Pulsatile Myocardial Tubes Fabricated With Cell Sheet Engineering

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Background—Tissue engineering approaches involving the direct transplantation of cardiac patches have received significant attention as alternative methods for the treatment of damaged hearts. In contrast, we used cardiomyocyte sheets harvested from temperature-responsive culture dishes to create pulsatile myocardial tubes and examined their in vivo function and survival.

Methods and Results—Neonatal rat cardiomyocyte sheets were sequentially wrapped around a resected adult rat thoracic aorta and transplanted in place of the abdominal aorta of athymic rats (n=17). Four weeks after transplantation, the myocardial tubes demonstrated spontaneous and synchronous pulsations independent of the host heartbeat. Independent graft pressures with a magnitude of 5.9±1.7 mm Hg due to their independent pulsations were also observed (n=4). Additionally, histological examination and transmission electron microscopy indicated that the beating tubes were composed of cardiac tissues that resemble the native heart. Finally, when myocardial tubes used for aortic replacement were compared with grafts implanted in the abdominal cavity (n=7), we observed significantly increased tissue thickness, as well as expression of brain natriuretic peptide, myosin heavy chain-α, and myosin heavy chain-β.

Conclusions—Functional myocardial tubes that have the potential for circulatory support can be created with cell sheet engineering. These results also suggest that pulsation due to host blood flow within the lumen of the myocardial tubes has a profound effect on stimulating cardiomyocyte hypertrophy and growth. These results demonstrate a novel approach for the future development of engineered cardiac tissues with the ability for independent cardiac assistance.

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Key Words: cardiac tube ■ cell sheet ■ transplantation

In cases of severe heart failure, various surgical methods, including cardiac transplantation, are currently accepted approaches for circulatory replacement or support. More recently, the direct injection of autologous bone marrow cells1 or skeletal myoblasts2 via the pericardium, endocardium, or coronary artery has also entered clinical trials, generating significant interest for the area of cardiac regeneration. Additionally, various tissue engineering approaches, such as the use of cardiomyocytes seeded into various polymer scaffolds3,4 or set and molded with liquid collagen,5 have been attempted previously, with improved cardiac function demonstrated after graft transplantation to infarcted hearts in animal models.3,6,7 In terms of cell sourcing, with many researchers focusing on the differentiation and expansion of bone marrow cells,8 embryonic stem cells,9 and cardiac stem cells10,11 into cardiomyocytes, novel alternatives will likely become available soon for the creation of these cardiac grafts.

For myocardial tissue reconstruction, we have developed an alternative method using neonatal rat cardiomyocyte sheets harvested from temperature-responsive culture dishes. With the layering of individual cardiomyocyte sheets, 3-dimensional synchronously beating tissues composed of only living cells and their own deposited extracellular matrix could be created and transplanted directly to subcutaneous tissues. On opening of the implant sites, vigorously pulsating tissues, with developed microvessels within the cardiac grafts, were observed.12 Additionally, when these layered cardiomyocyte sheet grafts were transplanted onto damaged hearts, they showed morphological communication with the host hearts via functional gap junctions, as well as significantly improved cardiac function, demonstrating their potential for myocardial tissue repair.13,14

In addition to the application of cardiac patches directly transplanted to host hearts, the next challenge for myocardial tissue regeneration is the creation of functional organlike structures with the ability to act as independent cardiac assist devices.15,16 In the present study we show the construction of pulsatile myocardial tubes using cell sheet engineering, with the potential for in vivo circulatory support.
Methods

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

All animal experiments were performed according to the Guidelines of Tokyo Women's Medical University on Animal Use, 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 Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

Preparation of Cardiomyocyte Sheets

Cardiomyocytes were isolated from the ventricles of 1-day-old Wistar rats (Charles River Japan, Tokyo, Japan) and cultured according to previously described procedures. Isolated cardiomyocyte suspensions were seeded at a density of \(4.8 \times 10^6\) cells per dish on 24-by-24-mm\(^2\) square-patterned temperature-responsive culture surfaces (CellSeed, Inc, Tokyo, Japan) and cultured at 37°C in a humidified atmosphere of 5% CO\(_2\).

Fabrication and Transplantation of Myocardial Tubes

After 4 days in culture, cardiomyocytes were transferred to another incubator set at 20°C for 1 hour, to release the cultured cells as intact sheets (Figure 1A). After detachment, a cardiomyocyte sheet was wrapped around a 1.5-cm portion of a resected thoracic aorta from an adult Wistar rat (8 weeks old, male, Charles River Japan), with surgical forceps (Figure 1B). The constructs were then incubated at 37°C for 30 minutes to allow for the cell sheet to adhere to the adventitia surface. Identical procedures were performed sequentially over the same region to create 6-layer constructs (Figure 1C).

Myocardial tubes were transplanted by microsurgery by the cuff method. Male F344 nude rats (8 weeks old, male, Charles River Japan) were anesthetized with the use of 4% inhaled isoflurane. After the abdomen was fully opened, a 1-cm portion of the host abdominal aorta was clamped just beneath the renal artery and above the site of bifurcation. The clamped portion was then resected with surgical scissors, and the ends of the aorta were passed through two 18-gauge cuffs, with the sleeves everted and secured by circumferential 6-0 silk sutures. Next, the edges of the engineered myocardial tube were gently slipped over the cuff and anastomosed to the host aorta with the use of circumferential 6-0 silk sutures. Blood flow was finally restored by clamp removal (n=17; Figure 1E).

For abdominal cavity implantation, animals were anesthetized as described above, and the abdominal cavity was opened. The engineered myocardial tubes were then inserted gently beneath the bowel and the overlying muscle layers. The skin incisions were then closed with 5-0 nylon sutures (n=7).

Four weeks after surgery, the replacement site was reopened, and 2 microelectrodes (100 \(\mu\)m in diameter; Unique Medical, Tokyo, Japan) were positioned over opposite ends of the myocardial tube, with a reference electrode placed on the abdominal skin. Skin surface electrograms were detected with round plate electrodes (11 mm in diameter; Nihon Kohden, Tokyo, Japan). To detect host ECGs, 3 electrodes were attached to the right upper chest, left subcostal, and right femoral regions. Electric potentials were amplified by bioelectric amplifiers (UA102, Unique Medical) and recorded by a data acquisition system (ML870 PowerLab 8/30, ADInstruments, New South Wales, Australia; n=4). For electric stimulation, a bipolar electrode (TF200–029, Unique Medical) was set at the edge of the myocardial tube, and monophasic pacing pulses (2 Hz, 5 ms, 3 to 5 V) were applied by an electric stimulator (UPS-801, Unique Medical).

Determination of Electric Potentials

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Graft Pressure Measurements

Four weeks after aortic replacement, a microtip catheter transducer (SPR-320, Millar Instruments, Houston, Tex) was inserted via the left femoral artery and passed to the base of the myocardial tube. Aortic pressure of the transplanted grafts was measured and recorded with the use of the same data acquisition system as described above (n=4).

Histological and Immunohistochemical Analyses

Resected tissues were fixed in 4% paraformaldehyde (Wako Pure Chemicals, Osaka, Japan) and routinely processed into 10-μm-thick paraffin-embedded sections. Hematoxylin and eosin and Azan staining were performed by conventional methods. For immunohistochemistry, deparaffinized sections were incubated with either a 1/100 dilution of anti–troponin T (Laboratory Vision, Fremont, Calif) or a 1/100 dilution of anti–connexin 43 (Chemicon International, Temecula, Calif) antibodies overnight at 4°C and with a 1/200 dilution of Alexa-Fluor-488–conjugated secondary antibodies (Invitrogen, Carlsbad, Calif) for 1 hour at room temperature. Sections were finally visualized by confocal microscopy (TCS-SP, Leica Microsystems AG, Wetzlar, Germany). For the detection of CD31 and von Willebrand factor, sections were incubated with either a 1/100 dilution of anti-CD31 (Research Diagnostics Inc, Flanders, NJ) or a 1/100 dilution of anti–von Willebrand factor (Dako Cytomation, Glostrup, Denmark) antibody, enzyme stained with the use of the LSAB2 kit (Dako) according to the manufacturer’s suggested protocol and visualized by light microscopy (ECLIPSE TE2000-U, Nikon, Tokyo, Japan). The same concentrations of corresponding nonspecific immunoglobulins were used as negative controls.

Transmission Electron Microscopy

Resected myocardial tubes (n=2) were fixed with 2% glutaraldehyde (Wako), washed 3 times with 0.1 cacodylic acid (Wako), and postfixed with 2% osmium tetroxide (Wako). Samples were dehydrated through a graded series of ethanol and embedded with the use of the TAAB EPON812 kit (TAAB Laboratories Equipment, Bearks, England). Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate (Wako) and examined at 80 kV by transmission electron microscopy (JEM2000EX, JEOL, Tokyo, Japan).

Graft Thickness Measurements

With the use of Azan-stained tissue sections, 15 random locations were selected from each sample, and the cardiac tissue thickness was determined from light microscopic photographs at a magnification of ×200 (n=3).

Laser Capture Microdissection and Gene Expression Analysis

Laser capture microdissection was performed with the use of procedures that were essentially the same as those described by Sgroi et al.18 Briefly, resected tissues were embedded in OCT compound (Sakura FineTek, Tokyo, Japan) and frozen in liquid nitrogen. Frozen sections 10-μm-thick were obtained by cryostat, stained with Alexa-Fluor-488–conjugated phalloidin (Invitrogen, Carlsbad, Calif), and dehydrated with Histogene (Takara Bio, Shiga, Japan). Air-dried sections were then laser microdissected with the use of the PixCell II system (Arcturus Engineering, Mountain View, Calif). Total RNA was then isolated with the PicoPure RNA isolation kit (Takara Bio) according to the manufacturer’s suggested protocol. Real-time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) was then performed with the use of the Perfect Real SYBR RT-PCR kit (Takara Bio) for 4 independent samples. To represent expression levels of each individual mRNA, we used an mRNA expression index that divided the value of the specific gene copies by the value for GAPDH.

Data Analysis

Data are expressed as mean±SD. An unpaired Student t test was performed to compare 2 groups. A probability value of <0.05 was considered significant.

Figure 2. Electric potentials and inner pressures from the beating tubes. Four weeks after implantation, electric potentials of the host heart (A) and the myocardial tubes (B) show independent excitations. C, Pressure measurements at the graft sites show that, initially, pressure changes cannot be distinguished from the host blood pressure. However, when the tubes are clamped (black arrow), an independent pressure derived from the graft pulsation is detected.
Figure 3. Histological analysis of myocardial tubes, 4 weeks after aortic replacement. A, Hematoxylin and eosin staining demonstrates the presence of stratified cardiac tissues surrounding the aortic vessel wall. Immunostaining for the cardiac isoform of troponin T (B) and connexin 43 (C) demonstrates the presence of cardiac muscle and diffuse gap junctions, respectively, throughout the resected grafts. The presence of endothelial cells, as well as neovascularization within the grafts, is also demonstrated by positive staining for CD31 (D) and von Willebrand factor (E). F, Azan staining also demonstrates microvessels with the presence of orange-stained red blood cells. Black arrows in D, E, and F indicate microvessels. Magnification: A, ×100; B, ×200; C, ×200; D, ×100; E, ×400; F, ×400.
Results

Myocardial Tubes Fabricated In Vitro Demonstrate Spontaneous Pulsation With Independent Graft Pressures After Aortic Replacement

Four weeks after transplantation, the implanted myocardial tubes demonstrated spontaneous and synchronized pulsations, distinct from the beating of the underlying vessels (see supplemental movie I). Detection of electric potentials confirmed that the spontaneous electric excitation spikes were independent of the host heartbeat (Figure 2A and 2B; n=4).

When inner pressure was measured at the center of the transplanted tube, any hemodynamic changes due to tube pulsation were initially indistinguishable from the host blood pressure. However, when the host abdominal aorta was clamped both above and below the ends of the myocardial tube, distinct inner pressures due to graft pulsation were detected (Figure 2B and 2C). The beating myocardial tubes were able to produce independent pressures, with an average of 5.9±1.7 mm Hg (maximal value of 8.1 mm Hg; n=4). Throughout the entire observation period, no thrombosis occurred in any of the animals.

Beating Myocardial Tubes Are Composed of Tissues That Resemble the Native Heart

Four weeks after aortic replacement, the resected myocardial tubes demonstrated the presence of stratified cardiac tissues lining the outer surface of the original blood vessels (Figure 3A). Immunostaining for troponin T revealed that the stratified, cell-dense tissues were composed of cardiomyocytes (Figure 3B). Additionally, the diffuse localization of connexin 43 throughout the cardiac tissues suggested gap junction formation within the grafts (Figure 3C). Staining with anti-CD31 and anti-von Willebrand factor antibody demonstrated the presence of endothelial cells and typical blood vessels within the myocardial tubes (Figure 3D and 3E). Finally, Azan staining (Figure 3F) and transmission electron microscopy (Figure 4A) confirmed the presence of functional microvessels containing red blood cells within the lumen, throughout the aortic replacement grafts. Results from transmission electron microscopy also showed the existence of well-differentiated myocardial tissues within the beating tubes, possessing numerous mitochondria, as well as myofilaments with elongated sarcomeres (Figure 4A and 4B). Desmosomes, which are indicative of cell-to-cell communication between neighboring cardiomyocytes, were also observed between adjacent cells (Figure 4C).

Aortic Replacement Enhances Cardiomyocyte Hypertrophy Within the Beating Tubes

To examine the effects of aortic replacement, engineered myocardial tubes were implanted inside the abdominal cavity for comparison. Four weeks after transplantation, the thickness of the aortic replacement tubes was 175±26 μm (n=3) and significantly higher than the myocardial grafts that were simply inserted into the abdominal cavity (80±30 μm; n=3), even though both groups consisted of 6-layer cardiomyocyte sheets wrapped around the resected aorta (Figure 5A to 5C). In addition, gene expressions of brain natriuretic peptide (BNP) and both myosin heavy chain-α (MHC-α) and myosin heavy chain-β (MHC-β) were significantly higher in the aortic replacement group (Figure 5D to 5F). These results indicated that the pulsations due to blood flow within the lumen of the aortic replacement tubes had an inductive effect on generating thicker, hypertrophic myocardial tissues.

Discussion

Previous approaches for myocardial reconstruction have focused generally on methods of engineering cardiac patches that can be transplanted directly to host hearts for tissue repair. In the present study we have successfully fabricated synchronously beating myocardial tubes producing inner pressures and showing characteristic structures of native cardiac tissues. These results demonstrate the next step of myocardial tissue reconstruction and a shift toward the fabrication of independently functioning cardiac structures, with the possibility of acting as tissue engineered cardiac assist devices. Although some efforts have been reported for the in vitro fabrication of myocardial tubes, our results are the first report confirming the in vivo function of bioengineered myocardial tubes that can produce inner graft pressures and have the potential for circulatory support.

An important factor in the development of the cell-dense myocardial tubes that resemble native cardiac tissues is the use of temperature-responsive culture dishes. Because of
The noninvasive harvest, the cell sheets can be simply wrapped and directly layered around the resected abdominal aorta, without the need for exogenous matrix materials or carriers, allowing for the observed synchronous pulsations of the engineered myocardial tubes. From both previous results and the present findings, we assert that the layering of cardiomyocyte sheets presents a significant advantage over scaffold-based tissue engineering in the creation of cell-dense, functional myocardial tissues.

In the cases of aortic replacement, the bioengineered myocardial tubes showed increased thickness with mature myofilaments and elongated sarcomeres. With aortic replacement, significantly higher gene expression levels of BNP, MHC-α, and MHC-β were also observed, in comparison with myocardial tubes implanted in the abdominal cavity. Because cardiomyocytes are known to rarely divide after birth, it is believed that the increases in myocardial tissue thickness and expression of genes related to cardiac development are mainly due to adaptive hypertrophy of the cardiomyocytes within the engineered tissues. Previous studies have demonstrated that mechanical stress has the ability to induce myocardial hypertrophy during both developmental and pathological states. In myocardial tissue engineering, researchers have applied these phenomena to accelerate cardiomyocyte hypertrophy by mechanical stretching. Similarly, in the present study pulsation due to host blood flow within the vessel lumen likely plays a role in the observed adaptive hypertrophy of the functional myocardial tubes. Therefore, the application of mechanical loads either in vitro or in vivo seems to be an essential factor in the engineering of functional tissues.

Because of the possibility of thrombosis and questions regarding graft compliance pertaining to small animals, we applied the use of the resected thoracic aorta in the present study. For future applications, the use of other materials, such as biodegradable polymers and artificial vessels, or combinatorial approaches with the use of tissue engineered blood vessels will likely allow for the exclusion of the resected aorta in the creation of the myocardial tubes.

**Figure 5.** Aortic replacement influences physiological cardiomyocyte hypertrophy. Azan staining of grafts used for aortic replacement (A) and those implanted within the abdominal cavity (B) reveals that the aortic replacement grafts possessed significantly thicker myocardial tissues (C). Magnification: A, ×100; B, ×100. Real-time quantitative RT-PCR for BNP (D), MHC-α (E), and MHC-β (F) shows that markers of cardiomyocyte hypertrophy are more highly expressed in the aortic replacement group. Data were assessed from the aortic replacement (black bars) and the abdominal cavity implant (white bars) grafts, respectively. Error bars indicate the SD (*$P<0.05$).
As the next stage, we are now trying to apply the use of newer and more advanced pacing devices to synchronize the graft beatings with host hearts to examine the effects on host hemodynamics. Furthermore, optimization of the pacing conditions in infarction models may be able to improve heart failure after myocardial damage. For future studies, we are also planning to examine potential cardiac support mechanisms, such as assisting blood flow to peripheral areas or increasing coronary artery flow via aortic arch pressure augmentation in the diastolic phase, analogous to intra-aortic balloon pumping.

A major obstacle in myocardial tissue engineering remains the insufficient oxygen perfusion into engineered 3-dimensional myocardial constructs, which limits construct thickness to \( \approx 100 \) to 200 \( \mu \)m. In the present study, although neovascularization was observed throughout the 6-layer myocardial tubes, graft necrosis may occur when additional layers are wrapped around the resected aorta in vitro. Therefore, to fabricate thicker, functional myocardial tissues, new technologies to control blood vessel growth are currently desired. Recently, as a possible technique, we have successfully fabricated \( \approx 1 \)-mm-thick myocardial patches using an in vivo approach of polysurgery.\(^{24}\) Future attempts with the use of either polysurgery or other methods, such as growth factor administration, gene transfer, or coculture with vascular progenitor cells to accelerate vascular network growth and formation,\(^{25}\) may contribute to the fabrication of thicker tissues. Overcoming the limits of passive diffusion should allow for the creation of more powerful myocardial tubes that can generate independent pressures that are sufficient for the circulatory support of damaged hearts. Further research is needed to elucidate these critical points.

Although several issues should be addressed regarding the future application of myocardial tubes, the present study shows the first development of a pulsatile myocardial tissue construct with the in vivo potential for circulatory support and proposes a new strategy that moves from tissue engineering toward organ engineering.

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**Disclosures**

Dr Okano is an investor in CellSeed, Inc, and is an inventor/developer designated on the patent for temperature-responsive culture surfaces.

**References**


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