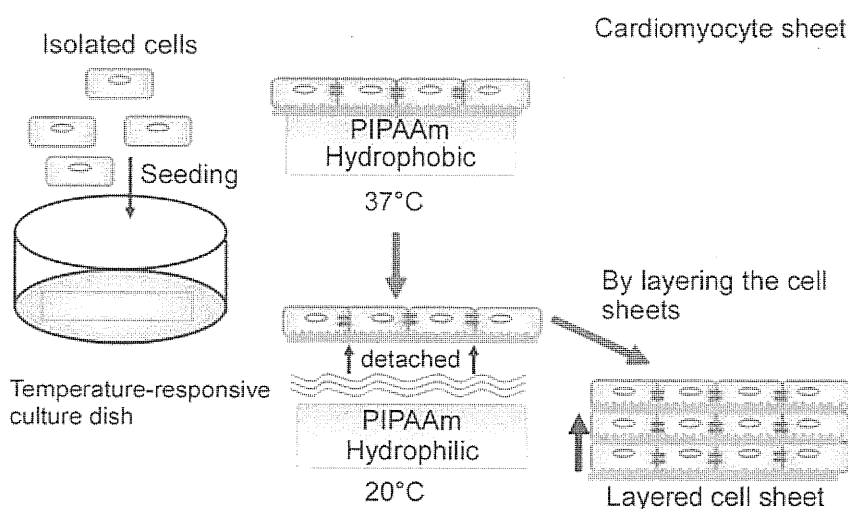


Fig 1. Diagram shows the method for engineering the cell sheets. Cell sheets harvested from a temperature-responsive culture dish can be layered to construct a 3-dimensional cardiac graft. (PIPAAm = poly(N-isopropylacrylamide).)

myocardium and communicate electrically with the host myocardium, as indicated by the presence of connexin43 and changes in the QRS wave and action potential amplitude, leading to an improvement in cardiac performance. Another report showed a similar electrical integration between a neonatal myocyte sheet and the host heart by electrophysiology [29]. Moreover, functional gap junctions and morphologic integration by "bridging cardiomyocytes" between the sheet and the host myocardium were detected [30]. These *in vitro* and *in vivo* studies clearly showed electric and morphologic coupling between the cell sheet and the host myocardium and revealed that the cell sheet may contract synchronously with the beating of the host heart and improve the regional systolic function.

A great disadvantage of this technique is that it is difficult to obtain thick cardiomyocyte sheets. For human applications, the one-cardiomyocyte-thick sheet is approximately 45- μm thick [24] and might not be strong enough to repair the tissue damage that is characteristic of end-stage heart failure. Thus, it is important to determine how many cardiomyocyte sheets can be layered *in vivo*. The limiting factor is that oxygen needs to be supplied to the cardiomyocyte sheet so that vascularization with the cardiac tissue can occur after implantation. A 4- or 6-layered neonatal cardiomyocyte sheet implanted into the subcutaneous tissue of athymic rats survived 1 year and showed angiogenesis, which was organized within a few days, and had a thickness of approximately 100 μm [25].

When more than 4 such grafts were implanted, however, central necrosis was observed instead of the rapidly organized microvasculature because of the insufficient oxygen supply [31]. To overcome the poor vascularization, a multistep polysurgical technique was developed in which thick-layered cardiomyocyte sheets were generated in ectopic tissue. This multistep transplant technique could recreate approximately 1-mm-thick myocardium with a well-organized vasculature network [31]. The problem now, however, was how to implant it into the damaged myocardium; that is, how to connect the microvasculature network in the thick-layered cardiomyocyte sheets to the host myocardium. Thus, although this method produced promising results in animal models, technical hurdles still need to be overcome before it can be clinically applied in humans.

In a study on the vascularization process after implantation, Sekiya and colleagues [32] reported that the cardiomyocyte sheet contains potential angiogenic factors such as the expression of angiogenesis-related genes and an endothelial cell network. Interestingly, the vasculature in the layered cardiomyocyte sheet comes from the sheet itself, and vessels extend from the sheet to the host myocardium to connect with the host vasculature [32]. An important study showed that added angiogenic factors, such as endothelial cells and some angiogenic growth factors [33], can enhance angiogenesis to improve the survival of thick-layered cardiomyocyte sheets in the damaged myocardium. On the basis of this report, Sekine and colleagues [34] performed a study showing

that a cocultured sheet of neonatal cardiomyocytes-endothelial cells developed enhanced vascularization and that the implanted sheet improved cardiac performance compared with a cardiomyocyte-only sheet. Such techniques designed to enhance angiogenesis may lead to a breakthrough for integrating thick-layered cardiomyocyte sheets, which have been incubated in ectopic tissue, with damaged myocardium in humans.

Myoblast Sheets

In the clinical setting, cellular cardiomyoplasty using dispersed cells is reported to have potential regenerative capability, and a method using skeletal myoblasts has been introduced in clinical trials and found to be relatively feasible and safe [35]. Skeletal myoblasts are the most likely cell source for clinical applications of tissue cardiomyoplasty at this time.

Memon and colleagues [36] demonstrated that the nonligature implantation of a skeletal myoblast sheet into a rat cardiac ligation model regenerated the damaged myocardium and improved global cardiac function by attenuating the cardiac remodeling by hematopoietic stem cell recruitment and growth factor release. Moreover, this system of cell delivery by cell sheet implantation showed better restoration of the damaged myocardium than needle injection [36]. In another study, the application of a skeletal myoblast sheet to a DCM hamster model resulted in the recovery of deteriorated myocardium accompanied by the preservation of α -sarcoglycan and β -sarcoglycan expression on the host myocytes and the inhibition of fibrosis [37]. They used a 27-week DCM hamster, which is at a moderate heart-failure stage (fractional shortening, 16%), and showed preserved function and histology of the distressed heart and prolonged survival.

In other studies, the grafting of skeletal myoblast sheets attenuated the cardiac remodeling and improved cardiac performance in a pacing-induced canine heart failure model [38] and in a porcine chronic infarction model [39]. In particular, the improvement in cardiac performance in the porcine chronic infarction model was maintained for at least 6 months, and the histology showed well-developed smooth muscle cells (not myoblasts) at the implanted site. Although these cells might have played a pivotal role in the myocardial regeneration after myoblast sheet implantation, their origin and characteristics are unknown. Further study will be needed to elucidate the function of these cells and clarify the mechanisms of myocardial regeneration.

These reports demonstrate that skeletal myoblast sheets can regenerate the deteriorated myocardium induced by coronary artery diseases and DCM in small and large animal models. Although these studies indicate that skeletal myoblast sheets have potential usefulness for treating moderate heart failure, their efficacy for end-stage heart failure is unknown and requires further study.

Mechanisms of Myocardial Regeneration After Cell Sheet Implantation in Heart Failure

The mechanism of recovery in the damaged myocardium treated with cell sheets has not been completely elucidated and may be very complicated. Although cytokine release and hematopoietic stem cell recruitment have been proposed as possible mechanisms of regeneration, other mechanisms are likely to be involved. For example, structural proteins may be restored by the relief of myocyte stretching, as evidenced by a reduction in the LV dimension or as a result of the action of growth factors. Skeletal myoblasts cannot beat synchronously with the host myocardium *in vitro* [40] or *in vivo* [41], and these implanted cells are not functionally integrated.

Originally, the main focus of cell sheet technology was to evaluate the potential of cell sheets applied to the epicardium to replace cardiomyocytes lost after myocardial infarction, but we still do not fully understand how cardiomyocyte and skeletal myoblast sheets integrate functionally with the diseased myocardium, and previous studies of the cardiomyocyte sheet and skeletal myoblast sheet cannot fully explain the functional integration with the diseased myocardium.

The histologic detection of massive angiogenesis in the implanted region is one of the critical factors for cardiac improvement, and we speculate that angiogenesis and the recovery of diastolic function [42] are major components of the regenerative mechanism in myoblast sheet implantation (Fig 2).

Other Types of Cell Sheets

A clinician can decide on the basis of the physiologic and pathologic characteristics of a disease which type of cell sheet is the most appropriate for treating it. In animals, the cell sheet technique has been applied using several different cell sources, such as skeletal myoblasts [36-38], mesenchymal stem cells (MSCs) [43], human smooth muscle cells [44], fibroblasts cocultured with endothelial

progenitor cells [45], and cardiomyocytes cocultured with endothelial cells [34].

In a rat myocardial infarction model, the growth of an MSC sheet on infarcted myocardium improved the anterior wall thickness, with new vessels and some differentiation of the implanted cells to cardiomyocytes [43]. Although an improvement in systolic function was observed, the differentiated cardiomyocytes might not have played a role because the incidence of differentiation from MSCs to cardiomyocytes was quite low. Rather, the breakthrough of this study was that a thick-layered sheet was obtained by self-incubating the cell sheet *in vivo*. Although the MSC sheet of the maximum thickness obtained, approximately 600 μm , is not strong enough to correct human end-stage heart failure [46], this method of self-incubation *in vivo* is a potential strategy for creating thick-layered sheets *in vivo*.

A cell sheet composed of 2 types of cocultured cells was developed to enhance angiogenesis in the rat [34, 45]. The cocultured cell sheet, which combined fibroblasts and endothelial progenitor cells, enhanced blood vessel formation and led to functional improvement [45]. This strategy might be effective for treating ischemic myocardium or peripheral arterial disease by increasing angiogenesis. In another study, the cocultured cell sheet combined fibroblasts and human smooth muscle cells and accelerated the secretion of angiogenic factors *in vitro* and increased blood perfusion *in vivo* by the formation of new vessels [44]. This enhanced effectiveness attained by coculturing 2 cell types is supported by another study in which the co-injection of bone marrow cells and myoblasts showed improved results over the transplantation of a single cell type in a canine model of ischemic cardiomyopathy [47].

Notably, the implantation of stem cells by needle injection induces little myogenesis because of the low incidence of their differentiation into cardiomyocytes *in vivo* [48] and the massive cell loss associated with the

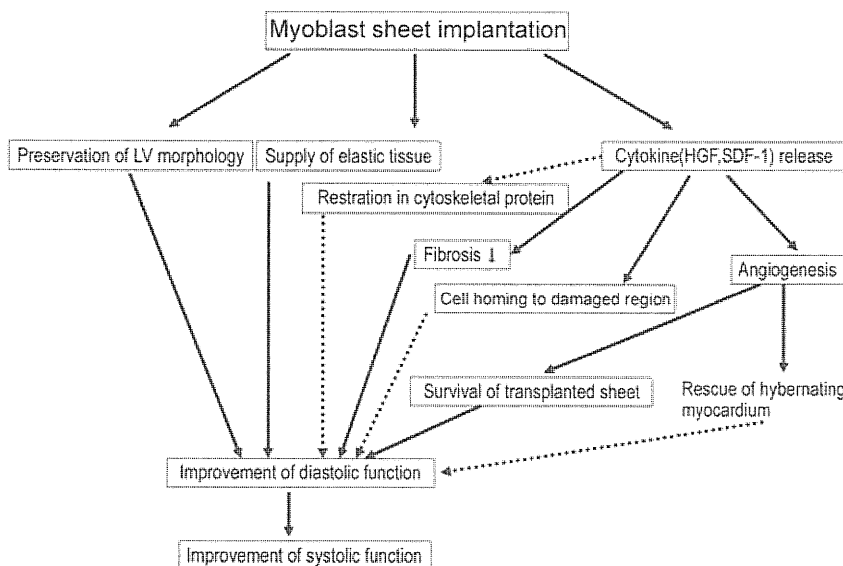


Fig 2. Possible regeneration mechanisms in myoblast sheet implantation. Myoblast sheets are proposed to preserve the left ventricular (LV) geometry, provide elastic tissue to a stiffened scar, and cause cytokine (HGF, SDF-1 etc) release. These cytokines may help restore cytoskeletal proteins, reduce fibrosis, increase angiogenesis, and promote cell migration to the damaged myocardium. These effects may induce improved regional diastolic function, leading to improved systolic function. Solid lines indicate evidence provided by published reports. Dotted lines indicate theoretical mechanisms. (HGF = hepatocyte growth factor; SDF-1 = stromal cell-derived factor 1.)

injection method [36]. Moreover, myoblast sheets cannot yet be used for myogenesis to replace myocardial scar tissue with functioning contractile tissue because there is no evidence that the myoblasts undergo synchronized beating *in vivo*. Unfortunately, we cannot yet use cardiomyocyte sheets clinically for myogenesis. The breakthroughs that will enable the clinical application of cell sheet technology for end-stage heart failure may include the development of a new cell source (contractile autologous cells) and the creation of a thick cardiac graft with great vessels.

Future Prospects for Cell Sheet Technology

In summary, cell sheet technology has been applied using many kinds of cells and has showed some functional impact on the failed heart of many animal models of heart disease. Although the improvement is mostly due to the paracrine effect of cytokines, the mechanisms by which the damaged myocardium heals are still incompletely elucidated.

Some questions remain to be answered before cell sheet technology can be applied to human patients. For example:

- How many cell sheets should be implanted to heal the severely failed heart?
- What severity of heart failure can be healed with cell sheets?
- What is the best method for implanting cell sheets with minimal cell loss?
- What is the best cell source for the cell sheet to regenerate the severely failed heart?
- How long can implanted cell sheets survive in the failed heart?

ECM remodeling in heart failure (excessive matrix degradation and myocardial fibrosis) contributes to LV dilatation and progressive cardiac dysfunction [49]. The most interesting matter in this field is about the effect of cell sheets on the distressed ECM in the failed heart. Moreover, can these implanted sheets normalize the wall stress in the injured region? Besides the changes of ECM after cell sheet implantation, the cell sheet is mainly composed of collagen, providing structural support and giving the heart properties that include stiffness and resistance to deformation. Myocardial tissue engineering should follow this concept to create physiologic scaffolds that could help to normalize cardiac wall stress in injured regions and improve strain distribution.

Finally, the data that comment about the superiority of cell sheets compared with conventional needle injection may be poor.

As mentioned, many drawbacks that should be elucidated could be addressed in this field, and the accurate answers for these queries about cell sheet technology have not been proposed yet. Thus, some studies that can answer these critiques should be performed.

Bioengineered Myocardial Grafts With a Scaffold

Many reports describe myocardial constructs that use artificial scaffolds combined with various kinds of cells (Table 1). Because several different cell types can be easily combined with scaffolds and many biocompatible materials have been created, there has been much progress in this field. However, which scaffold is the most appropriate for treating heart disease has been controversial. In this section, we will analyze several articles and discuss the merits and shortcomings of this method.

Bioengineered Cardiomyocyte Constructs With a Scaffold

Bioengineered cardiac grafts created by culturing fetal cardiac cells with porous alginate scaffolds [50] and Gelform (Pfizer Inc, New York, NY) [51] were developed and implanted into the infarcted myocardium of model animals. These approaches resulted in significant angiogenesis and attenuation of LV dilatation but caused no change in LV contractility [50, 51]. Engineered heart tissue was also developed by mixing embryonic chick cardiomyocytes with a collagen solution that resulted in a highly organized heart tissue-like structure [52].

On the other hand, Zimmerman and colleagues [53] created ring-shaped engineered heart tissue by mixing neonatal cardiomyocytes and collagen; this tissue demonstrated spontaneous beating and contraction force, which was improved by increasing the calcium concentration. They then developed a differentiated ring-shaped cardiac muscle construct by mixing neonatal rat cardiomyocytes with collagen I and matrix factors under mechanical overload [54]. This construct survived as an implanted graft that showed intense vascularization and differentiated heart muscle [55]. Histologic and electrical integration between the cardiac graft and host myocardium may be important for the regeneration of damaged myocardium. Zimmerman and colleagues [56] also reported that the implantation of large, 1- to 4-mm-thick, engineered heart grafts into a rat myocardial infarction model clearly improved the cardiac performance and that there was good electrical coupling between the grafts and host myocardium. However, the histologic integration (the presence of desmosomes and connexins between the graft and host myocardium) was not confirmed.

Drawbacks of Bioengineered Cardiomyocyte Constructs With a Scaffold

The major advantage of this technique is that the engineered heart tissue can be made into various shapes, including rings [54] and pouch-like forms [57], and into large grafts [56]. This technique is promising, but it may be difficult to apply to large animal models or humans. The problems include graft size and immunogenicity, because these grafts cannot survive *in vivo* without immunosuppression [55]. To reduce the immunogenicity, a new culture technique was developed without Matrigel (BD Biosciences, San Jose, CA), using serum-free medium with insulin and peptide growth factor [58].

Table 1. Biomaterials Used in Bioengineered Cardiac Grafts for Heart Failure

Author	Matrices	Cells	HF Model	Function
Kutschka	Collagen matrix/Matrigel/FGF or VEGF	H9c2 cell	Rat MI	↑
Simpson	Collagen type I	Human MSCs	Rat MI	↑
Birla	Silicon chamber/fibrin gel	CM	Rat FA	...
Yang	P4HB	Human pediatric aortic cells		...
Matsubayashi	PLA/PCL	Rat SMCs	Rat MI	↑
Kellär	...	Human dermal fibroblast	Mouse MI	↑
Papadaki	...	Neonatal rat CM		...
Fukuhara	PGAC/bFGF	Rat BMC	Rat MI	↑
Zimmermann	Collagen type I	Neonatal rat CM	Rat MI	↑
Kofidis	...	Mouse ES cell	Rat reperfusion model	↑
Van Luyn, Yost	...	Neonatal rat CM
Xiang	Glycosaminoglycan	MSCs
Kofidis	Collagen	Neonatal rat CM
Leor	Alginate	Neonatal rat CM	Rat MI	→
Siepe	PU	Rat SM	Rat MI	↑
McDevitt	PU/laminin	Neonatal rat CM
Alperin	PU/laminin/collagen IV/gelatin	ES cell-derived CM, mouse
Li	Gelatin	Fetal rat CM	Rat MI	→
Li	...	Fetal rat CM, SMC, FB, human CM
Akhyari	...	Human pediatric heart cell
Chachques	Collagen	Human BMC	Human MI	↑
Ishii, Shin	PCL	Neonatal rat CM
Zong	PLA/PGA	CM
Krupnick	PLA/PTFE/collagen I, IV	BM stem cells
Carrier	PGA	Neonatal rat CM
Park	PGA/PLA/PCL/collagen	Neonatal rat CM
Fujita	PGA/Collagen sponge	BM stem cells
Pego	TMC-DLLA copolymer	CM
Ott	Decellularized heart	Cardiac or endothelial cells
Kofidis	Collagen fleece	Neonatal rat CM
Radisic	Collagen sponge/matrigel	Neonatal CM, C2C12 cells
Gerecht-Nir, Radisic	...	Neonatal rat CM
Kofidis	Fibrin glue	Neonatal rat CM
Boublik	Hyaluronan benzyl ester/fibrin	Neonatal rat CM
Iyer	Polyethylene glycol	Neonatal rat CM, mouse FB, mouse EC

bFGF = basic fibroblast growth factor; BMC = bone marrow cell; CM = cardiomyocyte; DLLA = D,L-lactide; EC = endothelial cell; ES = embryonic stem; FA = femoral artery; FB = fibroblast; FGF = fibroblast growth factor; HF = heart failure; MI = myocardial infarction; MSCs = mesenchymal stem cells; P4HB = poly-4-hydroxy-butyrates; PCL = poly-ε-caprolactone; PGA = polyglycolic acid; PGAC = polyglycolic acid cloth; PLA = poly L-lactic acid; PTFE = polytetrafluoroethylene; PU = polyurethane; SM = skeletal myoblast; SMC = smooth muscle cell; TMC = 1,3-trimethylene carbonate; VEGF = vascular endothelial growth factor; ↑ = improved; → = no change.

Birla and colleagues [59] overcame the problem of limited blood supply to the cardiac tissue by developing a new method for creating contractile cardiac tissue, "in situ incubation of cardiac tissue." They implanted silicon tubes that were filled with fibrin gel and seeded with cardiomyocytes close to a vascular pedicle and found that living vascularized tissue with the characteristics of cardiac muscle developed around the great vessels [59]. This approach is quite different from the other

reported methods for generating cardiac tissue, and neither the maximum thickness of this cardiac tissue nor how to implant it into the failing myocardium is clear.

Several questions about cell scaffolds remain to be answered:

- How long do cells in these scaffolds survive in vivo?

- Do scaffolds have a bad influence on cell viability or cell survival after implantation?
- What is the best scaffold for cell survival and cell-host myocardium communication after implantation?
- How are oxygen and nutrition supplied to the implanted graft after implantation, and are the supplies disturbed by the scaffold?

An interesting report about appropriate scaffolds indicates that knitted poly-L-lactide fabric is more suitable for autologous cardiac grafts than gelatin and polyglycolic acid, because it has better cellular penetration, no thinning or dilatation *in vivo*, and elicits no inflammatory response [60].

Major obstacles to applying cardiomyocyte constructs with a scaffold include toxicity, immunogenicity, inappropriate elastic properties, and increased acidity *in vivo*, any of which could cause a severe inflammatory response. For example, polyesters such as polylactic acid and polyglycolic acid increase the acidity in the body, have irregular degradation kinetics that can lead to the sudden disappearance of the construct's mechanical properties [61], and are less flexible than heart tissue [62]. Although elastomeric polymers such as elastomeric polyurethane are more elastic, their degradation product, diisocyanate, is toxic. Extracellular derivatives such as collagen type I and fibronectin are good for cell viability, but they have rapid degradation kinetics and frail mechanical properties. Moreover, collagen, Matrigel, and glycosaminoglycan are immunogenic and have weak mechanical properties [63]. Other scaffolds, in which patches of polydioxanone were covered with Vicryl (Ethicon, Somerville, NJ) or poly-3-hydroxybutyrate, increased C-reactive protein messenger RNA after implantation into the rat stomach [64].

Although we have only described cell-containing artificial scaffolds, recent innovative work has examined the use of biologic scaffolds. Ott and colleagues [65] reported that decellularized hearts can be reseeded with cardiac and endothelial cells and that the reseeded hearts have pump activity [65]. This reseeded heart still needs to be analyzed *in vivo* for its ability to activate recellularization and other properties. This technique has the potential to create a whole heart, although immunity after implantation might still be a problem.

Bioengineered Cell Scaffolds With Other Types of Cells

Bioengineered cardiac muscle grafts have been developed using human heart cells and gelatin-matrix scaffolds [66], neonatal rat cardiomyocytes and collagen matrix [67], and cardiomyocytes and biodegradable polyurethane films [62]. All of these grafts showed spontaneous beating, but the outcomes after implantation into the myocardium were not reported. Xiang and colleagues [68] created collagen-glycosaminoglycan scaffolds containing adult bone marrow-derived MSCs and implanted them into an ischemic reperfusion rat model. The scaffolds were degraded and the MSCs survived in the scar

without acute inflammation, but there was no comment about the improvement of cardiac function [68]. Simpson and colleagues [69] demonstrated that the implantation of a collagen type I matrix seeded with human MSCs into a rat infarction model induced functional improvement and prevented remodeling, without detecting the human MSCs. This finding predicts that the functional improvement does not require long-term graft survival.

Several other studies also showed improved cardiac function after the implantation of a bioengineered cardiac graft with scaffold into injured myocardium. In a coronary ligation rat model, LV systolic function was improved and LV dilatation attenuated after the implantation of vascular smooth muscle cell-seeded poly-L-lactide (a sponge polymer of epsilon-caprolactone-co-L-lactide reinforced with knitted poly-L-lactide fabric), 4 weeks after injury, as assessed by echocardiography and a Langendorff apparatus [70]. Bone marrow cell-seeded polyglycolic acid cloth containing basic fibroblast growth factor improved cardiac function and supported angiogenesis in a rat ligation model assessed using a Langendorff apparatus [71]. In addition, tissue composed of human dermal fibroblasts cultured on a knitted Vicryl mesh induced angiogenesis in a mouse ligation model [72] and improved systolic function, as measured by the Millar conductance catheter system [73].

Many different cell scaffolds have been reported and are summarized in Table 1. The advantage of cell scaffolds is that thick grafts can be obtained and the shape of the graft is flexible [55]. Moreover, the characteristics of the material can be chosen according to the purpose of the construct, for example, cell transport, enhanced angiogenesis, cell survival, or certain degradation kinetics [63].

Clinical Trial of a Scaffold-Containing Bioengineered Construct

A clinical trial using collagen matrix seeded with bone marrow cells was performed in 20 patients presenting with LV postischemic myocardial scars [74]. Improvements in New York Heart Association functional class, LV end-diastolic volume, scar area and thickness, and ejection fraction were observed in comparison with the pretreatment values. However, when compared with bone marrow cell implantation alone, only the change in LV end-diastolic volume was significant. Because the implantation was performed concomitantly with coronary artery bypass grafting, the effectiveness of the cell therapy is still unclear. However, this trial may indicate that the treatment is feasible and safe because no deaths or related adverse events have been reported to date.

The application of the cell-seeded construct to the epicardium cannot replace the infarcted myocardium or reshape the ventricle. The removal of the scar and remodeling of the ventricle will provide an opportunity to impact a cell-seeded scaffold in a ventricle that has a normal size and shape and does not have heart failure. The cell-seeded construct could then be used to prevent recurrent dilatation. Recurrence is a major problem after surgical ventricular restoration, and the use of a cell-seeded graft rather than Dacron (DuPont, Wilmington,

DE), polytetrafluoroethylene, or pericardium might offer significant advantages.

Biomaterial Implantation

The two main purposes of implanting biomaterials are to enhance angiogenesis and to create cardiac tissue in situ by inducing stem cell migration to the scaffold. Supporting this strategy, several reports have shown that resident cells recruited to the injured heart can create new muscle and vascular tissue [75-77].

Gaballa and colleagues [78] reported that implanting collagen type I into a rat myocardial infarction model induced neoangiogenesis and reduced LV remodeling. Biodegradable polyester urethane urea implantation into a rat infarction model improved systolic function and the thickness of the LV wall, and smooth muscle cells with a histologically contractile phenotype were found in the implanted site [79]. The implantation of a porous collagen scaffold enhanced the recruitment of neural crest cells to the implanted patches, even though the frequency of cardiogenic stem cells in the cryoinjured heart is rare compared with the intact heart. Moreover, Western blot analysis showed the expression of smooth muscle and endothelial cell markers but not of cardiomyocytes [80].

Ota and colleagues [81] implanted an ECM patch made from porcine urinary bladder into the porcine right ventricle (RV) wall and detected factor VIII-positive and α -smooth muscle actin-positive cells. An ECM patch derived from porcine urinary bladder was also implanted into the RV free wall in dogs, and the remodeled tissue contained approximately 30% cardiomyocytes [82], but it is not known whether they represented the recruitment of residual stem cells or of stem cells derived from bone marrow.

Interestingly, Robinson and colleagues [83] implanted an ECM patch derived from urinary bladder into a porcine infarction model and detected α -smooth muscle actin-positive cells 1 month after implantation, but at 3 months, the cells derived from the ECM patch were similar to those of normal myocardium by flow cytometry analysis. The mechanism, improvement in cardiac function, and long-term results were not reported. An ECM-derived myocardial patch was implanted into a canine RV with a defect extending the full thickness of the RV wall, which led to improvements in regional systolic and diastolic function, compared with a Dacron patch, and to the presence of cardiomyocytes [84].

Acellular bovine pericardium with a porous structure affixed to genipin was implanted into the RV of a rat model, and histologic examination revealed smooth muscle cells, neomuscle fibers, neoglycosaminoglycans, and neocapillaries in the implanted tissue, but myocytes were not seen [85]. This strategy may interest researchers in the mechanisms of stem cell migration and differentiation.

In this review, we surveyed many exciting topics in myocardial generation therapy in which cardiac surgeons are currently interested. Remarkable progress has been

made in cell-based treatments, including cellular cardiomyoplasty and tissue cardiomyoplasty, in a very short period of time. Many researchers and clinicians are enthusiastic about developing new technologies to treat currently intractable heart disease and examining the mechanisms of these techniques from the physiologic, cellular, histologic, and functional points of view. Owing to such studies, some techniques have already been applied clinically, but much remains to be learned, particularly because the technology is still rudimentary at the basic research and clinical levels. The field of clinical myocardial regenerative therapy is just emerging, however, and now is the time for cardiac surgeons to examine ways to promote the assimilation of basic achievements into routine clinical therapy.

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Impaired Myocardium Regeneration With Skeletal Cell Sheets—A Preclinical Trial for Tissue-Engineered Regeneration Therapy

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Background. We hypothesized that autologous skeletal cell (SC) sheets regenerate the infarct myocardium in porcine heart as a preclinical trial.

Methods and Results. The impaired heart was created by implantation of ameroid constrictor on left anterior descending for 4 weeks. SCs isolated from leg muscle were cultured and detached from the temperature-responsive domain-coated dishes as single monolayer cell sheet at 20°C. The following therapies were conducted: SC sheets (SC group, n=5); sham (C group n=5). Echocardiography demonstrated that cardiac performance was significantly improved in the SC group 3 and 6 months after operation (fractional area shortening, 3 months; SC vs. C=49.5±2.8 vs. 24.6±2.0%, $P<0.05$) and left ventricle dilatation was well attenuated in the SC group. Color kinesis index showed that distressed regional diastolic and systolic function in infarcted anterior wall was significantly recovered (SC vs. C=57.4±8.6 vs. 30.2±4.7%, $P<0.05$, diastolic: 58.5±4.5 vs. 35.4±6.6%, $P<0.05$, systolic). Factor VIII immunostains demonstrated that vascular density was significantly higher in the SC group than the C group. And % fibrosis and cell diameter were significantly lower in the SC group. And hematoxylin-eosin staining depicted that skeletal origin cells and well-developed-layered smooth muscle cells were detected in the implanted area. Positron emission tomography showed better myocardial perfusion and more viable myocardial tissue in the distressed myocardium receiving SC sheets compared with the myocardium receiving no sheets.

Conclusions. SC sheet implantation improved cardiac function by attenuating the cardiac remodeling in the porcine ischemic myocardium, suggesting a promising strategy for myocardial regeneration therapy in the impaired myocardium.

Keywords: Cells, Heart failure, Myocardial infarction, Tissue, Transplantation.

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Despite the recent remarkable progress in medical and surgical treatments for heart failure, end-stage heart failure has been still a major cause of death worldwide. After myocardial infarction, the myocardium is capable of a limited regenerative capacity and no medication or procedure used clinically has shown efficacy in regenerating myocardial scar

tissue with functioning tissue. Thus, there is a need for new therapeutics to regenerate damaged myocardium.

Recent developments in tissue engineering show promise for the creation of functional cardiac tissues without the need for biodegradable alternatives for the extracellular matrix (1). And we reported that cardiomyocyte sheets have been developed by using temperature-responsive culture dishes and these sheets survived in the back of nude rats and showed a spontaneous contraction over a long period of time (2). Recent reports suggested that cardiomyocyte sheets integrated with the impaired myocardium and improved cardiac performance in a rat model of ischemic myocardium (3).

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And more recently, in the aim of clinical application, nonligature implantation of skeletal myoblast sheet regenerated the damaged myocardium and improved global cardiac function by attenuating the cardiac remodeling in the rat ligation model (4) and dilated cardiomyopathy hamster model (5). This cell delivery system by using cell sheets implantation showed better restoration of damaged myocardium compared with needle injection (4, 5). Moreover, grafting of skeletal myoblast sheets attenuated cardiac remodeling and improved cardiac performance in pacing-induced canine heart failure model (6).

Given this body of evidence, we hypothesized that the autologous skeletal cell (SC) sheet implantation might remodel the chronic heart failure caused by ischemic injury.

Therefore, this preclinical study using Swine model was designed to test therapeutic effectiveness.

MATERIALS AND METHODS

Myocardial Infarction Model

"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 Laboratory Animal Resource and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985). This animal experiment was approved by the Animal Care Committee of Osaka university graduate school of medicine. We induced acute myocardial infarction of 10 swine (20 kg, KEARI, Japan) by the following method. Swine were preanesthetized by intramuscular injection of ketamine hydrochloride 20 mg/kg (Ketalar, Sankyo, Japan) and xylazine 2 mg/kg (Seractar, Bayer). Animals were positioned spine and a 22-gauge indwelling needle (Surflo F&F, Terumo, Tokyo, Japan) was inserted in the central vein of the auricle. A three-way cock (Terufusion TS-TR2K, Terumo, Tokyo, Japan) was attached to the external cylinder of the indwelling needle, and an extension tube was connected for continuous anesthetic injection. The animals were intubated with an endotracheal cannula (6 Fr, Sheridan) using a pharyngoscope and then connected to an artificial respirator (Harvard, USA) by the cannula. Artificial respiration was implemented at a stroke volume of 200 to 300 mL/stroke and a stroke frequency of 20/min. The animals were continuously drip injected with propofol 6 mg/kg/hr (Diprivan, AstraZeneca) and vecuronium bromide 0.05 mg/kg/hr (Musculux, Sankyo Yell Yakuhin Co., Ltd., Japan) using a syringe pump (Terufusion TE-3310N, Terumo, Japan). The animal was then fixed in a recumbent position, so that the left thorax was exposed, and the outer layer of skin and muscles between the third and fourth ribs were dissected. After confirming the cutting into the thoracic cavity, the distance between the third and fourth ribs was widened with a rib spreader to allow a direct view of the left auricle and the LAD coronary artery. The pericardium was dissected along the LAD from the upper part of the left auricle (~6 cm) to expose the myocardium around the LAD. LAD on the proximal side below the left auricle from the myocardium was exfoliated for approximately 1 cm, and then a small amount of lidocaine hydrochloride jelly (Xylocaine jelly, AstraZeneca) was applied to allow for anesthetizing the area. An ameroid constrictor (COR-2.50-SS, Research Instruments) was then fit using No. 1 or 2 suture. The chest cavity was closed to end the procedures. The animals were randomly divided into two treatment groups: the first received autologous SC sheet implantation (SC group, n=5). For control, we have performed sham operation (C group, n=5).

Preparation of Skeletal Cell Sheets for Grafting

One week after implantation of ameroid constrictor on LAD, skeletal muscle weighing approximately 5 g was removed from the pretilial region with the porcine under general anesthesia. Following the addition of trypsin-ethylenediaminetetraacetic acid (Gibco, Grand Island, NY), excessive connective tissue was carefully removed to minimize the content of contaminating fibroblasts, and the muscle tissue was minced until the

fine pieces formed a homogeneous mass. The specimens were then incubated at 37°C in shaker bath with 0.5% type 1 collagenase (Gibco) in Dulbecco's modified Eagle's medium (Gibco). After brief placement, the fluid was collected, and the same volume of culture medium, SkBM (Cambrex, Walkersville, MD) supplemented with fetal bovine serum (Thermo Trace, Melbourne, Australia), was added to halt the enzymatic digestion process. The cells were collected by centrifugation, and the putative SCs were seeded into 150 cm² polystyrene flasks after removal of fibroblasts by sedimentation for a few hours and cultured in SkBM at 37°C. During the culture process, we maintained cell densities at less than 70% confluence by carrying out passaging of cells for one time to prevent SCs from premature differentiation and fusion process resulting in myotubes formation. When the cells become approximately 70% confluent after 10 to 11 days cultivation, the cells were dissociated from the flasks with trypsin-ethylenediaminetetraacetic acid and reincubated on 100 mm temperature-responsive culture dishes (Cellseed, Tokyo, Japan) at 37°C with the cell numbers adjusted to 1 × 10⁷ per dish. More than 90% of these cells were desmin positive (Fig. 1). After 4 days, the dishes were removed to refrigerator set at 20°C, and left there for approximately 30 min. During that time, the SC sheets detached spontaneously from the surfaces. Each sheet had a diameter of 30 to 40 mm and consisted of layers of SCs; the sheets were approximately 100-µm thick in cross-sectional views (Fig. 1). Approximately 10 sheets were obtained from the 5 g of skeletal muscle.

Implantation of Skeletal Cell Sheets

Autologous SC sheet implantation was performed in the swine 4 weeks after LAD ligation. Swine were anesthetized as mentioned above. The swine were exposed through the sternum. The infarct area was identified visually on the basis of surface scarring and abnormal wall motion. In the SC group, we implanted 10 SC sheets into the infarcted myocardium. The control group was treated similarly but received no SC sheets. Because piling up four or more sheets caused the central necrosis of the myoblasts presumably because the lack of in oxygen supply, we decided to pile two or three layers of the SC sheet over the broad surface of the impaired heart.

Measurement of Cardiac Function

Swine were anesthetized as mentioned above. Cardiac ultrasonography was performed with a commercially available echocardiograph, SONOS 5500 (PHILIPS Electronics, Tokyo, Japan). A 3-MHz annular array transducer was placed on a layer of acoustic coupling gel that was applied to the left hemithorax. Swine were examined in a shallow left lateral decubitus position. The heart was first imaged in the two-dimensional mode in short-axis views at the level of the largest left ventricle (LV) diameter. The calculation of the LV volume was based on the LV short-axis area using AQ system (7). And fractional area shortening (FAS) of the LV diastolic was calculated as follows:

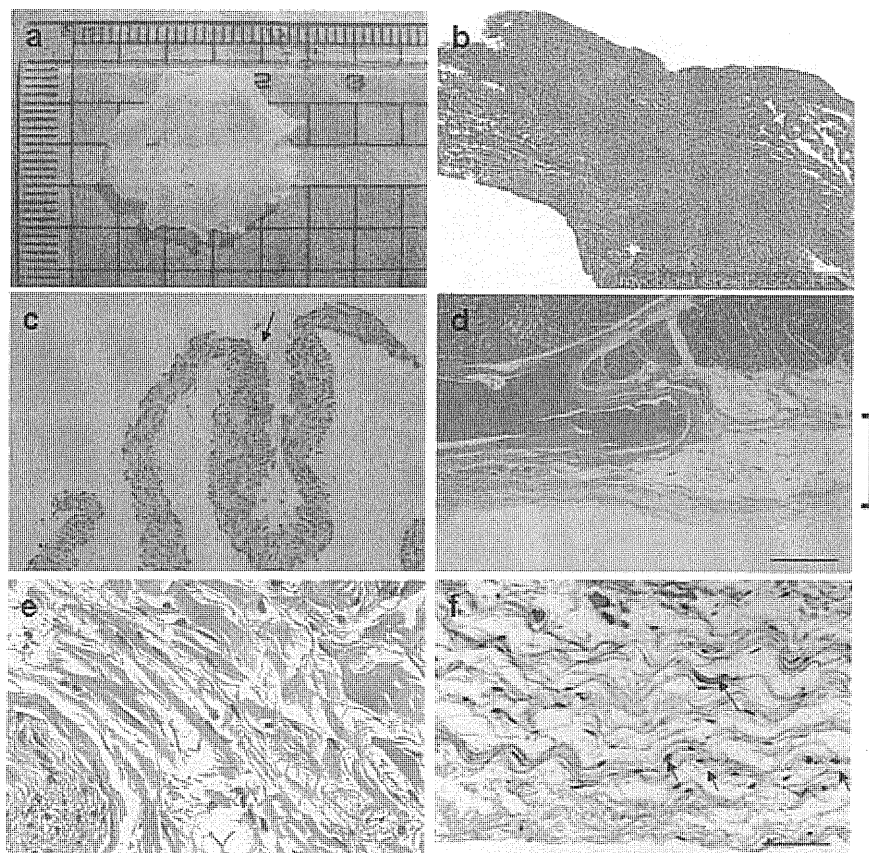
$$\text{FAS (\%)} = \frac{(\text{LV end-diastolic area} - \text{LV end-systolic area [ESA]})}{\text{LV end-diastolic area}} \times 100$$

These data are presented as the average of measurements of two or three selected beats.

Quantification of Regional Diastolic and Systolic Function by Color Kinesis

Diastolic CK images were obtained using a commercially available ultrasound system (SONOS 5500, Philips Medical Systems) from the LV midpapillary short-axis view for the determination of wall motion asynchrony as previously reported (8). CK examined every image pixel within the region of interest, which was drawn around the LV cavity, classifying it as blood or tissue based on integrated backscatter data. During diastole, each pixel was tracked into the next frame, and pixel transitions from endocardium to blood were detected and interpreted as diastolic endocardial motions. These pixel transitions were encoded using a color hue specific to each consecutive video frame, so that each color represents the excursion of that segment during a 33-ms period of time. The sites of regional LV diastolic wall motion or regions of interest were set on the basis of standard segmentation models: anterior, lateral, posterior, inferior, anteroseptal wall. The CK diastolic index was defined as the LV segmental filling fraction

FIGURE 1. Histological characteristics of skeletal cell (SC) sheet. (a) SC sheet detached from the Poly (*N*-isopropylacrylamide)-grafted polystyrene by lowering the temperature. Its size is approximately 3 cm × 2 cm². (b) Hematoxylin-eosin (H&E) stain; cross-sectional views of SC sheet in vitro. SC sheet demonstrates homogeneous heart-like tissue. (c) Not so many smooth muscle cells were detected in the SC sheets. The arrow indicates the smooth muscle cells in the SC sheet. (d) H&E stain revealed that SC sheets attached on the surface of epicardium. Left square bracket indicates implanted SC sheets. (e) Oval-shaped cells that showed positive for eosin in cytoplasm were detected in the SC group microscopically in some layers over epicardium. (f) Elastica Masson Goldner showed that oval-shaped cells that supposed to origin from skeletal tissue exist in the transplantation site. Arrows indicate oval-shaped cells that suppose to be originated from skeletal tissue.



during the first 30% of the diastolic filling time (LV segmental cavity area expansion during the first 30% of diastole, divided by the segmental end-diastolic LV cavity area expansion, expressed as a percentage). We introduced the use of color kinesis method that displays endocardial motion in real time to evaluate the regional systolic function (8).

Histopathology

LV myocardium specimens were obtained 6 months after the SC sheet implantation. Each specimen was fixed with 10% buffered formalin and embedded in paraffin. A few serial sections were prepared from each specimen and stained with hematoxylin-eosin (H&E) stain and elastica Masson-Goldner for histological examination or with Masson's trichrome stain to assess the collagen content.

To label vascular endothelial cells so that the blood vessels could be counted, immunohistochemical staining of factor VIII-related antigen was performed according to a modified protocol. Frozen sections were fixed with a 2% paraformaldehyde solution in phosphate-buffered saline (PBS) for 5 min at room temperature, immersed in methanol with 3% hydrogen peroxide for 15 min, then washed with PBS. The samples were covered with bovine serum albumin solution (DAKO LSAB Kit DAKO CORPORATION, Denmark) for 10 min to block nonspecific reactions. The specimens were incubated overnight with an Enhanced Polymer One-Step Staining (EPOS)-conjugated antibody against factor VIII-related antigen coupled with horseradish peroxidase (DAKO EPOS Anti-Human Von Wille brand Factor/HRP, DAKO, Denmark). After the samples were washed with PBS, they were immersed in diaminobenzidine solution (0.3 mg/mL diaminobenzidine in PBS) to obtain positive staining. Ten different fields at 200× magnification were randomly selected, and the number of the stained vascular endothelial cells in each field was counted under a light microscope. The result was expressed as the number of blood vessels per square millimeter.

The following antibodies against smooth muscle cells and skeletal myosin (slow) were used to evaluate the existence of SCs: primary antibodies, anti-

smooth muscle actin (clone 1A4, DAKO) antiskeletal myosin (slow) (clone NOQ7.5.4D, Sigma); secondary antibodies, anti-mouse Ig biotinylate (DAKO).

Picro-sirius red staining for the assessment of myocardial fibrosis or periodic acid-Schiff staining for that of cardiomyocyte hypertrophy was performed as described (9).

Positron Emission Tomography Procedure

We performed positron emission tomography (PET) studies on pigs which were transplanted SC sheets and control by using ¹⁵O-water and ¹⁸F-FDG. The pigs were anesthetized by the introduction of pentobarbital followed by continuous inhalation of propofol (4 mg/kg/hr) and were placed supine on the bed of the scanner. PET was performed using a HEADTOME-III tomograph (Shimadzu, Kyoto, Japan) and data were analyzed as described elsewhere (10).

Holter Electrocardiography

To evaluate arrhythmia we used Holter electrocardiography (ECG) for 24 hours. We checked arrhythmia by checking the number of ventricular premature beat after SC sheet implantation in myocardial infarction porcine (n=3).

Data Analysis

Data are expressed as means ± SEM and subjected to multiple analysis of variance (ANOVA) using the StatView 5.0 program (Abacus Concepts, Berkeley, CA). Echocardiographic data were first analyzed by two-way repeated measurement ANOVA for differences across the whole time course, and one-way ANOVA with the Tukey-Kramer posthoc test was used to verify the significant for the specific comparison at each time point. To assess the significance of the differences between individual groups concerning other numeral data, statistical evaluation was performed with an unpaired *t* test. Statistical significance was determined as having a *P* value less than 0.05.