reported that skeletal myoblasts prevented ventricular dilation and preserved the matrix architecture in a remote region through paracrine effects.[23] These mechanisms require further elucidation in future studies.

In the MAGIC trial only the high-dose group (8.0×10^8 cells) showed beneficial effects on improving diastolic function,[7] while cardiac reverse remodeling occurred in a dose-dependent manner in a rat myocardial infarction model.[24] While the number of injected BMSCs was fairly consistent among the four patients in our study, this was not the case for the myoblasts, the number of which dramatically varied from 58 to 248 million. It is currently unclear as to what number of cells is optimal for such treatment, and it is technically difficult to maximize the number and purity of myoblasts in elderly patients, making it difficult to interpret the outcomes. Therefore, the cell ratio may be critical, and an age limit should be considered for myoblast therapy.

The preoperative specimen from patient 4 obtained from the target area showed scar-like tissue, in which a low number of cells was found (Fig. 5E). The postoperative biopsy specimen showed similar findings (Fig. 5F), in contrast to the postoperative specimen obtained from patient 1 (Fig. 5D). Therefore, we consider that a minimum number of pre-existing cells is needed for cell therapy to provide paracrine effects, such as repair and reinforcement.

Concerning arrhythmia, we did not detect a lethal arrhythmia during the entire period of clinical study (6 months). Although cell transplantation by injection was suspected to cause lethal arrhythmias by re-entry circuit formation, no sustained VTs were identified during 24-h telemetry, and no VTs longer than triplets were recorded by the Holter ECG. We speculated that the amount of implanted myocytes in this study might not have been sufficient to confer electrical activity to cause an arrhythmia, and the mechanical unloading provided by the LVAD may also have prevented an arrhythmia.

In summary, this study suggested that combined cell therapy is feasible, but a large-scale study without LVAD is required to examine the effects of cell transplantation alone and to identify patients who are likely to respond to this therapy.

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Allogenic Skeletal Myoblast Transplantation in Acute Myocardial Infarction Model Rats

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Background. The limitations of syngenic cell therapy include patient safety and quality control of the source cells. Therefore, it is important to develop and assess procedures using allogenic cells. We investigated the impact of allogenic skeletal myoblast (SMB) transplantation on acute myocardial infarction with respect to immune response, donor cell survival, and therapeutic efficacy.

Methods. Female Lewis rats underwent proximal left anterior descending coronary artery ligation. Fifteen minutes later, they underwent major histocompatibility (MHC)-matched Lewis SMB transplantation (group S) and MHC-mismatched ACI SMB transplantation (group A), or treated with buffer injection as a control (group C).

Results. Flow cytometry showed that the SMBs expressed MHC antigens and B7 signal molecules in vitro. In group A, transcription levels of interleukin-2 receptor and interferon-γ were significantly increased 7 days after transplantation, and the area surrounding the donor SMBs was intensely infiltrated with CD4- and CD8-positive cells. Estimation of the number of donor cells in the recipient left ventricular chamber revealed that except for day 0, group A had fewer donor SMBs, which disappeared faster, compared with group S. Echocardiography demonstrated that the ejection fraction (EF) of group A was lower than that of group S.

Conclusion. MHC-mismatched allogenic SMB transplantation in infarcted myocardium induces the immune response and acceleration of donor cell clearance, decreasing the therapeutic effect. Donor cell survival and inflammation may play important roles in the therapeutic mechanism of SMB transplantation therapy for acute myocardial infarction.

Keywords: Allogenic, Cell transplantation, Skeletal myoblast, Donor cell survival, Immune rejection, Acute myocardial infarction.

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Recently, clinical trials using autologous cell therapy to treat ischemic heart failure have drawn worldwide attention. Randomized, controlled clinical trials have demonstrated that cell therapy is safe and promising, but it is not yet sufficiently effective in recovering cardiac function in patients after myocardial infarction (MI) (*1–4*). Its limitations include potential injury to patients during cell harvesting and the difficulty of obtaining a stable supply of uniform quality cells from patients of various backgrounds. More refinements are needed to use cell therapy effectively in clinical applications, including the use of allogenic cells rather than autologous cells.

Allogenic bone marrow mesenchymal stem-cell (MSC) transplantation is reported to show little immunogenicity and can even exert immunomodulating effects (*5–7*). We also reported that allogenic bone marrow MSC transplantation is feasible and effective for treating acute MI (AMI), partly because of its low immunogenicity (*8*). Skeletal myoblasts (SMBs) are another powerful cell source for treating ischemic heart failure (*9*). For treating Duchenne muscular dystrophy, however, allogenic SMB transplantation has been largely inefficient, because of the host's immune response to the donor SMBs (*10, 11*). There is evidence that the sensitivity of the immune reaction in allogenic transplantation is affected not only by histocompatibility matching but also by the combi-

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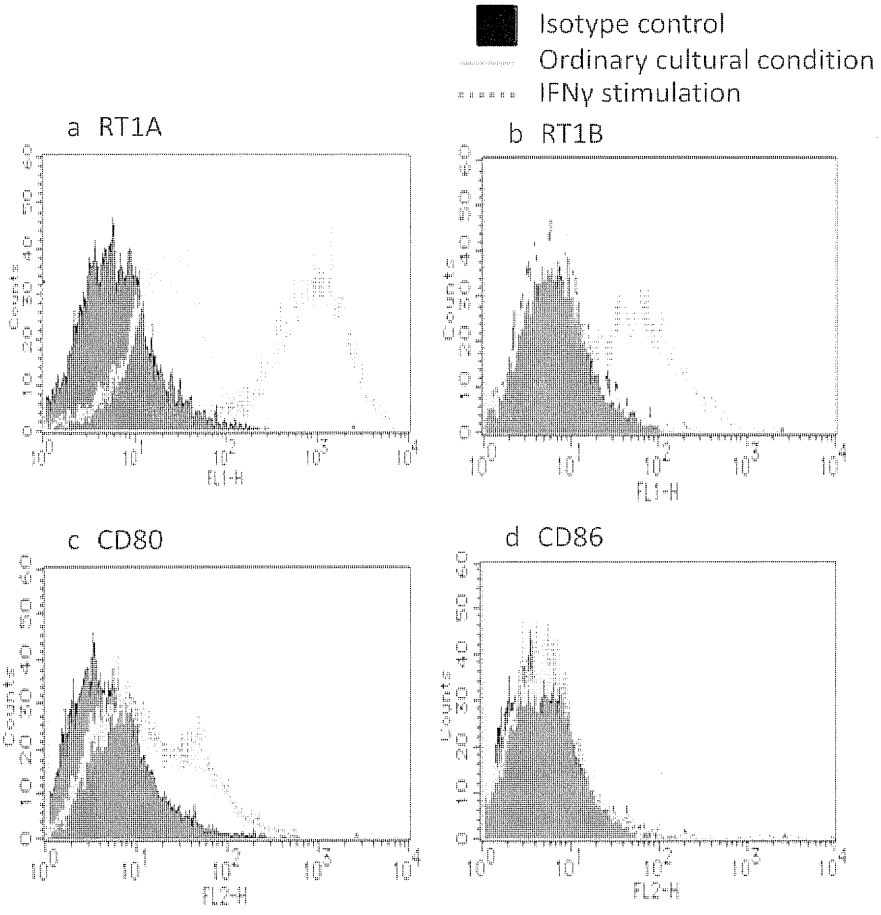

FIGURE 1. Expression of immunologically important cell surface molecules on skeletal myoblasts (SMBs). RT1A (major histocompatibility [MHC] class I), RT1B (MHC class II), CD80 (B7.1), and CD86 (B7.2) were analyzed by flow cytometry. (a) The SMBs were positive for RT1A expression, which was enhanced by interferon (IFN)-γ stimulation. (b) The SMBs were usually negative for RT1B, but they became RT1B positive with IFN-γ stimulation. (c) The cells were positive for CD80. (d) The cells were negative for CD86 regardless of INF-γ stimulation. *Filled curves* show SMBs stained with isotype-matched controls. *Solid-line curves* show SMBs grown under normal culture conditions and stained with each of the antibodies. *Dotted-line curves* show SMBs with INF-γ stimulation and stained with each of the antibodies.

nation of the donor source and transplantation site (*12–15*). For future clinical applications of allogenic SMB transplantation into infarcted myocardium, it is important to evaluate the basal donor cell survival, immune reaction, and therapeutic efficacy. Such studies may also shed more light on the mechanisms underlying its therapeutic effect and indicate ways to improve the therapeutic effect of conventional SMB transplantation therapy.

In this study, our aim was to evaluate the immune reaction after allogenic SMB transplantation into ischemic myocardium and its impact on graft survival and therapeutic effect. We transplanted major histocompatibility (MHC)-mismatched allogenic SMBs into left anterior descending coronary artery-occluded rats without using immunosuppressive drugs and analyzed the immune reaction, donor cell survival, and therapeutic efficacy.

RESULTS

In Vitro Analysis of SMB Immunogenicity

SMBs harvested from 3-week-old ACI and Lewis (LEW) rats were examined for immunity-related cell surface markers by flow cytometry. There were no differences in the marker expressions between the SMB origins of the strains. SMBs expressed the MHC class I molecule RT1A. When SMB immunogenicity was enhanced by interferon (IFN)-γ, the level of MHC class I increased (Fig. 1a). SMBs did not express the MHC class II molecule RT1B under ordinary culture conditions, but they became positive for it under IFN-γ stimulation (Fig. 1b). Furthermore, B7.1 (CD80) was expressed and B7.2 was not, and these expressions were not affected by IFN-γ stimulation (Fig. 1c and d). The SMBs were negative for CD45, indicating that there was no contamination or hematopoietic cell differentiation (data not shown).

Immunoreactions Induced by Allogenic SMB Transplantation in AMI Model Rats

To examine the immune response after allogenic SMB transplantation, the transcription levels of interleukin-2 receptor (IL-2R) and IFN-γ, which reflect the level of activated T-cell infiltration, were measured in recipient hearts using quantitative reverse-transcriptase polymerase chain reaction (PCR; Fig. 2). On day 7, group A showed significantly higher IL-2R expression than groups C and S, and it was still higher on day 28 (Fig. 2a). The IFN-γ expression also indirectly reflects the level of immune rejection. The changes in IFN-γ expression in group A coincided with the IL-2R expression pattern, which was significantly increased on days 7 and 28 compared with group C. In group S, IFN-γ increase was also observed on days 7 and 28 (Fig. 2b).

Infiltration of T Cells Into the Cell Transplantation Area

Immunohistochemical analysis was performed 7 days after SMB transplantation, when the transcription levels of

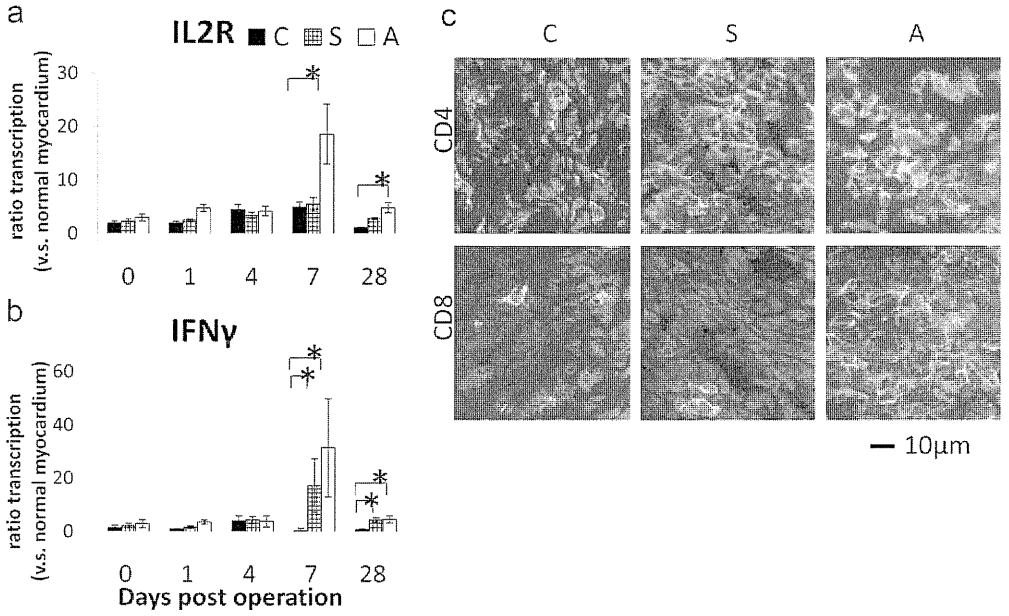

FIGURE 2. Evaluation of the immune response in recipient hearts. Transcriptional levels of (a) interleukin-2 receptor (IL-2R) and (b) interferon (IFN)-γ in recipient hearts 0, 1, 4, 7, and 28 days after transplantation were determined by quantitative reverse-transcriptase polymerase chain reaction assays. The values were normalized to the endogenous control GAPDH and represented as a ratio to the transcription level in normal myocardium. (a) In group A, the level of IL-2R was significantly increased 7 and 28 days after transplantation compared with groups C and S. (b) The IFN-γ level showed a similar pattern of transition to that of IL-2R. In group A, IFN-γ was increased 7 and 28 days after transplantation. *Filled columns* show group C, *checkered columns* show group S, and *open columns* show group A. *P less than 0.05. (c) T-cell infiltration into the cell transplantation area. Representative images from each group are shown. The level of CD4-positive cell infiltration was the same between groups S and A and increased compared with group C (*upper*). The infiltration of CD8-positive cells was especially enhanced in group A (*lower*). *Blue*: nuclei, *red*: implanted skeletal myoblasts, and *green*: CD4 or CD8. Bar=10 μm.

IL-2R and IFN-γ were significantly increased. A strong infiltration of CD4-positive T cells was observed around the donor SMBs of groups A and S compared with group C (Fig. 2c, upper). In contrast, many more infiltrated CD8-positive T cells were observed in group A than in groups S and C (Fig. 2c, lower).

Time Course Analysis of Donor Cell Number in Infarcted Left Ventricle

We evaluated the number of donor cell survival in the recipient myocardium by quantitative PCR for donor-specific *sry* gene and calculated from a standard curve. In group C, no specific signals for *sry* were detected at any time point. Fifteen minutes after transplantation, 0.5×10^6 or fewer cells were detected in groups S and A, which was approximately 10% of the prepared donor cells. In group A, the number of initially delivered donor cells was the same as in group S. Thereafter, the number of allogenic SMBs was lower in group A than in group S; half of the donor cells in group A disappeared within 7 days, and no cells were detected 28 days after transplantation (Fig. 3a). In group S, the donor cells proliferated nearly 5-fold from day 1 to 4, then decreased thereafter. Cell proliferation at day 4 was also confirmed by bromodeoxyuridine incorporation (see **Figure, Supplemental Digital Content 1,** http://links.lww.com/TP/A337). At 28 days after transplantation, the number of residual donor cells in the infarcted heart tissue in group S was nearly half of the initial count.

Detection of Donor Cells in Recipient Hearts

We performed histologic analyses 4 days after transplantation to identify the donor cells. In group S, large numbers of donor SMBs were detected in the scar area and in the border zone between the scar and normal areas (Fig. 3b and c). In group A, only a few donor SMBs were detected compared with group S (Fig. 3f and g), which confirmed the PCR findings (Fig. 3a). The donor cells had a fibroblastic shape but were not cardiomyocytes and did not have a capillary component (Fig. 3d and e).

Echocardiography Findings After Allogenic SMB Transplantation in AMI Model Rats

Cardiac performance was determined 8 weeks after cell transplantation using echocardiography (Fig. 4). There were no significant differences for body weight, heart rate, or blood pressure among the experimental groups (data not shown). The left ventricular (LV) end-diastolic dimension (Fig. 4a) and LV end-systolic dimension (LVDs; Fig. 4b) were not significantly different among the three groups, although the LVDs in group S was relatively small (P=0.067 vs. group A; Fig. 4b). The LV ejection fraction, which represents contractility, was calculated from the LV end-diastolic dimension and LVDs. The LV ejection fraction value in group A was the same as in group C, whereas it was significantly higher in group S (Fig. 4c).

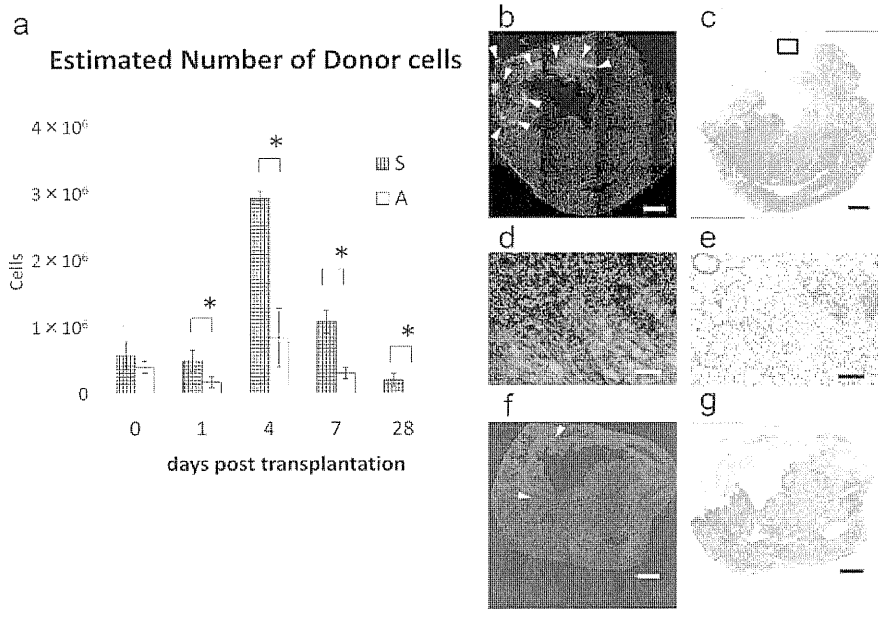

FIGURE 3. Detection of donor skeletal myoblasts (SMBs) in recipient hearts. (a) The number of donor cells was significantly lower in group A than in group S on days 1, 4, 7, and 28. *Checkered columns* show group S, and *open columns* show group A. **P* less than 0.05. (b–g) Recipient hearts were analyzed by histology 4 days after PKH26-labeled SMB transplantation. (b) In group S, abundant SMBs were detected. (c) Serial section of the image in b, showing that most of the donor cells were surrounded by infarcted area. (d and e) High-magnification images of the *square* in c, showing the surviving donor cells with a fibroblastic shape. (f) Fewer donor cells were detected in group A than in group S. (g) Serial section of the image in f showing donor cells surrounded by infarcted area. (b–e) Group S; (f and g) group A; (b, d, and f) 6-diamidino-2-phenylindole staining; and (c, e, and g) hematoxylin-eosin staining. *Blue*: nuclei, *red*: fluorescent dye-labeled donor SMBs. *Arrowheads*: donor SMBs. (b, c, f, and g) Bar=1 mm and (d and e) Bar=100 μm.

DISCUSSION

In this study, we evaluated allogenic SMB transplantation in AMI model rats with a focus on the implanted cell survival and immune response. SMBs potentially expressed both MHC and B7 signal molecules (Fig. 1), which suggested that allogenic SMBs might be targeted by the host's immune response. Indeed, our in vivo analyses showed increased IL-2R and IFN-γ transcription in the recipient myocardium tissues (Fig. 2a and b). Seven days after transplantation, the IL-2R and IFN-γ expressed by immunocytes were notably increased in group A, indicating that a higher level of inflammation with immune rejection was induced by allogenic than by syngenic SMB transplantation. In addition, histologic analysis showed that the grafted allogenic SMBs were surrounded by CD4- and CD8-positive T cells (Fig. 2c, right). Immune rejection may also affect the donor cell clearance rate. There were fewer allogenic SMBs in the recipient hearts than syngenic ones, and their number decreased over time, disappearing within 28 days, when syngenic SMBs still remained (Fig. 3a). Histologic analysis of the transplanted labeled SMBs showed that the allogenic SMB aggregates were small and sparse compared with those of the syngenic SMBs (Fig. 3b–g). The higher inflammation level and lower rate of donor cell survival may indicate a loss of therapeutic benefit from allogenic cell transplantation for AMI (Fig. 4). This is the first report to estimate the number of implanted allogenic and syngenic SMBs and to assess the immune reaction in the infarcted myocardium over time.

Both the innate and adaptive immune systems are implicated in the destruction of allogenic cells. Both syngenic and allogenic transplanted cells are destroyed by the innate immune system shortly after cell transplantation. Then $CD4^+$ and $CD8^+$ T cells infiltrate locally around the alloantigens. As shown by flow cytometry analysis, the SMBs expressed both MHC class I and costimulatory signal molecules (Fig. 1). $CD4^+$ and $CD8^+$ T cells activated by antigens of the allogenic SMBs may mediate the immunologic rejection. Guo et al. (*16*) demonstrated that allogenic SMB transplantation at 1 week after MI had a comparable effect to autotransplantation when an immunosuppressive reagent was used. In allogenic SMB transplantation for MI, modulation of the immune responses and prolong donor cell survival by immunosuppressive reagents usage or gene modification of donor SMBs may be a strategically effective approach.

An increase in IFN-γ at the transcriptional level, and CD4-positive cell infiltration, was observed in group S at 7 days after transplantation. Although we used LEW rats for both the donors and recipients in our syngenic transplantation model, a T-cell response was still observed. This response is directed against minor histocompatibility antigens (*17*, *18*). In particular, the male-specific H-Y antigen from a donor is known to induce an immune response in female recipients, mainly by CD4-positive T-cell activation (*17*, *19*). Sakakida et al. (*20*) reported that sex-mismatched skin grafts in LEW rats survive, which suggests that the H-Y antigen is not so critical in transplantations between LEW rats. We performed sex-mismatched transplantation to quantify the male donor cells in female recipient hearts. Our findings support the idea that major and minor histocompatibility-matched

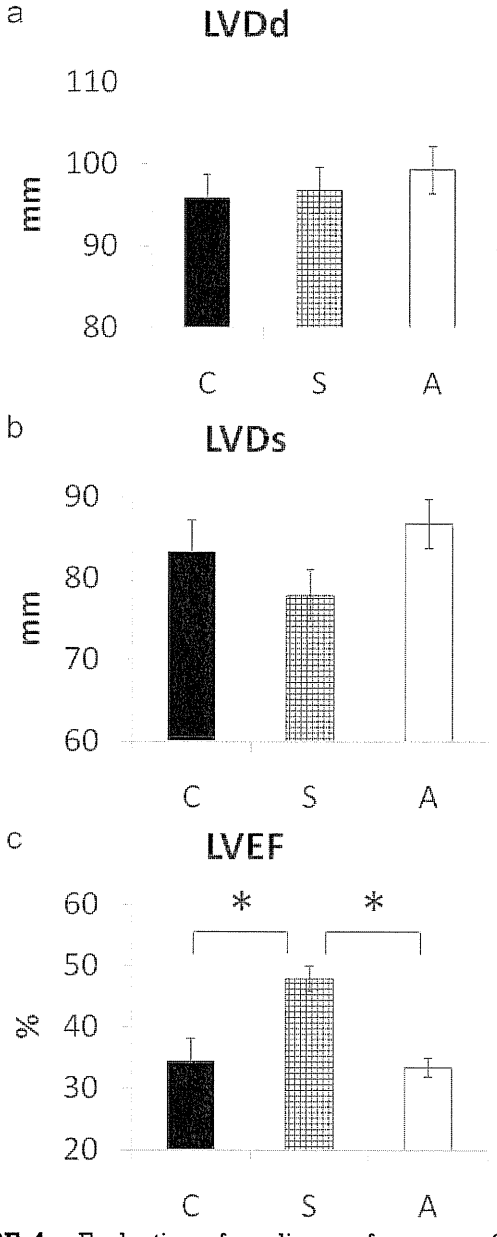

FIGURE 4. Evaluation of cardiac performance. (a) The left ventricular end-diastolic dimension (LVDd) and (b) left ventricular end-systolic dimension (LVDs) were determined by echocardiography 8 weeks after cell transplantation. (c) The left ventricular ejection fraction (LVEF) was calculated as LVEF(%)=(LVDd^3−LVDs^3)/LVDd^3×100. 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; *checkered 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: $1.71\times10^5\pm84617$ vs. $4.52\times10^4\pm13061$). 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^{avl}, 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	TCTCCTCAGAAATTCCACCACAGTTGCTG
SRY	GCCTCAGGACATATTAATCTCTGGAG	GCTGATCTCTGAATTCTGCATGC	AGGCGCAAGTTGGCTCAACAGAATCC
GAPDH	CCATCACTGCCACTCAGAAGAC	TCATACTTGGCAGGTTTCTCCA	CGTGTTCCTACCCCCAATGTATCCGT
IL-2R	CCCTCAGGTGTTTCTTGAGCTT	CTTTCCCAGAGAGTGAGGCTTC	TGGCCACTGCTACCTGATACTCCTTTGTGA
IFN-γ	ATCGAATCGCACCTGATCACTA	TTCTTATTGGCACACTCTCTACCC	AACAACCCACAGATCCAGCACAAAGC

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

(LEW/Sea: RT-1[l], 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% CO_2 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 μL of Hank's buffered salt solution; Sigma-Aldrich) obtained from a male ACI or LEW rat, or 200 μL 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-μm 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 μg/mL, Life Technologies). The samples were then counterstained with 6-diamidino-2-phenylindole (1 μg/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±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;
continuos-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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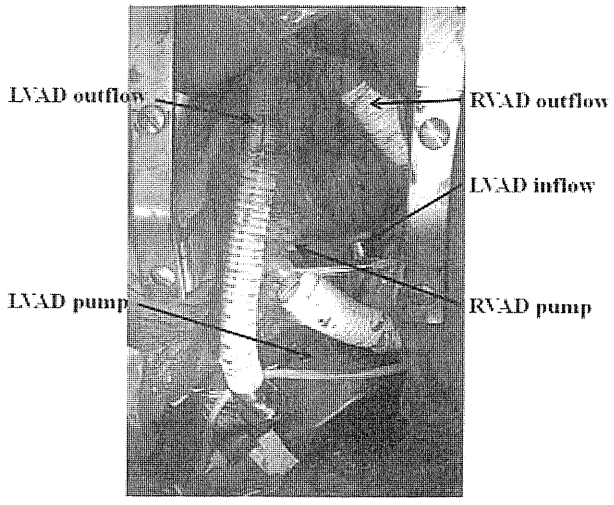


Figure 1 The inflow cannula of DuraHeart left ventricular assist device (LVAD) is inserted in the LV apex and the outflow graft is anastomosed to the ascending aorta. The Jarvik 2000 right ventricular assist device (RVAD) is within the RV, and the outflow graft is anastomosed to the pulmonary trunk. 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.

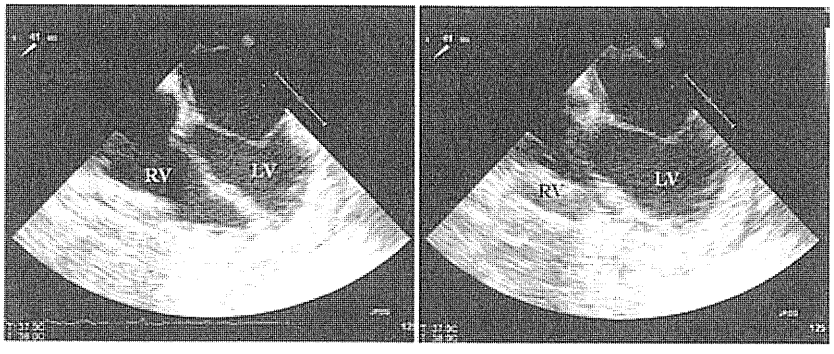
Weaning from CPB was somewhat difficult because it required a delicate adjustment of the BiVAD flow. Even though the Jarvik 2000 RVAD flow rate was set at the minimum (dial 1: 8,000 rpm), the RV was sucked down, and an adequate forward flow could not be obtained initially. Transesophageal echocardiography (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



A B

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.

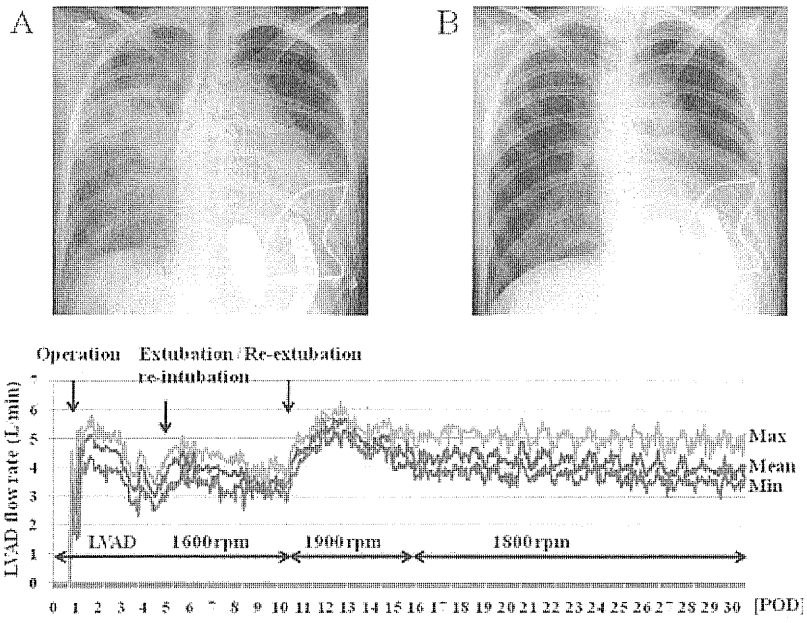


Figure 3 (A) Severe pulmonary congestion was observed after extubation. (B) The pulmonary congestion was improved immediately after reintubation. (C) Left ventricular assist device (LVAD) flow increased after extubation. Although dynamic flow change occurred in the acute phase, the flow rate was stabilized 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 mode[8] 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 device results in immediate onset of pulmonary edema. Rousel et al[10] 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). Postoperatively, 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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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 microtissue 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

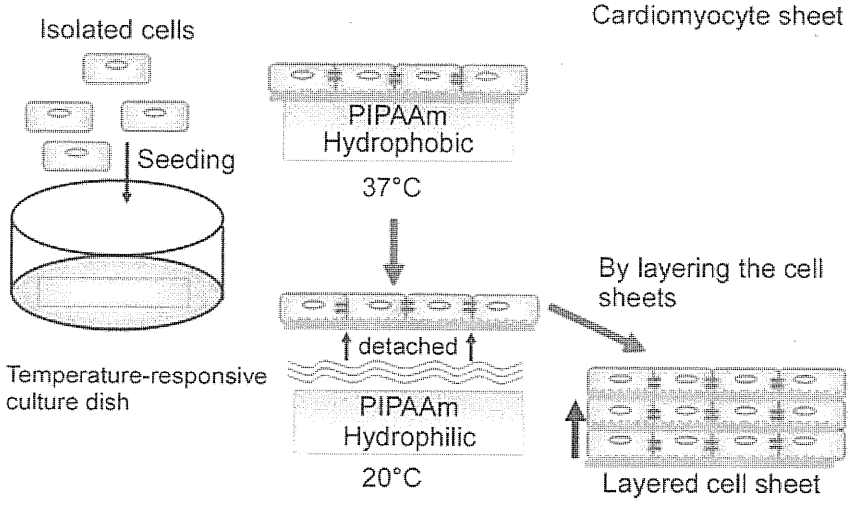

Fig 1. Diagram shows the method for engineering the cell sheets. Cell sheets harvested from a temperature-responsive culture dish can be layered to construct a 3-dimensional cardiac graft. (PIPAAm = poly(N-isopropylacrylamide.)

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

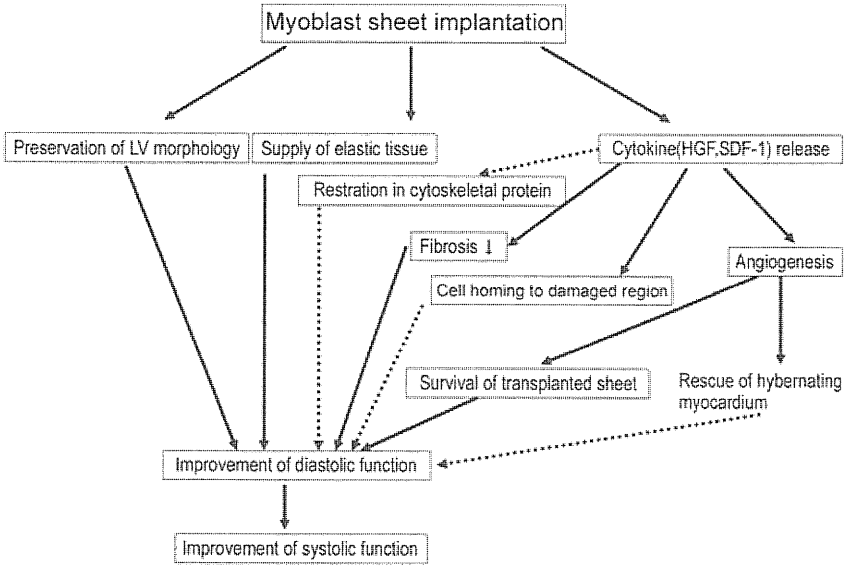

Fig 2. Possible regeneration mechanisms in myoblast sheet implantationMyoblast sheets are proposed to preserve the left ventricular (LV) geometry, provide elastic tissue to a stiffened scar, and cause cytokine (HGF, SDF-1 etc) release. These cytokines may help restore cytoskeletal proteins, reduce fibrosis, 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	↑
Kellar	...	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-butyrate; PCL = poly-e-caprolactone; PGA = poly glycolic 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,