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**this study.**

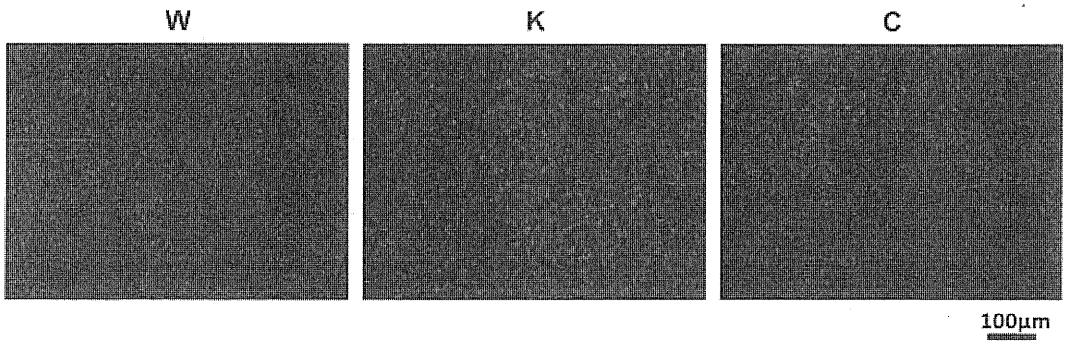
| | Reverse Primer | Probe |
|---|---|---|
| GTGTTC | GATGCCTGCTTCACCACCTT | CCGCCTGGAGAAACCTGCCAAGTATG |
| TCTGTCTACT | TTGGTGGTTTGCTACGACGT | CCCAGACCCTCACACTCAGATCATCTTC |

**Supplementary figure legends**

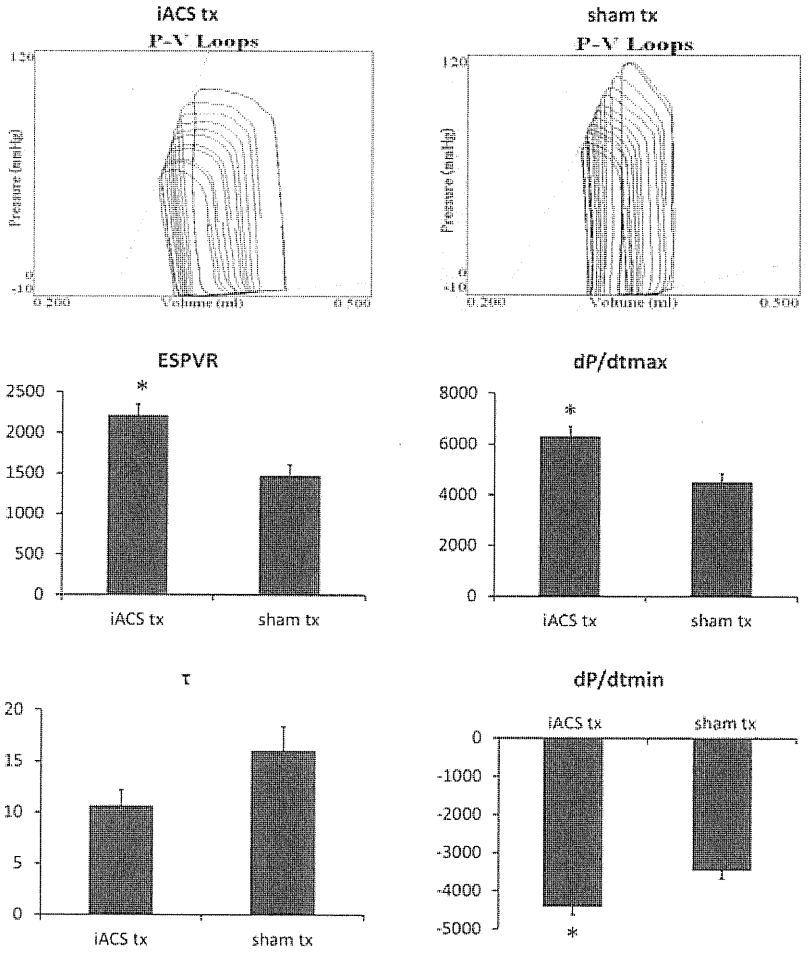
**Supplementary figure 1. Capillary formation in the iACS-treated heart.** Four weeks after infarction and iACS transplantation, CD31 staining was performed to assess angiogenesis. Representative images from each group are shown. The density of CD31-positive capillaries was the same among the groups. Green, CD31; blue, nuclei.

**Supplementary figure 2. Hemodynamic effects of WT-iACS transplantation in rat AMI.** In the iACS-treated group, the $dP/dt_{max}$ was significantly higher and the $dP/dt_{min}$ significantly lower than in the control ($P<0.05$ v.s. sham-treated group, *unpaired t* test). The $\tau$ value was not significantly different between the groups, although it was smaller in the iACS-treated group ($P=0.12$ v.s. sham-treated group, *unpaired t* test). The ESPVR value was significantly higher in the iACS-treated group. tx, treatment.

Supplementary figure 1



Supplementary figure 2



# Novel regenerative therapy using cell-sheet covered with omentum flap delivers a huge number of cells in a porcine myocardial infarction model

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**Objective:** A key challenge to applying cell transplantation to treat severely damaged myocardium is in delivering large numbers of cells with minimum cell loss. We developed a new implantation method using skeletal myoblast (SMB) sheets, wrapped with an omentum flap as a blood supply to deliver huge numbers of SMBs to the damaged heart. We examined whether this method could be used to deliver a large amount of cells to deteriorated porcine myocardium.

**Methods:** Cell sheets were obtained by culturing mini-pig autologous SMB cells on temperature-responsive culture dishes. Myocardial infarction was induced by placing an ameroid constrictor around the left anterior descending artery. The mini-pigs were divided into 4 treatment groups (n = 6 in each): cell sheets with omentum, cell sheets only, omentum only, and sham operation. Each animal implant consisted of 30 cell sheets ($1.5 \times 10^7$ cells per sheet). Six 5-layer constructs were each placed on a different area, immediately adjacent to but not overlapping one another, to cover the infarct and border regions.

**Results:** The new regenerative cell delivery system using SMB sheets covered and wrapped with omentum resulted in (1) a significantly reduced infarct size causing, at least in part, a thin scar with thick well-vascularized cardiac tissue; (2) increased angiogenesis, as determined by a significantly higher vascular density; and (3) improved cardiac function, as determined by echocardiography, compared with the conventional method (SMB sheet implantation).

**Conclusions:** This cell delivery system shows potential for repairing the severely failed heart. (J Thorac Cardiovasc Surg 2011;142:1188-96)

Heart failure is a frequent and life-threatening disorder, despite recent medical and surgical advances. Myocardial regenerative therapy is gaining interest as a means for improving left ventricular (LV) function in patients with end-stage heart disease.[1-3] However, a recent clinical trial of cell transplantation by needle injection reported slightly disappointing results.[2-4] The main drawbacks of cell transplantation by needle injection appear to be poor retention and survival of the injected cells, local mechanical myocardial damage owing to injury by the needle itself, and the potential for lethal arrhythmias. We have been investigating cell-sheet techniques for delivering cells to severely damaged myocardium more efficiently, without damaging the myocardium, and, consequently, more effectively. This technique provides better improvement of cardiac function than obtained with the needle cell-injection method.[5-7]

The greatest advantage of the cell-sheet technique is that the sheet consists only of cells, which produce an extracellular matrix without requiring an artificial scaffold. The cell sheet has a high ability to integrate with native tissues, because the adhesion molecules on its surface are preserved.[5-7] The layered grafts must be carefully prepared to avoid tearing, but they themselves are strong, flexible, and easy to work with.

It has been suggested that an increased number of implanted skeletal myoblast (SMB) sheets is related to better results, such as improved cardiac function and angiogenesis, less fibrosis, and less hypertrophy, with the amounts of secreted cytokines dependent on the number of cell sheets used.[7] However, cell sheets with more than 5 layers show areas with disorganized vasculature, presumably because of insufficient supplies of blood, oxygen, and nutritients.[7,8] Thus, in applying cell transplantation to the severely damaged myocardium, a key challenge is in improving the blood perfusion of the implanted cells so

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**Abbreviations and Acronyms**

LAD = left anterior descending coronary artery
LV = left ventricular
LVEDV = left ventricular end-diastolic volume
LVEF = left ventricular ejection fraction
LVESV = left ventricular end-systolic volume
MI = myocardial infarction
O group = omentum only
RT-PCR = real-time polymerase chain reaction
S group = cell sheets only
SMB = skeletal myoblast
SO group = cell sheets wrapped with omentum
STAT3 = signal transducer and activator of transcription 3
VEGF = vascular endothelial growth factor

that large numbers of regenerative cells can be delivered with minimal cell loss.

The omentum is reported to potentially provide revascularization for the ischemic myocardium,[9] release a number of angiogenic cytokines,[10,11] supply stem cells, and attenuate inflammation.[12] The omentum was once commonly used in surgical revascularization to treat ischemic heart disease; however, omentopexy alone is not very effective for supporting the angiogenesis needed in the infarcted area for rapid recovery.[9] On the basis of those findings, we speculated combining SMB sheets with omentum might enhance survival of the implanted cells by improving angiogenesis. Thus, as a novel method for implanting large amounts of cells, we developed a cell-delivery system using SMB sheets wrapped and covered with omentum flap as an external source for blood flow. We hypothesized that this method could replace the myocardial infarction (MI) scar with cell-sheet–based cardiac tissue in the pig heart.

## MATERIALS AND METHODS

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

### Animal Models and Study Protocol (Figure 1)

Thirty-seven female mini-pigs (8-10 months old; Japan Farm Co Ltd, Kagoshima, Japan) weighing 20 to 25 kg were used in these experiments.

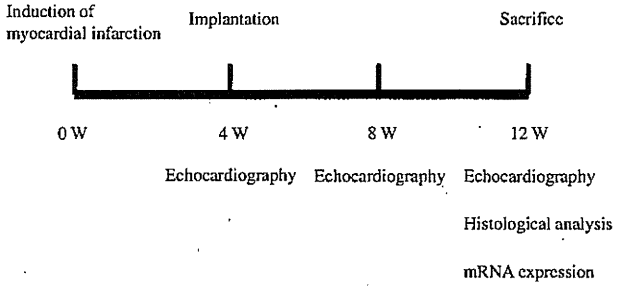


**FIGURE 1.** Study protocol for the assessment of cardiac function and histologic analysis.

The mini-pigs were anesthetized with an intravenous administration of ketamine (6 mg/kg) and sodium pentobarbital (10 mg/kg) for endotracheal intubation and then maintained with inhaled sevoflurane (15%-2%). The pericardial space was exposed by left thoracotomy through the fourth intercostal space. The distal portion of the left anterior descending coronary artery (LAD) was directly ligated as ischemic preconditioning to reduce the occurrence of lethal ventricular arrhythmia, followed by placement of an ameroid constrictor around the LAD just distal to the left circumflex coronary artery branching.[5,13] The muscle and skin were closed in layers, and the mini-pigs were then taken off the anesthetics. Eleven (30%) of the study animals died in the early postoperative period. This technique produced an ischemic cardiomyopathy model that reflected clinical relevance and can be used for appropriate preclinical studies with minimal procedure-related mortality.

Computer-generated random allocation generated 4 randomized study groups 1 week after MI. Autologous cells were isolated and grown in culture for 3 weeks to prepare samples for implantation. Four weeks after MI induction, the mini-pigs were again placed under general anesthesia for echocardiography followed by either cell-sheet implantation or a sham operation. Two mini-pigs whose LV ejection fractions (LVEFs) were above 40% before treatment, as measured by transthoracic echocardiography using the Simpson method, were excluded from the study. At 4 and 8 weeks after either cell-sheet implantation or sham operation, the mini-pigs again underwent general anesthesia for echocardiographic examination. The mini-pigs were humanely killed after the 8-week echocardiography measurements for histologic and biochemical analyses of the heart tissue.

### Preparation and Grafting of SMB Cell Sheets

Autologous skeletal muscle weighing approximately 10 to 15 g was removed from the quadriceps femoris muscle, and purified autologous SMB cells were cultured for 3 weeks to prepare them for implantation, as described previsously.[5] Autologous SMBs are precursor cells of adult myofibers and feature several advantages, including autologous origin, high in vitro scalability, lack of tumorigenicity (owing to their myogenic lineage restriction), and strong resistance to hypoxia after ischemia. The cells were incubated in 60-mm temperature-responsive culture dishes (UpCell; Cellseed, Tokyo, Japan) at 37°C for 24 hours ($1.5 \times 10^7$ cells per dish). The dishes were then transferred to another incubator, set at 20°C, for 1 hour to release the cultured cells as intact cell sheets. Under this protocol, the SMBs spontaneously detached from the plate as a free-floating monolayer cell sheet.

### Grafting the SMB Cell Sheet Wrapped With Omentum

The mini-pigs with MI were divided into 4 treatment groups ($n = 6$ in each): cell sheets wrapped with omentum (SO group), cell sheets only (S group), omentum only (O group), and sham operation (sham group). Each animal in the SO and S groups received approximately 30 cell sheets ($1.5 \times 10^7$ cells per sheet) with the total cell number being $4.5 \times 10^8$.

ET/BS

In the S group, a median sternotomy was performed. The cell sheets were placed on the epicardium of the ischemic area (LAD region) and stitched in place around the edge. In the SO group, a small upper midline laparotomy and median sternotomy were performed to move omentum from the peritoneal space into the mediastinal space, preserving the arch structure of the left gastroepiploic artery. We created a hole in the diaphragm and passed the omentum flap through the hole into the pericardial cavity. Five-layer cell sheets of SMBs were wrapped with omentum and then covered with the harvested omental flap, because a maximum of 5 cell-sheet layers can be implanted in one place.[7,8] Six 5-layer constructs were each placed on a different area to cover the infarct and border regions. The operations were performed with the animals under general anesthesia.

## Histologic and Immunohistochemical Analyses

Eight weeks after the treatment, the hearts were dissected and embedded in optimum cutting temperature compound, snap-frozen in liquid nitrogen, and cut into 5-$\mu$m thick sections. The paraffin-embedded sections were fixed in 4% paraformaldehyde and stained with hematoxylin–eosin (Figure 2) or Masson trichrome (Figure 3). The percentage of infarct area was quantified as the positively stained LV area/total LV area. The stained and total LV areas were obtained by tracing using Image J software at the mid-LV level, where the base of the papillary muscles is clearly depicted.

So that vascular density could be evaluated in the border area, the cryosectioned samples were immunolabeled with anti–von Willebrand factor antibody (1:250 dilution; Dako, Glostrup, Denmark) (Figure 4). The number of positively stained capillary vessels that were 5 to 10 $\mu$m in diameter in the peri-infarct border-zone myocardium in 16 individual randomly selected fields per heart was counted under high-power magnification (×200). The numbers from the 16 fields were averaged to determine the vascular density (per square millimeter). The stained slides were viewed on a BioZero laser scanning microscope (Keyence, Osaka, Japan).

The following antibodies were used to identify SMBs: the primary antibodies were anti-smooth muscle actin, anti-vimentin, anti-desmin, and anti-skeletal myosin (fast) (all from Dako); the secondary antibodies were goat anti-mouse and anti-rabbit immunoglobulin G (Invitrogen, Leek, The Netherlands).

## Analysis of mRNA Expression

Total RNA was extracted from cardiac muscle tissue, reverse transcribed into cDNA using TaqMan reverse transcription reagents (Applied Biosystems, Stockholm, Sweden), and real-time polymerase chain reaction (RT-PCR) was performed with an ABI PRISM 7700 machine (Applied Biosystems).[14] For each gene, RNA samples were prepared and assayed in triplicate. RT-PCR was used to determine the expressions of vascular endothelial growth factor (VEGF) and signal transducer and activator of transcription 3 (STAT3) in our in vivo experiments. The average copy number of gene transcripts for each sample was normalized to that for glyceraldehyde-3-phosphate dehydrogenase.

## Echocardiography: Measurements of Global LV Function

Global cardiac function was assessed using commercially available echocardiographic equipment with a 4.0-MHz transducer (Aplio; Toshiba, Otawara, Japan) before and 4 and 8 weeks after cell-sheet implantation. Echocardiographic measurements included LV end-diastolic and end-systolic volumes (LVEDV and LVESV, respectively) and LVEF. LVEF was calculated as follows: $\text{LVEF}\ (\%) = 100 \times (\text{LVEDV} - \text{LVESV})/(\text{LVEDV})$.

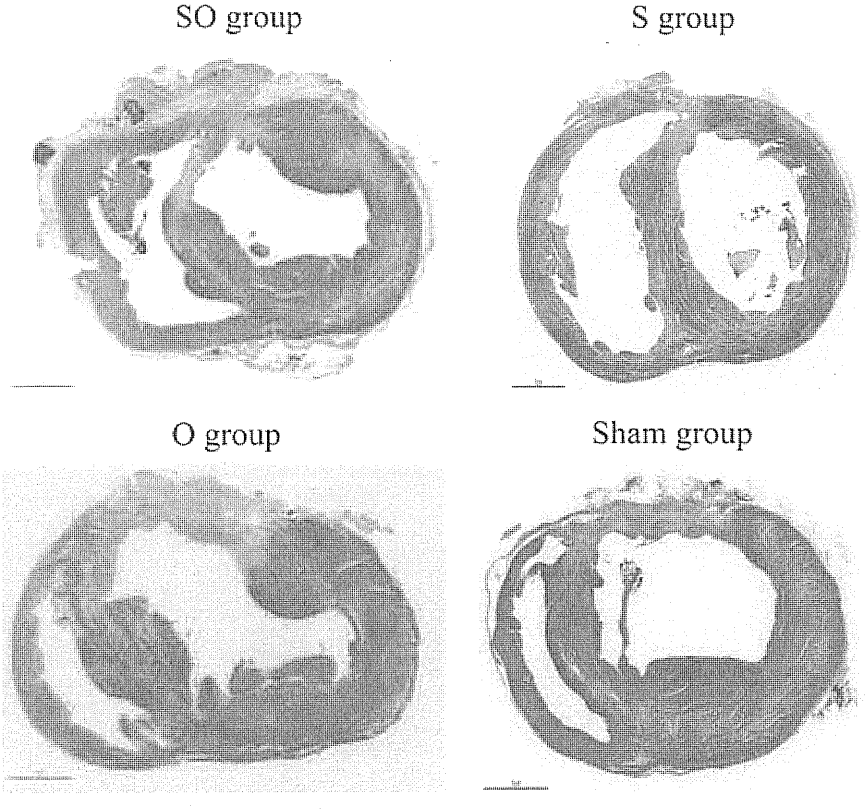


**FIGURE 2.** Macroscopic (×40) views of the heart in the 4 groups (hematoxylin–eosin staining). *SO group,* skeletal myoblast (SMB) sheets wrapped with omentum; *S group,* SMB sheets only; *O group,* omentum only; *sham group,* sham operation.

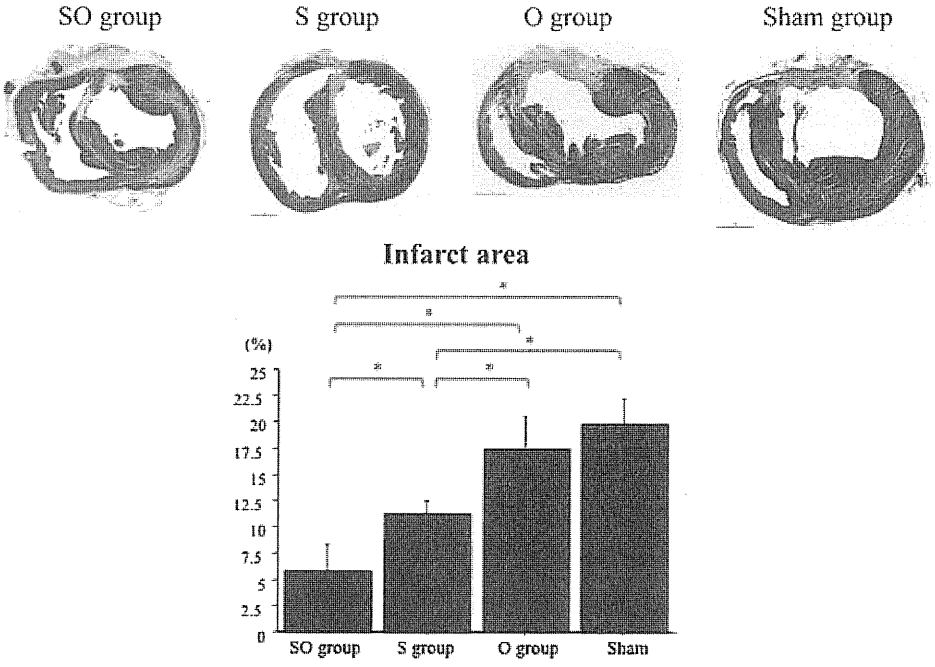


**FIGURE 3.** Macroscopic (×40) views of the heart in the 4 groups (Masson trichrome staining). Infarct area: The SO group showed a significant improvement in the infarct area of the whole heart. Abbreviations as in Figure 2. *$P < .05$.

### Statistical Analysis

SPSS software (version 11.0, SPSS, Inc, Chicago, Ill) was used for statistical analyses. Continuous values are expressed as the mean ± standard deviation. The significance of differences was determined using a 2-tailed multiple *t* test with Bonferroni correction following analysis of variance for individual differences.

## RESULTS

### Modulation of Myocardial Structure

Myocardial structural components, including fibrosis and vascularity, were assessed by hematoxylin–eosin staining, Masson trichrome staining, and immunohistochemistry

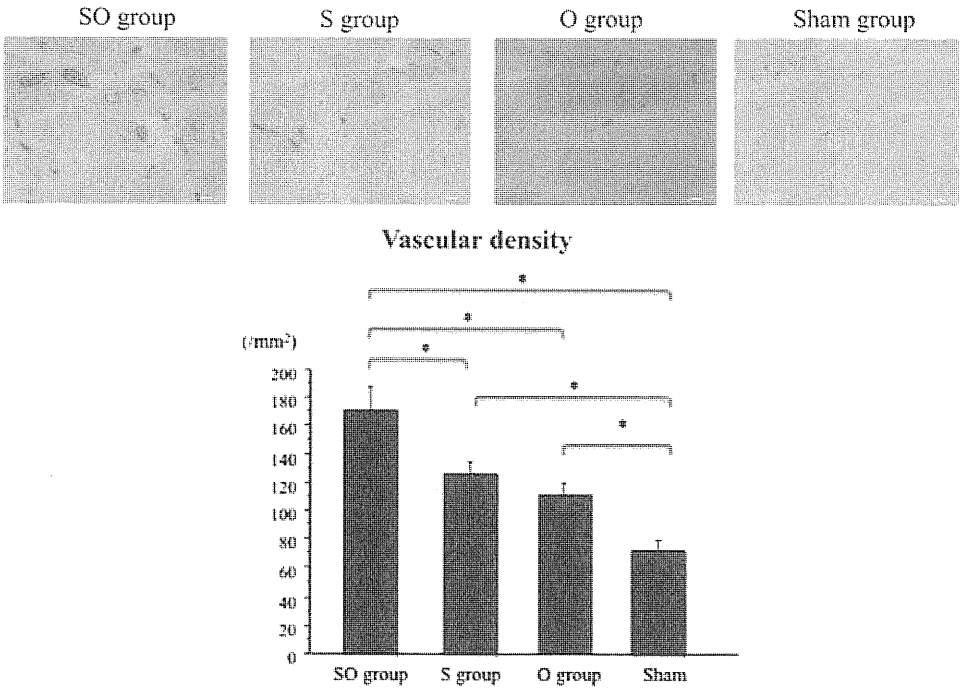


**FIGURE 4.** Microscopic (×200) views of sections of the peri-infarct border-zone region stained with the von Willebrand factor antibody (factor VIII) in the 4 groups (*bar* = 20 μm). Vascular density: The SO group showed a significant improvement in vascular density, as assessed by anti–von Willebrand factor antibody. Abbreviations as Figure 2. N > 4 in each group. *$P < .05$.

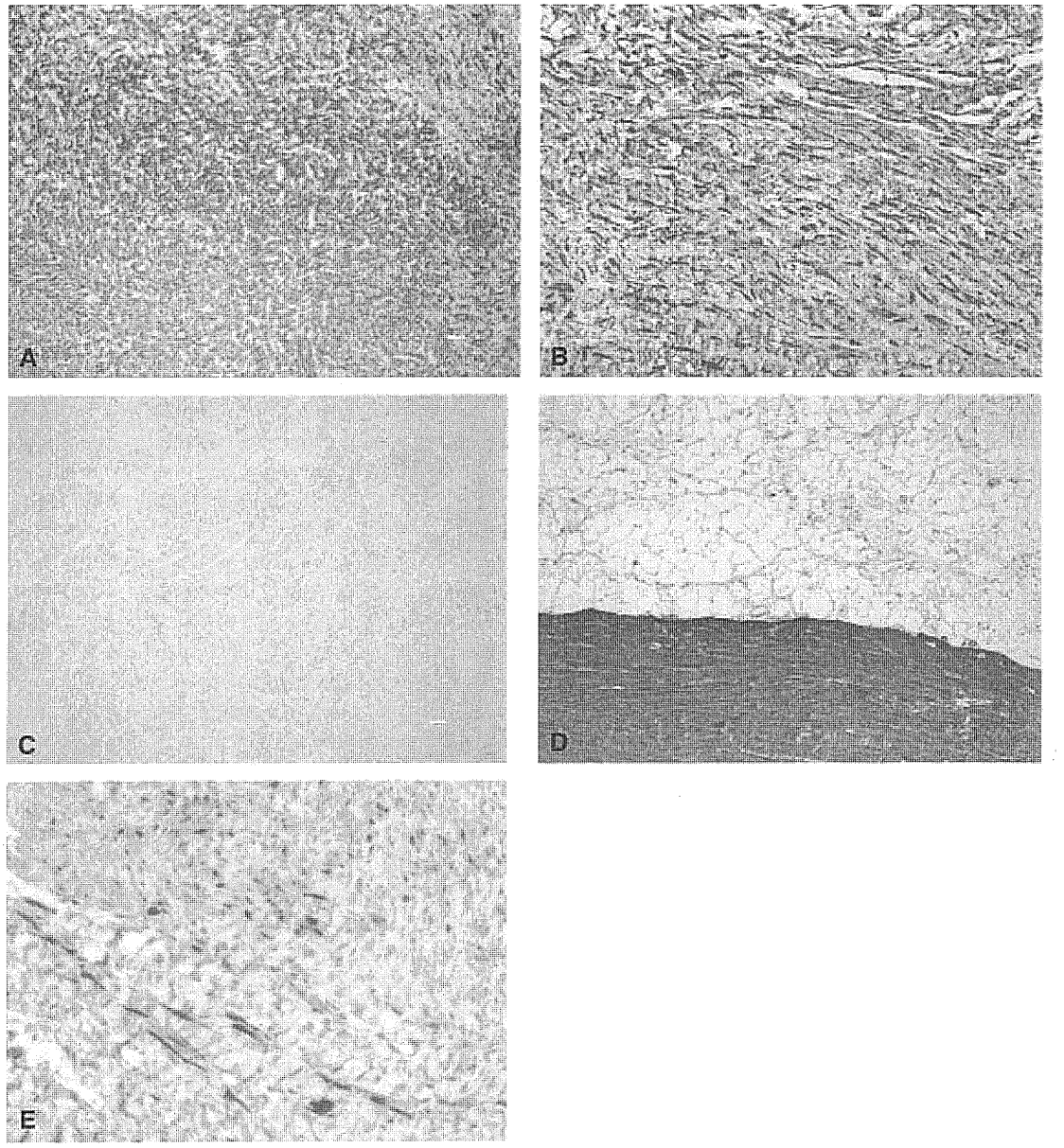


FIGURE 5. A, Microscopic (×200) views of the infarct region in the SO group (alpha smooth muscle actin staining). B, Microscopic (×200) views of the infarct region in the SO group (vimentin staining). C, Microscopic (×200) views of the infarct region in the SO group (fast type myosin heavy chain staining). D and E, Microscopic (×200) views of the heart (desmin staining). *SO group*, SMB sheets wrapped with omentum.

for anti–von Willebrand factor 8 weeks after treatment. The cavity of the LV was enlarged in the O and sham groups, whereas the global myocardial structure was well maintained in the SO and S groups, as assessed by hematoxylin–eosin staining (Figure 2). Collagen was densely accumulated in the infarct area and globally distributed in the remote area after the sham operation, whereas less collagen had accumulated in both the infarct and remote areas in the other 3 groups compared with the sham group, as assessed by Masson trichrome staining. The size of the infarct area, quantitatively assessed by computer-based planimetry of Masson trichrome–stained hearts, was significantly smaller in the SO group than in the other 3 groups (Figure 3). The vascular density, assessed by immunohistochemistry for anti–von Willebrand factor, was significantly greater in the SO group than in the other 3 groups (Figure 4).

Many alpha-smooth muscle actin- and vimentin-positive cells were present in the implanted sheets in the SO group; these cells were negative for fast-type myosin heavy chain and desmin (Figure 5, *A–D*). However, a few desmin-positive cells were also detected at the implanted site (Figure 5, *E*). These results suggested that most of the

detected cells had the phenotype of myofibroblasts, whereas a few expressed the phenotype of SMBs. Although these cells might have played an important role in the myocardial regeneration after SMB sheet implantation, their origin and characteristics have not been fully evaluated. Further study will be needed to elucidate the function of these cells and clarify the mechanisms of myocardial regeneration.

### mRNA Expressions After Cell-Sheet Implantation

The expression of a variety of molecules that are intramyocardially expressed and potentially related to reverse LV remodeling was assessed by RT-PCR. Among them, the relative expression levels of VEGF and STAT3 mRNA were highest in the SO group, suggesting that the combination of SMB sheets and omentum accelerated the secretion of angiogenesis-related cytokines (ie, VEGF and STAT3) from the SMB sheets in vivo (Figure 6).

### Cardiac Functional Recovery

Serial changes in the global systolic and diastolic LV function after cell-sheet implantation were assessed by conventional echocardiography. After the sham operation, LVEDV and LVESV tended to increase until 8 weeks, whereas the LVEF and anterior wall thickness did not change. In contrast, LVEDV did not change in the SO group, but LVESV tended to decrease in the SO and S groups. LVEF increased significantly at 4 and 8 weeks after treatment in the SO group compared with the baseline value. At 4 and 8 weeks after treatment, LVESV was significantly smaller and LVEF significantly greater in the SO group than in the S group, whereas there was no significant difference in LVEDV between them. The anterior wall thickness increased significantly at 8 weeks after treatment in the SO group compared with its baseline value; this change was significantly larger than that in the sham group (Figure 7).

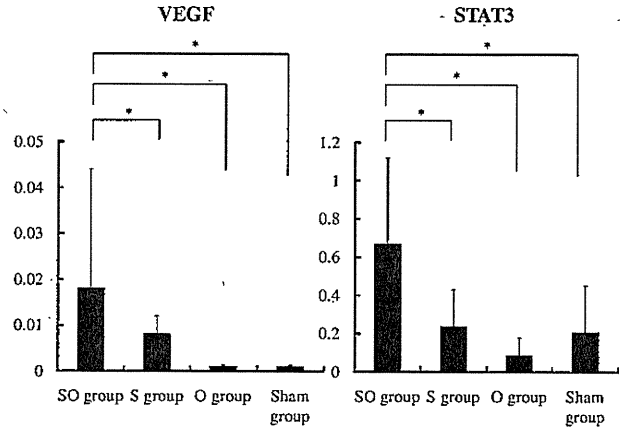


**FIGURE 6.** mRNA expressions in implanted infarcted hearts were determined by real-time PCR using porcine primers. The expressions of vascular endothelial growth factor *(VEGF)* and signal transducer and activator of transcription 3 *(STAT3)* mRNA were significantly increased in the SO group compared with the other groups. Abbreviations as in Figure 2. N > 4 in each group. *$P < .05$.

## DISCUSSION

The major findings of this study were that a new regenerative cell delivery system using SMB sheets covered and wrapped with omentum resulted in the following benefits compared with the conventional method (ie, SMB sheet implantation): (1) a reduced infarct area that led, at least in part, to a thin scar with thick well-vascularized cardiac tissue; (2) angiogenesis induction; and (3) improved cardiac function. Our data suggest that this new cell-delivery system acted in large part by enhancing the paracrine effect of the SMBs.

The mechanism of the restoration of damaged myocardium by SMB sheet implantation is complex, involving many pathways.[5-7] Recent reports describe the beneficial results of SMB sheet implantation in several animal experimental models and patients with heart failure. These benefits were primarily attributed to the secretion of cytokines from the implanted cell sheets (ie, a paracrine effect). It has been suggested that regenerative therapy for the severely damaged myocardium, in which large numbers of cells must be delivered with minimal cell loss, may be achieved by improving the blood perfusion in the region of implanted cells.[7,8] The general intention is to protect the implanted cells from ischemic injury and necrosis by inducing cardiac protective responses (eg, angiogenesis and attenuation of inflammation). A previous study[15] reported that angiogenic factors, such as endothelial cells and some angiogenic growth factors, can enhance angiogenesis to improve the survival of thick-layered cardiomyocyte sheets applied to the damaged myocardium. Shimizu and associates[8] reported that transplanted 3-layered cardiomyocyte sheets could be vascularized in subcutaneous tissue without necrosis, but sheets with 4 or 5 layers had areas with disorganized vasculature and primary ischemia. To overcome this limitation, they performed repeated transplantations of triple-layer grafts, which created a thick myocardium with a well-organized microvascular network.[8] However, technical hurdles still need to be overcome before a similar method can be clinically applied in humans.

In the present study, we demonstrated that the implantation of SMB sheets in combination with omentum was superior to conventional SMB-sheet implantation. The use of an omentum flap to provide revascularization within the ischemic myocardium was previously performed in patients with ischemic heart disease.[9] Although omentopexy alone was not effective for eliciting a rapid recovery, recent basic research studies have suggested a mechanism by which omentum tissue induces angiogenesis.[10,11]

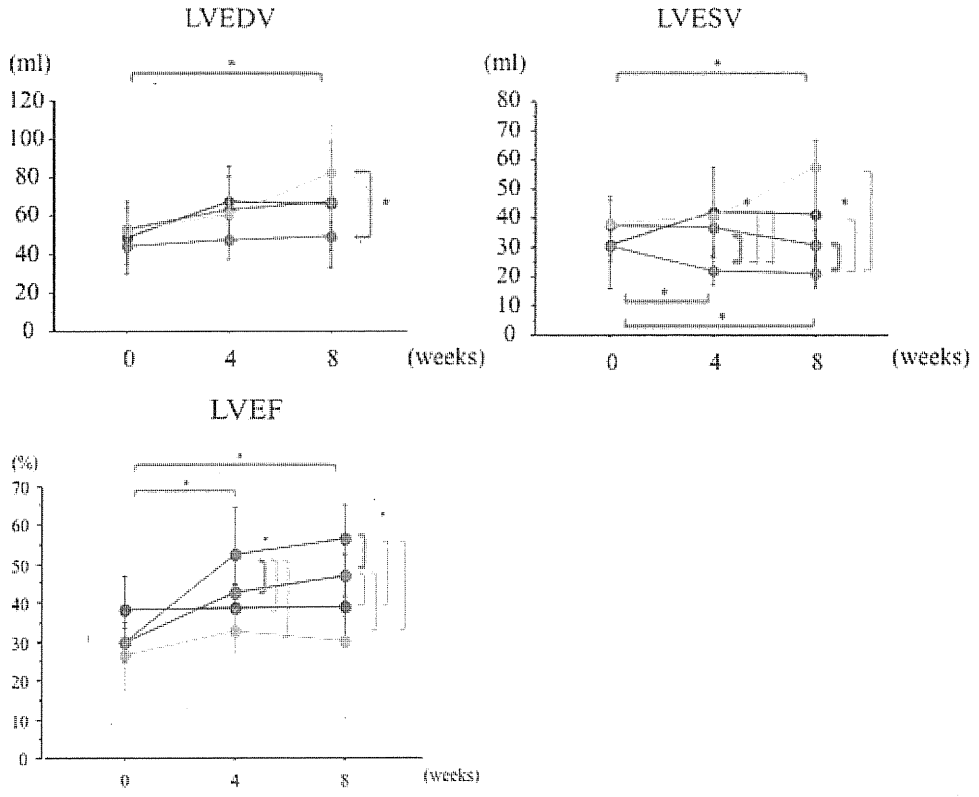


**FIGURE 7.** Echocardiographic analysis. The SO group showed less ventricular remodeling and greater degrees of improved anterior wall thickness and cardiac function than did the S group. *Pink line,* SO group; *red line,* S group; *blue line,* O group; *green line,* sham group; *LVEDV,* left ventricular end-diastolic volume; *LVESV,* left ventricular end-systolic volume; *LVEF,* left ventricular ejection fraction; other abbreviations as in Figure 2. N = 6 in each group. $*P < .05$.

Omentum flap has often been used by cardiothoracic surgeons to stimulate revascularization. Omentum also has the potential to supply fat-derived stem cells[9] and various cytokines[10,11] and to attenuate inflammation.[12] The adipocytes in omentum release a number of angiogenic growth factors, such as VEGF, suggesting that omentum flap can act as a physiologic exogenous source of multiple angiogenic factors that act synergistically to promote arteriogenesis.[16] Our results support the idea that the interposed omentum enhances the cardiac protection provided by the superimposed SMB sheets, such as by inducing angiogenesis, increasing the blood flow, and prolonging cell survival (in part by attenuating inflammation and protecting the cells from apoptosis). Other experimental studies aimed at enhancing the effects of omentopexy have been reported. Ruel and associates[16] demonstrated that a gastric submucosal patch has excellent angiogenic effects when used as an endogenous source of growth factors in a swine model of chronic myocardial ischemia. Kanamori and colleagues[17] showed that omentopexy enhances the angiogenic effect of cell therapy in a swine model of acute MI. Compared with these studies, our new method seems safer and less invasive because it does not require gastrectomy or bone marrow aspiration.

On the basis of our present results, we speculate that the myocardial functional recovery obtained with the combined method was associated with the upregulation of angiogenic cytokines (eg, VEGF, STAT3) and with increased angiogenesis and blood flow. In addition, the reduced infarct area, as determined by evaluating Masson trichrome–stained hearts, indicates that the animal model used in the present study may be one of hibernating myocardium rather than of chronic infarction.[13] Together with the paracrine effects of the implanted SMB sheet, humoral substances from the omentum might have had beneficial effects on the native cardiomyocytes and viable surrounding muscle cells, preventing global myocardial remodeling. One possible mechanism to explain our results is that the SMB sheet with omentum implantation therapy induced the release of cytokines and enhanced the development of the microvasculature (ie, microcirculation, which is particularly vulnerable to injury during ischemia), which on reperfusion rescued the hibernating myocardium, thereby enhancing the recovery of myocardial performance. A recent report mentioned a possible role of stromal cell–derived factor 1 in recruiting stem cells. Because stromal cell–derived factor 1 is secreted in muscle tissue, and muscle satellite cells express functional CXCR4 receptors, the implanted SMBs may serve

to recruit stem cells to the site of damage, where they promote heart repair.[6] Nevertheless, it remains to be determined what cytokines play a major role in generating therapeutic effects among the many complex molecular and cellular mechanisms involved.

We could find no ventricular premature beat by Holter electrocardiographic analysis after the treatment (data not shown). We[5] previously reported that rat and porcine MI models show less arrhythmia after being treated with SMB-sheet implantation than with needle injection. We speculate that the needle damages the myocardium, which may induce arrhythmia. In contrast, the SMB sheet implantation technique does not normally injure the myocardium. Moreover, we did not observe any cases of lethal ventricular arrhythmia in animals implanted with SMB sheets wrapped with omentum.

## CONCLUSIONS

A new cell-delivery method using SMB sheets combined with omentum allowed us to implant large numbers of SMBs in a porcine MI model. The implanted sheets became well-vascularized cardiac tissue, with less scarring and improved cardiac function over that of the animals receiving SMB sheets alone. This method may be applicable for repairing the severely failing heart.

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## Discussion

**Dr Vivek Rao** *(Toronto, Ontario, Canada).* I have no relevant financial disclosures.

I thank the authors for the courtesy of providing me with a copy of their manuscript well in advance of the meeting. I congratulate them as well for a very elegantly performed and executed study, and I will confirm with the audience that the results shown here are just a small tip of the iceberg of the complete results presented in the manuscript. Most important, the manuscript contains a lot of functional data that, unfortunately, owing to time constraints, Dr Shudo was unable to present.

Having said that, I have a few questions with regard to this study. In the manuscript, it was not quite clear to me whether the number of cell sheets delivered were different between the omentum group (O group) and the cell-sheets-only group (S group). You mentioned that to achieve the omentum wrapping you had a limitation of 5 sheets in the omentum-plus-cell group (SO group). Was that different from the total number of cell sheets implanted in the cell-sheets-only group (S group)?

**Dr Shudo.** Thank you for your useful comments, Dr Rao.

In this study we used 30 cell sheets total. On the basis of our results in the previous studies, the increase in the number of implanted cell sheets was related to the favorable results, such as enhancing the paracrine effect and improving cardiac function. However, these previous studies also suggested that more than 5 layers of SMB sheets had disorganized vasculature in the implanted site, maybe owing to the primary ischemia. Therefore, in this study, at most 5 layers were attached in one place and covered and wrapped with the omentum to support the angiogenesis.

**Dr Rao.** I will point to some of the LV function data that were presented in your manuscript. You measured LV function both at 4 weeks and 8 weeks after LAD ligation. What was striking to me was that in most models of LAD ligation there is continued deterioration in LV function in the control groups. In contrast, in your study, the 3 relative control groups had stabilization of their LV

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function from 4 to 8 weeks with a continued improvement in LV function in the omentum-plus-cell-transplant group (SO group). Can you explain why the LV function appeared to stabilize in your model.

**Dr Shudo.** In this study, MI was induced by first ligating the distal portion of the LAD for ischemic preconditioning, followed by placement of the ameroid constrictor around the main trunk of the LAD, just distal to the left circumflex artery branching. It has been reported that the ameroid ring was estimated to occlude inside completely about 2 weeks after being placed in vivo. As you mentioned (and as shown in one slide), LVEF seems to be unchanged 1, 2, and 3 months after placement of the ameroid ring, whereas LVEDV and LVESV indices significantly increased gradually after induction of MI. Therefore, I would like to mention that LV remodeling was successfully obtained in this model in this study.

**Dr Rao.** I have 1 final question. You showed that the cell retention was approximately double that with the addition of the omentum wrapping, 60% cell retention versus 30% with the cell sheet alone. Yet for almost all of your end points there was absolutely no benefit to cell sheet implantation alone, which is obviously in contrast to many of the studies done previously with SMBs showing a benefit in function, angiogenesis, et cetera. Can you explain why your cell sheet transplant alone had absolutely no benefit in this model.

**Dr Shudo.** That seems to be a bit confusing. We have obtained good results even in the cell-sheet-only group (S group), too. However, we would like to focus on the preferable results of the new cell-implantation method.

Thank you again for your comments and good questions.

**Dr Paul Kurlansky** *(Miami, Fla).* I have no disclosures.

This was very interesting work. I have just 2 questions. First, what exact cells or mixture of cells are you putting into the cell sheets? Second, have you looked histologically at the hearts to see if you actually have myocardial regeneration or if you just have hypertrophy and preservation of the residual border zone myocardium?

**Dr Shudo.** Thank you so much. Regarding the first question, although this is unpublished information, we performed the experimental study about SMBs mixed with adipose tissue, derived mesenchymal stem cells. This model shows priority to the SMB cell-sheet-only group (S group).

Regarding the analysis of border zone, our study using tissue Doppler echocardiography and speckle tracking echocardiography data showed that regional functional recovery was significantly greater in the infarct border region. This result may support the idea that cell-sheet-with-omentum therapy prevented progression of MI by supporting the hibernating myocardium.

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SURGERY TODAY


*Original Article*

# Clinical Impact of Combined Transplantation of Autologous Skeletal Myoblasts and Bone Marrow Mononuclear Cells in Patients with Severely Deteriorated Ischemic Cardiomyopathy

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## Abstract

***Purpose.*** Simultaneous injection of autologous bone marrow cells and skeletal myoblasts has been demonstrated to improve cardiac function in animal models. We evaluated the potential application of this combination cell therapy in patients with severe ischemic cardiomyopathy who required left ventricular assist device (LVAD) implantation.

***Methods.*** Four patients (age range, 43–69 years) who required LVAD implantation due to severe ischemic cardiomyopathy were studied. Skeletal myoblasts were obtained from the thigh, while bone marrow mononuclear cells were collected and purified at the time of the operation. These cells were directly injected in a serial manner into the damaged myocardium.

***Results.*** No fatal arrhythmias or major complications were observed. The number of injected skeletal myoblasts ranged from $2.7 \times 10^7$ to $3.0 \times 10^8$, and their purity ranged from 25% to 96%. Two patients showed decreased brain natriuretic peptide levels and echocardiographic improvements in the transplanted areas, as well as increased perfusion revealed by $H_2{}^{15}O$ positron emission tomography, of whom one was successfully weaned from LVAD. Histological findings at autopsy of the other patient showed a small amount of skeletal muscle in the injected area. Only marginal improvements were observed in the other two patients.

***Conclusions.*** Combined cell transplantation is feasible for patients with severe ischemic cardiomyopathy, and functional recovery is anticipated in selected patients.

**Key words** Cell transplantation · Device · Heart failure · Cardiomyopathy

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## Introduction

Regenerative medicine is expected to provide an alternative treatment for end-stage heart failure, by providing methods to regenerate heart muscle and recover cardiac function in affected patients. Bone marrow stem cells (BMSCs) have been used in a number of clinical trials, as they are expected to differentiate into cardiac muscle or capillary cells to regenerate cardiac tissue.[1–4] The cells seem to be useful to restore lost functionality due to acute myocardial infarction by improving ejection fraction (EF) and exercise tolerance, or reducing infarcted areas through angiogenesis.[1–4] However, no clinical studies have shown BMSCs to be useful for chronic heart failure.

On the other hand, skeletal muscle has shown a capacity for self repair, because of a resident population of proliferative muscle cells known as myoblasts.[5] Once activated, skeletal myoblasts can form new muscle to restore lost functionality. Menasché et al. were the first to use cultured skeletal myoblasts in a study of ten patients with ischemic cardiomyopathy,[6] in whom they transplanted cells by direct injection to infarcted areas. Those skeletal myoblast injections in combination with surgical treatments increased the EF by improving local myocardial thickening, and consequently, improved their New York Heart Association (NYHA) classification. However, four of their patients required treatment with an internal cardioventricular defibrillator (ICD) due to ventricular tachycardia. Although a large randomized study (MAGIC trial) performed thereafter did not find significant improvements in cardiac function or postoperative arrhythmic events, high-dose myoblast transplantation showed the capacity to improve diastolic function through paracrine effects.[7] Furthermore, Dib et al. found survival and engraftment of transplanted myoblasts and improvement of clinical cardiac data, which suggested the feasibility and safety of autologous myoblast transplantation.[8]

Our preclinical data, obtained with a canine chronic myocardial infarction model, demonstrated that a combination of autologous skeletal myoblasts and BMSCs provided better cardiac performance than did either cell type alone, because of their induction of both myogenesis and angiogenesis.[9] Ott et al. also suggested that combination cell therapy may be clinically relevant by merging the beneficial effects of each cell line, based on data obtained from a rat model.[10] The first clinical case also confirmed the relevance of combined cell therapy;[11] therefore, the objectives of the present study were to assess the safety and feasibility of combination cell therapy in a clinical setting.

All patients recruited for the present study had end-stage heart failure due to ischemic cardiomyopathy (ICM) requiring a left ventricular assist device (LVAD). This patient group was considered unlikely to recover their cardiac function by mechanical unloading.[12] Cell transplantation was the only procedure performed, and cardiac function was assessed before and after the operation.

## Patients and Methods

### *Patient Selection*

Patients were considered eligible if all of the following criteria were met: (1) age between 18 and 70 years old, (2) a history of myocardial infarction and subsequent alteration of left ventricular function, and (3) end-stage heart failure with symptoms of NYHA functional class IV with intravenous catecholamine, with or without optimal contemporary medical management, including angiotensin-converting enzyme inhibitors and β-blockers requiring an LVAD, or an LVAD already being utilized. Regardless of the indications for heart transplantation, the LVAD was implanted into the patients who met the above criteria and were suggested not to survive without LVAD implantation due to end-stage heart failure. The major exclusion criteria were peripheral muscular dystrophy, malignancy, pregnancy, alcoholism, positive serologic test results for human immunodeficiency virus, hepatitis, human T lymphotropic virus type 1, and syphilis. The study was approved by the Ethical Committee and Internal Review Board (IRB) of Osaka University, and informed consent was obtained from each participant.

### *Study Registration*

This study was registered in the UMIN Clinical Trials Registry (UMIN-CTR) and the registration number is UMIN000001859. The date of protocol fixation was April 1, 2004, and first patient was enrolled on July 5, 2004.

### *LVAD Implantation and Cell Transplantation*

Prior to cell transplantation, all patients underwent implantation of an extracorporeal pneumatic LVAD (Toyobo, Tokyo, Japan) through a median sternotomy. The interval between LVAD implantation and cell transplantation was 155 ± 86 days (range 101–284 days). Cell transplantation was performed by injection through a left thoracotomy with a 1-ml syringe that contained myoblasts or bone marrow mononuclear cells (BM-MNCs). Prior to the injection, cultured autologous myoblasts and freshly extracted autologous BM-MNCs were separately suspended in 3 ml of Dulbecco's modified Eagle's medium solution and separated into 10 1-ml syringes for each cell type. The cells were injected into the intramyocardial area with a 26-gauge needle, anterior to the lateral and posterior myocardium as far as possible. Maximum technical care was taken to avoid loss of cells.

### *Myoblast Isolation Procedure*

For each patient, a muscle biopsy specimen was obtained from the tibialis anterior and/or medial vastus muscle (weight range, 3.8–22 g), then washed in ice-cold normal saline solution. Each muscle specimen was immediately packed in a resealable container filled with preservation medium and transferred to the Cell Processing Center (Medical Center for Translational Research, Osaka University Hospital), where the myoblasts were isolated and cultured according to the method of Dib et al.[8] The cell culture was continued until an adequate number of cells (>300 × $10^6$) was obtained. Purity was confirmed, and cell counting was performed using a fluorescence-activated cell sorter (FACS) analysis with a CD56 antibody (BD, Rockville, MD, USA). Once the target number of cells was reached, the cultured cells were frozen in liquid nitrogen until transplantation. The interval between myoblast harvesting and cell transplantation was 69 ± 45 days (range 41–136 days). These cell processing procedures were safely performed following the ordinance of Ministry of Health, Labour and Welfare of Japan regarding good manufacturing practices, even though it was not mandatory.

### *Extraction of BM-MNCs*

A total of 200–300 million bone marrow (BM) cells were aspirated from the ileum with a BM harvest needle just before cell transplantation. Bone marrow-MNCs were then sorted using a CS3000-Plus blood-cell separator (Baxter, Deerfield, IL, USA) according to the method reported by Tateishi-Yuyama et al.[13] Bone marrow aspiration was performed until more than 100 × $10^6$ cells were obtained.

*RhythmM onitoring*

All patients were hospitalized during this study and monitored by 24-h telemetry, and Holter electrocardiograms (ECG) were obtained at 1, 2, 3, and 6 months postoperatively.

*Assessment of Cardiac Function*

Cardiac function was assessed by measuring the serum brain natriuretic peptide (BNP) levels, while the left ventricular ejection fraction (LVEF) was determined by two-dimensional echocardiography. While the patient was supported by LVAD, LVEF was measured using the LVAD off-study technique.[12]

*Color Kinesis*

Diastolic color kinesis images performed 1 month after cell transplantation were obtained from the left ventricle in the mid-papillary short-axis view. The timing of color encoding was set to begin at the first frame, in which outward endocardial motion was noted, and its duration was set to the maximum value (26 frames). The regional fraction area change was considered to be the regional incremental fraction area that changed during diastole in regard of the percentage of regional end-diastolic area in the short-axis view. The color kinesis diastolic index (CK-DI) was defined as the left ventricular (LV) segmental filling fraction during the first 30% of the diastolic filling period, and was used to assess regional LV active relaxation.[14]

*Histopathology*

Histopathology samples were obtained from the LV apex at the time of LVAD implantation or from an anterolateral lesion at the site of transplantation. Standard histological examinations were performed with hematoxylin–eosin staining, with Masson's trichrome staining used to visualize fibrosis. Immunostaining of the fast-isoform myosin heavy chain (Sigma, St. Louis, MO, USA) was performed to reveal transplanted myoblasts at the site of transplantation. Immunohistochemical staining using factor VIII-related antigen (DAKO EPOS Anti-Human Von Willebrand Factor/HRP; DAKO, Glostrup, Denmark) was performed to visualize vascular density.

## Results

*Patients*

Four patients met the criteria for enrollment in this study, and underwent LVAD implantation and cell transplantation. Patient demographics are shown in Table 1. Their age ranged from 43 to 69 years old. The interval from myocardial infarction and cell transplantation ranged from 4 to 9 months. All patients had end-stage heart failure with symptoms of NYHA functional class IV and required Toyobo extracorporeal LVAD implantation prior to cell transplantation, and none of them showed functional recovery between the LVAD implantation and cell transplantation. The two younger patients were considered to be eligible for heart transplantation, but the other two were not, due to their advanced age.

*Cell Culture*

There were no serious complications related to the muscle biopsy procedures and no problems encountered during cell culturing at the Cell Processing Center. In addition, there was no bacterial or fungal contamination, as determined by US Pharmacopeia sterility, mycoplasma testing, and by examining the levels of endotoxin in the culture media. Cell cultures were terminated when the cell count reached more than $300 \times 10^6$ cells. The culture periods ranged from 20 to 23 days. The viability of cells ranged from 93% to 98% at the time of cell transplantation. However, the purity was different in each patient, thus the actual numbers of surviving myoblasts injected were $138 \times 10^6$ and $248 \times 10^6$ in the two younger patients (patients 1 and 2), respectively, and $77 \times 10^6$ and $58 \times 10^6$ in the two older patients (patients 3 and 4), respectively. On the other hand, an adequate number (more than $200 \times 10^6$ cells) of BM-MNCs was obtained from each patient.

*Adverse Events*

No deaths or arrhythmias occurred during the cell injections, and minimal bleeding was observed from the injection sites. All patients were in stable condition postoperatively with LVAD support. There was no sustained ventricular tachycardia (VT) or ventricular fibrillation (Vf) recorded postoperatively by 24-h electrocardiogram telemetry. The Holter ECG revealed that only $141 \pm 177$ ventricular premature beats (VPBs)/day were recorded and $1.8 \pm 2.5$ couplets/day were recorded. No VTs or Vfs were detected. Although the LVAD was successfully removed from patient 1 at 360 days after cell transplantation, he died due to recurrence of heart failure after refusing reintroduction of LVAD at 351 days after LVAD removal (Table 2). In addition, another patient died due to sepsis at 346 days after cell transplantation, and two from cancer at 211 days and 379 days after cell transplantation, respectively. There were no major transplantation-related adverse events.

**Table 1.** Patients' demographics

| Patient | Age (years) | Myocardial infarction: Age (months) | Myocardial infarction: Location | NYHA Class | Mechanical support | Pre-Tx EF (%) | Culture period (days) | Cell transplantation: No. of cells injected ($\times 10^6$) | Cell transplantation: Viability of cells (%) | Cell transplantation: Purity of myoblasts (%) | Cell transplantation: No. of myoblasts injected ($\times 10^6$) | Cell transplantation: No. of bone marrow mononuclear cells ($\times 10^6$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 53 | 7 | LMT | IV | LVAD | 22 | 22 | 300 | 98 | 47 | 138 | 200 |
| 2 | 43 | 4 | LMT | IV | LVAD | 17 | 20 | 300 | 93 | 89 | 248 | 300 |
| 3 | 69 | 5 | LMT | IV | LVAD | 10 | 23 | 300 | 95 | 27 | 77 | 240 |
| 4 | 67 | 9 | LMT | IV | LVAD | 37 | 20 | 300 | 96 | 20 | 58 | 250 |

LMT, left main trunk; NYHA, New York Heart Association; LVAD, left ventricular assist device; Tx, transplantation

*Functional Assessment*

Left ventricular ejection fraction was improved in three patients, and the patients who received a larger number of cultured myoblasts (patients 1 and 2) showed more than 10% improvement (Fig. 1). These improvements were detected at the 1-month follow-up examination and were amplified at 6 months after the procedure in patient 2 and at 1 year in patient 1, who showed 21% improvement at the 1-year follow-up examination, which led us to wean him off the LVAD. Improvement in EF was marginal in patient 3, while patient 4 showed no improvement in the LVEF.

Three patients (1, 2, and 3) underwent color kinesis analysis. The mean CK-DI was improved from 33% to 53% in patient 1 and from 23% to 41% in patient 2 (Fig. 2). In detail, the regional improvements in CK-DI were remarkable in the lateral, anterior, and posterior regions where cell transplantation was performed, while other areas also showed improvement in regional LV active relaxation in those patients. On the other hand, patient 3 did not show any improvement of diastolic function, even in the transplanted area. Patients 1 and 2 also showed remarkable decreases in BNP levels after the operation (Fig. 3), whereas patients 3 and 4 did not. When data for LVEF, diastolic function, and BNP level were combined, patients 1 and 2 showed improvement in cardiac function after cell transplantation, whereas patients 3 and 4 did not.

*Histological Analysis*

Post-transplantation biopsies were performed in 3 patients, at the time of LVAD removal in patient 1, and at autopsy in patients 2 and 4, while the family of patient 3 refused the autopsy procedure. Immunostaining of the fast-isoform myosin heavy chain detected a very small number of myoblasts in patient 2 (Fig. 4A), and the positive control confirmed that the stained cells were of skeletal muscle origin (Fig. 4B). No myoblasts were detected in patients 1 and 4. In patient 2, factor VIII immunostaining showed that the microvasculature network was increased following the operation (Fig. 5A) as compared to before transplantation (Fig. 5B). The preoperative biopsy specimen obtained from patient 1 showed global infarction and tissue damage (Fig. 5C), while that obtained postoperatively from the transplanted area at the time of LVAD removal showed well-settled muscle cells (Fig. 5D). On the other hand, the preoperative biopsy specimen obtained from patient 4 showed severe ischemic changes and a necrotic myocardium (Fig. 5E), and that obtained postoperatively at autopsy showed scarring and very few surviving cells (Fig. 5F). Therefore, postoperative biopsy results supported other evidence showing that patient 1 had recovered cardiac function, while patient 4 did not.

**Table 2.** Patients' outcomes after transplantation

| Patient | Sustained VT or Vf | LVAD removal | Days after transplantation | Current status | Survival (days) | Cause of death |
|---|---|---|---|---|---|---|
| 1 | No | Yes | 360 | Deceased | 711 | Heart failure |
| 2 | No | No | — | Deceased | 345 | Sepsis |
| 3 | No | No | — | Deceased | 221 | Pharyngeal cancer |
| 4 | No | No | — | Deceased | 360 | Bladder cancer |

VT, ventricular tachycardia; Vf, ventricular fibrillation; LVAD, left ventricular assist device

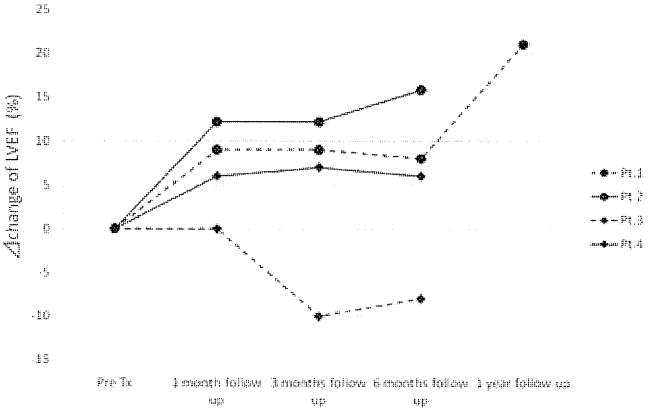


**Fig. 1.** Changes in baseline left ventricular ejection fraction (*LVEF*) at 1 month, 3 months, 6 months, and 1 year after the procedure. Ejection fractions were obtained at the time of left ventricular assist device (LVAD) implantation. *Pt*, patient; *Pre Tx*, before transplantation

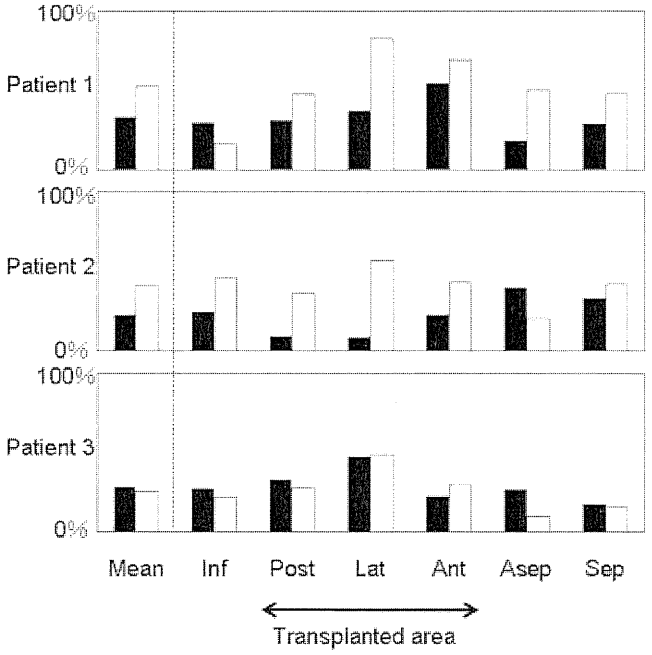


**Fig. 2.** Changes in diastolic color kinesis index (CK-DI) between the preoperative baseline (*black bars*) and the postoperative follow-up (*white bars*) examinations. CK-DI expresses the expansion speed of the left ventricle during the diastolic phase, which implies LV diastolic function. The left ventricle was divided into six segments as defined in the American Society of Echocardiography guidelines. Cell transplantation was performed lateral to the posterior and anterior regions

### *Study Limitations*

Only four patients were enrolled in this study, therefore the data were insufficient to evaluate their statistical significance. Second, analysis of the effects of cell transplantation alone was difficult, because the mechanical unloading effect of LVAD may have been significant.

## Discussion

This study is the human trial to show the possibility of combination therapy with autologous bone marrow cells and cultured myoblasts. Bone marrow stem cells are reported to have a high capacity for angiogenesis,[15] as well as the potential to be mobilized and differentiate into cardiomyocytes and induce new capillary formation and proliferation of the preexisting vasculature network.[16] Although Orlic et al. suggested that c-kit positive cells could generate de novo myocardium,[17] Balsam et al. and Murry et al. reported different conclusions.[18,19] On the other hand, experiments with skeletal myoblasts have shown that they can differentiate into myotubes and improve postinfarction cardiac function.[20,21] Thus, is reasonable to consider that implanta-

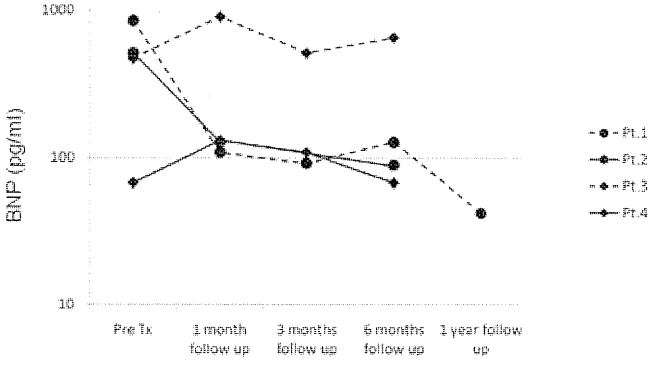


**Fig. 3.** Changes in baseline brain natriuretic peptide (*BNP*) levels at 1 month, 3 months, 6 months, and 1 year after the procedure

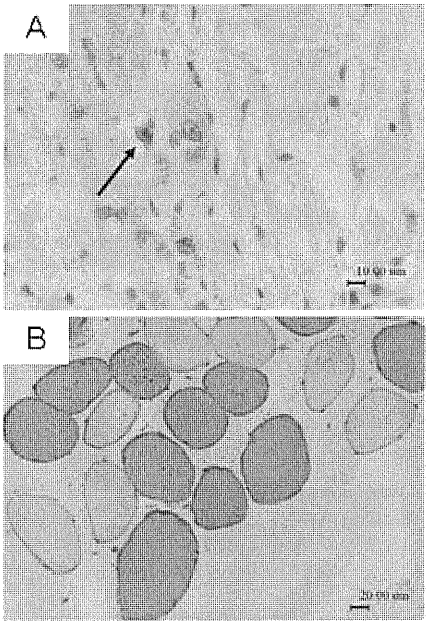


**Fig. 4.** **A** Biopsy specimen obtained from patient 2 from the transplanted area at the time of autopsy (fast-isoform myosin heavy chain staining). **B** Skeletal muscle tissue was used as a positive control

tion of contractile cells into areas of postinfarction scarring could functionally rejuvenate those areas.

In the present study, two of the patients had EF improvement greater than 10%, as well as improved diastolic function in both transplanted and nontransplanted areas, and decreased BNP levels after cell transplantation. Patient 2 showed a slightly increased vasculature network, although very few myoblasts were noted (Fig. 5A and B), as described in our previous case report,[11] while patient 1 did not show any engrafted myoblasts postoperatively. Therefore, as experimental studies have shown that a very low number of cells (in the range of 1%) are engrafted within a few weeks after transplantation, paracrine effects, rather than myogenesis, are considered to be the crucial mechanisms involved in cell therapy, in which transplanted cells may trigger an endogenous pathway to activate and/or protect existing cardiomyocytes by angiogenesis and cytokine delivery.[22] Although these effects may be conferred by BMSCs solely, additional beneficial effects of skeletal myoblasts were also expected based on animal experiments.[9] Moreover, the improvement in global diastolic function in the present patients may also be explained by paracrine effects, as Farahmand et al.

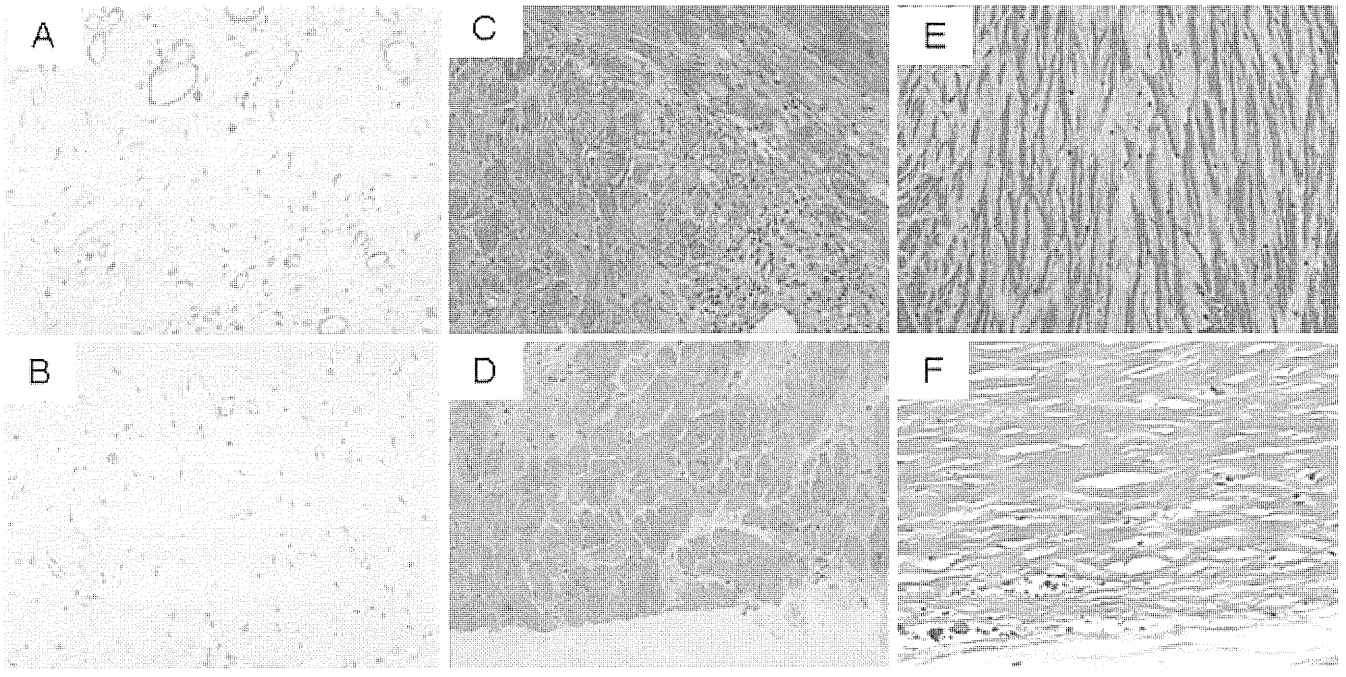


**Fig. 5A–F.** Histological results. **A** Biopsy specimen obtained from the transplanted area in patient 2 at the time of autopsy showing rich vasculature. **B** Poor vasculature in a tissue specimen from the LV apical core removed at the time of LVAD implantation. Both specimens **A** and **B** were stained using a Factor VIII antibody. **C** Surgical specimen obtained from the LV lateral wall in patient 1 at the time of LVAD implantation showing acute myocardial infarction. **D** Biopsy specimen obtained from the same site as **C** at the time of LVAD removal, showing well-settled muscle tissues. **E** Surgical specimen obtained from the LV apex of patient 4 at the time of LVAD implantation, showing few surviving cells as compared to the biopsy specimen from the same site obtained at autopsy from patient 1 (shown in **D**). The specimens shown in **C**, **D**, **E**, and **F** were stained with hematoxylin–eosin using a customary technique