unpaired t-test. Statistical significance was determined as having a p-value less than 0.05.

Results

Characteristics of myoblast sheet

We obtained mono-layered myoblast sheets by lowering the temperature, which released them from the Poly(*N*-isopropylacrylamide)-grafted polystyrene. It's size is about 3cm×2cm square (Fig. 1*a*). HE staining demonstrated that SC sheet contained a lot of SCs and SC sheets had an appearance of homogenous tissue、which thickness of one SC sheet was about 100μm (Fig. 1*b*). Some Smooth muscle cells are detected in the SC sheets, but those cells are not majority (Fig. 1 *c*)

Histological assessment

HE staining demonstrated that transplanted skeletal cell sheets were attached in the epicardium (Fig.1d) and oval-shaped cell that showed positive for Eosin in cytoplasm were detected in the SC group microscopically in some layers over epicardium (Fig. 1e). Elastica Masson

Goldner showed that oval-shaped cells that supposed to origin from skeletal tissue exist in the transplantation site (Fig. 1f). These cells were not seen in the control group. And the SC group demonstrated decrease in the cross-sectional LV area compared with the C groups (Fig. 2a). Masson Trichrome staining showed that clustered skeletal cells were detected in the center of the scar, while clustered skeletal cells were not detected in the C group (Fig. 2a,b). Many clusters of well developed smooth muscle cells exist in the center of the whole scar in the SC group, while in the C group smooth muscle cells which formed vasculature exist in the scar (Fig 2c,d,e,f). Although slow type myosin positive cells exist only on the endocardium and epicardium, those cells were not detected in the center of scar (Fig.2 g,h). So these figures depict that the skeletal muscle cells which exist in the center of the scar is not residual myocyte after infarction.

Quantificatin of histopathology

In the SC group, vascular density was found to be significantly higher than in the C groups (SC vs C= 217.1±30.2 vs 114.2±18.2 /field; $P < 0.05$) (Fig. 3b).

Picoro-sirius red staining demonstrated that % fibrosis was significantly

reduced in the SC group compared with the C group (SC vs C= 1.6±0.2 vs 3.1±0.3%; $P < 0.05$) (Fig. 3b). PAS staining showed that cell diameter was significantly shorter in the SC group than the C group (SC vs C= 10.7±0.3 vs 18.3±1.4 μ m ; $P < 0.05$) (Fig. 3b).

These histological findings were universally identified in the native myocardial tissue without distinction of distance from the grafted region.

Functional assessment of the Infarcted Myocardium

The Fractional area shortening (FAS) and left ventricle end- systolic area (ESA) scores at baseline were not significantly different between the two groups.

Three months after the implantation, 2D echocardiography showed significant improvement of the FAS (Fig. 4a) in the SC group compared with the C group (SC vs C=49.5±2.8 vs 24.6±2.0%, $P<0.05$). These functional improvements were preserved 6 months after implantation (SC vs C=50.8±6.4 vs 25.3±2.8%, $P<0.05$). The ESA was significantly smaller in the SC group than in the C group 3 months after the implantation (SC vs C=4.3±0.5 vs 9±1.3cm^2, $P<0.05$) (Fig. 4b). These attenuation of LV dilatation were preserved 6 months after implantation (SC vs C=4.9±0.8 vs

7.5±1.4cm^2, P<0.05). During this long-term observation, all SC sheet-treated animals were alive and exhibited no malignant arrhythmia assessed by 24-hour Holter ECG once a week (data not shown).

Before treatment, diastolic dysfunction was observed in the infarction area of myocardium and the regional delayed relaxation was detected in the remote site of infarction by Color Kinesis. After 3 months after implantation, CK-diastolic index in the lateral (SC vs C=61.7±6.4 vs 43.7±4.8 %, P<0.05), anterior (SC vs C=57.4±8.6 vs 30.2±4.7 %, P<0.05), and antero-septal (SC vs C=59±6.6 vs 38.4±6.6%, P<0.05) segment were significantly ameliorated in the SC group compared with the C group, and regional systolic function in transplanted site were significantly improved in the SC group while not in the C groups (SC vs C: lateral, 59.8±3.3 vs 43.6±5.4%, P<0.05; anterior, 58.5±4.5 vs 35.4±6.6%, P<0.05; antero-septal, 59.8±3.3 vs 43.6±5.4%, P<0.05) respectively (Fig. 5).

We could detect no ventricular premature beat for 24 hours by the Holter ECG in 3 myocardial infarction porcines received skeletal cell sheets.

Regional Myocardial Blood Flow and residual myocardial tissue

PET study by using ^{15}0-water showed that the myocardial water-perfusable

tissue fraction (PTF) and myocardial blood flow (MBF) were higher in the anterior wall where skeletal cell sheets were implanted compared with the myocardium receiving no sheets. This data depicts that MBF was better and microcirculation in the infacted myocardium was preserved in the skeletal cell sheets implanted myocardium. PET study by using ^{18}F-FDG revealed that more viable myocardial tissues were preserved in the skeletal sheet implanted myocardium compared with the myocardium receiving no sheets. Coronary angiography revealed that LAD was completely occluded by the ameroid constrictor in both cases (Fig.6).

Discussion

Over the past several years, increasing awareness of the shortcomings of heart transplantation and left ventricular assist system implantation has led cardiovascular surgeons to consider alternative means of treating end-stage heart failure. In clinical setting, cellular cardiomyoplasty has been reported to have the potential of fundamental regenerative capability and has already been introduced in clinical trials with skeletal myoblast [11] or bone

marrow mononuclear cells (BM-MNCs) [12], and results suggest that it is a relatively feasible and safety therapy as a therapeutic angiogenesis. In this setting, cardiac tissue implantation was proposed to the treatment of end staged heart failure as a new concept of regenerative therapy and experimentally some groups depicted it's effectiveness in the damaged myocardium [13, 14]. We also reported that cell sheets have great impacts on restoration of damaged myocardium in the rat infarction model [3,4] and dilated cardiomyopathy hamster [5]. In order to convince the effectiveness of cell sheets in pre clinical trial, we examined whether autologous skeletal cell sheets implantation might become one of the armamentarium of regenerative therapy for chronic heart failure caused by myocardial infarction in the porcine model.

The potential added advantages of the cell sheet implantation method include the implantation of a high number of cells with minimum cell loss. In contrast, the injection method is associated with a high loss of cells or surface proteins due to the trypsin treatment. In spite of a high number of cell loss in needle injection, the cell sheet implantation method might provide the advantages of a higher number of cell implantation without

cellular community destruction, leading the more improvement of cardiac performance rather than cell injection method [4]. In case of needle injection, inflammation accompanied with destruction of myocardium induced by needle injection promotes graft death after cell transplantation [15].

To examine the effects of the skeletal cell sheet implantation therapy, we analyzed cardiac function and performed a histological assessment of the infarcted heart after skeletal cell sheet transplantation in a swine infarction model. Skeletal cell sheet implantation therapy significantly induced angiogenesis, reduction of fibrosis histologically. And cell diameter of host myocyte was significantly attenuated its hypertrophy compared with the no treatment group. PET study revealed the better regional blood perfusion and better regional myocardial viability in the myocardium receiving cell sheets compared with the myocardium receiving no sheets.

Moreover, skeletal cell sheet implantation induced functional recovery of damaged myocardium. Especially, we demonstrated the regional diastolic and systolic dysfunction was well recovered in the sheet implanted group. Before treatment, diastolic dysfunction of infarcted area and regional

delayed relaxation of non-infarcted site were detected by Color Kinesis in the porcine infracted myocardium. After treatment, diastolic dysfunction of infarcted site was significantly recovered and the phenomenon of regional delayed relaxation in non-infarcted site was not seen. Presumably, implanted elastic myoblast sheets and a large quantity of well-developed smooth muscle cells, which are detected in the center of the scar, improved the regional diastolic dysfunction of implanted site. Although skeletal cell sheet can not contract in vivo after implantation, this recovery of diastolic disassociation of LV might result in the recovery of systolic dysfunction.

To the best of our knowledge, this is the first report in which tissue-engineered skeletal cell sheets implantation was successfully used to improve cardiac performance in a large animal model of ischemic myocardium according to the Raplase's theory.

The mechanisms of the restoration of damaged myocardium by skeletal cell sheet implantation might be quite complicated and many pathways might affect the recovery of ischemic myocardium. Recent reports depicts that cell sheets enhance the recruitment of hematopoietic stem cells through the release of stromal-derived factor 1 [4]. The fact of thicker anterior wall

and the improvement of regional function might depend on both the recruitment of cytokine releasing stem cells, survival of grafted cells, and well-developed smooth muscle cells. And these cells might have good elasticity and these elastic cells and tissues softened the stiffness of anterior wall in association with the attenuating fibrosis even in the infarct area. This reduced stiffness of anterior wall might lead to the improvement of the diastolic dysfunction. Transplanted skeletal cells cannot differentiate into cardiomyocyte anymore, but regional systolic function improved in the transplanted site. Probably the improvement of regional diastolic function due to elastic cells might be responsible for the restoration of regional systolic dysfunction. Recent reports demonstrated that regional left ventricular myocardial relaxation was closely related to regional myocardial contraction [16] and the improvement of regional myocardial relaxation leads to the recovery of global diastolic function [17]. Moreover the improvement of regional systolic function is closely related global systolic function [18]. We assume that this theory about the relationship between diastolic and systolic function is one of the mechanisms about the improvement of diastolic and systolic function in the cell sheet transplanted

myocardium.

Question is why the well-developed smooth muscle cells exist in the center of the scar in the SC sheet group after transplantation in spite of a small quantity of smooth muscle cells in the SC sheet? Does a small quantity of smooth muscle cells in the SC sheet proliferate after transplantation? Do progenitor cells in the SC sheet differentiate to smooth muscle cells? Do progenitor cells or smooth muscle cells in the host myocardium migrate to the implanted site and proliferate? To the regret, there is no data to answer these questions exactly in this paper and more detailed studies are needed to elucidate this important question.

Some reports depicted that the expression of Hepatocyte growth factor (HGF) in the myoblast sheet transplanted ischemic myocardium is quite higher compared with the non transplanted ischemic myocardium [4]. HGF has an antifibrotic activity both through the activation of a matrix degradation pathway [19], restoration of cytoskeletal proteins on cardiomyocyte [20], and induce angiogenesis in the ischemic myocardium [21]. Our study demonstrated that % fibrosis was significantly reduced in the skeletal cell sheet transplanted group. This paracrine secretion of HGF

from skeletal cell sheets might attribute the reduction of %fibrosis. In our study, much more Factor VIII positive cells are detected in the skeletal cell sheet transplanted myocardium. This might be induced by paracrine secretion of HGF and angiogenesis might rescue the ischemic host cardiomyocyte and bring about the improvement of the distressed function of host cardiomyocyte. The distressed cytoskeletal proteins on the cardiomyocyte in the ischemic myocardium might be re-organized by the HGF secreted form skeletal sheet and the restoration of cytoskeletal proteins might lead to the improvement of cardiac function. And some reports demonstrated that myoblast sheets maintain the distressed cytoskeletal proteins on the host cardiomyocyte in the DCM hamster model [5]. Consequently, cell sheet treatment is appropriate for recovery of ischemic cardiomyopathy. Recent research works demonstrated that several regenerative factors such as IGF-1 [22] and Thymisine b4 [23] were highly expressed in the rat ischemic myocardium model after myoblast sheet implantation by RT-PCR analysis (data not shown). After myoblast sheet transplantation to ischemic myocardium, several regenerative factors are highly expressed in the transplanted site, and these long term and low

dosed expressed regenerative factors might cooperatively restore the damaged myocardium.

We could no ventricular premature beat analyzed by Holter ECG after skeletal cell sheet implantation. We have already proved that in the rat infarction model arrhythmia is less in the skeletal cell sheet implantation group compared with the needle injection group and this work represented that more MCP-1 postive cells and CD11b (Macrophage marker) positive cells were detected in the needle injection group compared with skeletal cell sheet implantation (data not shown). We speculate that needles destroy the myocardium and this destroyed myocardium may induce the inflammation and this inflammation may induce the arrhythmia. On the other hand, skeletal cell sheet implantation technique normally doses not destroy the myocardium when they are implanted to recipient heart. Moreover skeletal cell sheet will survive on the epicardium and electrical wave originated from implanted myoblasts may not deliver to the recipient myocardium directly. But when we implant myobalsts by needle injection, implanted myoblasts survive in the center of the myocardium and electrical wave will deliver to the myocardium directly, leading to the arrhythmia.

In conclusion, we have preclinically demonstrated skeletal cell sheets produced histologically and functionally apparent prevented the deterioration of the impaired myocardium in the swine model. These data provide a basis for attempting clinical cell-sheet implantation in ischemic disease as the armamentarium to promote the regeneration of chronic heart failure caused by myocardial infarction.

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Figure legends

Figure 1. Histological characteristics of skeletal cell sheet

a. Skeletal cell sheet detached from the Poly(*N*-isopropylacrylamide)-grafted polystyrene by lowering the temperature. It's size is about 3cm×2cm square

b. HE stain; Cross-sectional views of skeletal cell sheet in vitro. Skeletal cell sheet demonstrates homogeneous heart like tissue.

c. Not so many smooth muscle cells were detected in the skeletal cell sheets. The arrow indicate the smooth muscle cells in the SC sheet.

d. HE stain revealed that skeletal cell sheets attached on the surface of epicardium. [indicates implanted skeletal cell sheets.

e. Oval-shaped cells that showed positive for Eosin in cytoplasm were detected in the SC group microscopically in some layers over epicardium.

f. Elastica Masson Goldner showed that oval-shaped cells that supposed to origin from skeletal tissue exist in the transplantation site. Arrows indicate oval-shaped cells that suppose to be originated form skeletal tissue.

Figure 2. The detection of a large quantity of skeletal cells in the center of the scar

a,b Masson trichrome staining reveals that some layered muscles are detected in the center of the scar in the SC sheet transplantation group, while not in the control.

c,d,e,f Smooth muscle actin staining demonstrated that well developed smooth muscle cells occupied in the center of the scar in the SC sheet transplantation group, while only smooth muscle cells which are formed vasculature are detected in the control.

g,h Slow type myosin staining showed that no positive cells exist in the center of the scar. This means that skeletal cells which are detected in the center of the scar are not the residual myocyte after infarction.

Figure 3. Macroscopic images of impaired myocardium receiving skeletal cell sheets and Histological evaluation

a. In the SC group, the anterior wall has recovered compared with the C group. In the SC group, the short axis area of the LV is small compared with the C groups. In contrast, the C group shows a dilated LV and the anterior wall is thinner than in the SC groups.

b. Histological evaluation

Vascular density

The SC group showed a significant improvement in vascular density as assessed by immunostaining for the Factor VIII -related antigen. *: $p < 0.05$ vs C.

The ratio of fibrosis-occupied area (% fibrosis) at a site remote from the infarcted heart region

Picoro-sirius red staining demonstrated that % fibrosis at a site remote from the infarcted heart region was significantly reduced in the SC group compared with the C group. *: $p < 0.05$ vs C.

The diameter of cardiomyocyte

The diameter of cardiomyocyte is significantly shorter in the SC group than the C group. *: $p < 0.05$ vs C.

Figure 4. Global functional effects of infarcted myocardium receiving the implant

Global systolic function assessed by the FAS (Fig.4a) was significantly improved in the SC group 3 months after transplantation, and these functional improvements were preserved 6 months after skeletal cell sheet