Cardiac Grafting of Engineered Heart Tissue in Syngenic Rats

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Background—Cell grafting has emerged as a novel approach to treat heart diseases refractory to conventional therapy. We hypothesize that survival and functional and electrical integration of grafts may be improved by engineering cardiac tissue constructs in vitro before grafting.

Methods and Results—Engineered heart tissue (EHT) was reconstituted by mixing cardiac myocytes from neonatal Fischer rats with liquid collagen type I, matrigel, and serum-containing culture medium. EHTs were designed in circular shape (inner/outer diameter: 8/10 mm; thickness: 1 mm) to fit around the circumference of hearts from syngenic rats. After 12 days in culture and before implantation on uninjured hearts, contractile function of EHT was measured under isometric conditions. Baseline twitch tension amounted to 0.34±0.03 mN (n=33) and was stimulated by Ca⁺⁺ and isoproterenol to 200±12 and 185±10% of baseline values, respectively. Despite utilization of a syngenic model immunosuppression (mg/kg BW: azathioprine 2, cyclosporine A 5, methylprednisolone 2) was necessary for EHT survival in vivo. Echocardiography conducted 7, 14, and 28 days after implantation demonstrated no change in left ventricular function compared with pre-OP values (n=9). Fourteen days after implantation, EHTs were heavily vascularized and retained a well organized heart muscle structure as indicated by immunolabeling of actinin, connexin 43, and cadherins. Ultrastructural analysis demonstrated that implanted EHTs surpassed the degree of differentiation reached before implantation. Contractile function of EHT grafts was preserved in vivo.

Conclusions—EHTs can be employed for tissue grafting approaches and might serve as graft material to repair diseased myocardium. (Circulation. 2002;106[suppl 1]:I-151-I-157.)

Key Words: cells ■ transplantation ■ contractility ■ echocardiography ■ tissue

Cell based therapy has emerged as a novel and potentially curative approach for the replacement of impaired myocardium.¹ In general, 2 approaches have been employed: Implantation of isolated cells by direct injection into the myocardium²–¹⁰ or percutaneous application¹¹ and construction of cardiac muscle constructs in vitro that can be surgically attached to the myocardium.¹²–¹³ A most elegant approach has been employed by Orlic and coworkers who could demonstrate that stem cells can be liberated from bone marrow, home to infarcted myocardium, and transdifferentiate into cardiac myocytes after injection of cytokines.¹⁴ Most cell implantation procedures resulted in an improved myocardial function in animal models of myocardial infarction¹⁵–¹⁶,¹³ and Menasche et al. have reported first results from successfully grafted myoblasts into the myocardium of patients undergoing bypass surgery.⁷ Percutaneous application of bone marrow cells has been performed by Strauer and coworkers.¹¹ Despite these promising results, several questions remain unresolved at present and may provide arguments for the development of techniques to reconstitute 3D cardiac tissue grafts in vitro. Some of the most pertinent questions concern cell survival, integration, differentiation, functional effects, and electrical coupling.¹⁵ At present it is unclear whether cardiac tissue grafts provide any advantage. Yet, in analogy to cardiac tissues currently used in pediatric cardiac surgery, 3D engineered cardiac tissue grafts are expected to provide directed contractile function and to survive and electrically couple to the host myocardium. In addition, they can be designed in appropriate shape and size. For this purpose, tissue constructs should (1) have clearly defined contractile properties, (2) be large in size, (3) become vascularized and integrated into the host myocardium, and (4) improve contractile function of diseased myocardium.

At present various biomaterials have been employed.¹²,¹³,¹⁶,¹⁷ However, coherent and directed contractions...
of these cardiac tissue constructs with measurable force development have not been reported so far. We have developed heart muscle constructs, termed engineered heart tissue (EHT), that fulfill some prerequisites of graft material for replacement therapy approaches.\textsuperscript{18–21} EHTs display morphological, electrophysiological, and contractile properties of native heart muscle preparations and can be designed in different geometrical shapes and sizes.\textsuperscript{19–21} Survival of EHTs in vivo has been tested previously when implantation was performed underneath the peritoneum.\textsuperscript{22} In the present study, we examined the applicability of EHT grafting on the heart of syngenic rats.

Methods
All procedures were approved by the local animal protection authority (Ansbach GZ 621-2531.31-2/00) and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication 85–23, revised 1985). Cardiac myocytes were isolated from neonatal Fisher 344 rats. Syngenic rats (150 to 250 g) were used as recipients. Collagen type I was prepared from tails of Fisher 344 rats.

Engineered Heart Tissue
EHTs were prepared as previously described with some modifications.\textsuperscript{21} Briefly, ventricles of neonatal hearts were digested with trypsin/collagenase according to the manufacturers protocol (Worthington Biochemical Corp, Freehold, NJ). After repeated washes with serum-containing culture medium (DMEM, 10% horse serum, 2% chick embryo extract, 100 U/mL penicillin, 100 μg/mL streptomycin) isolated cells were mixed with collagen type I, a basement membrane protein mixture (matrigel; Tebu, Frankfurt, Germany), and concentrated serum-containing culture medium (2xDMEM, 20% horse serum, 4% chick embryo extract, 200 μL/mL penicillin, 200 μg/mL streptomycin); pH was neutralized by titration with NaOH (0.1 mol/L). The reconstitution mix was pipetted into circular casting molds of different diameter to optimize EHT size for grafting applications (Figure 1). EHT culture was performed as described earlier.\textsuperscript{21,22}

Force Measurement
After 12 days EHTs were transferred into thermostatted organ baths filled with Tyrode’s solution and subjected to isometric force measurement as described previously.\textsuperscript{21} After 30 minute equilibration without pacing, EHTs were electrically stimulated with rectangular pulses (2 Hz, 5 ms, 80 to 100 mA). Preload was adjusted to Lmax, that is, the length were EHTs developed maximal active force. For general characterization of Fischer 344 EHTs concentration response curves for Ca\textsuperscript{2+} (0.2 to 2.8 mmol/L) and isoprenaline (0.1 to 1000 nmol/L) were generated. Before implantation isotropic responses were analyzed by bolus stimulation with Ca\textsuperscript{2+} (2.8 mmol/L), isoprenaline (1 μmol/L), and carbachol (1 μmol/L). Twitch tension and twitch duration (time from 10% of peak force development to 90% relaxation) were evaluated by BMON software (Ingenieurbüro Jacckel, Hanau, Germany).

EHT Implantation
Rats were anesthetized with isoflurane (4%) and intubated for continuous ventilation with room air supplemented with oxygen and isoflurane (1%). Hearts were exposed through a left lateral thoracotomy. After removal of the pericardium 2 EHTs were sutured (5 Ethibond\textsuperscript{®}, Ethicon) onto a recipient heart. Tardomyocel\textsuperscript{®} (12,500 IU penicillin/kg and 15.5 mg streptomycin/kg, intramuscular injection; Bayer) and buprenorphine hydrochloride (0.1 mg/kg, intraperitoneal injection) were injected during surgery. For immunosuppression cyclosporine A (5 mg/kg), azathioprine (2 mg/kg), and methylprednisolone (2 mg/kg) were administered daily by subcutaneous injection.

Figure 1. EHT Design for Tissue Replacement Therapy. (a) EHTs of different sizes, adjusted in diameter to fit around the circumference of an adult rat heart. (b) Stars indicate EHT placed on a Langendorff perfused heart ex vivo. Bar: 1 cm.

Echocardiography
Left ventricular (LV) function was evaluated by transthoracic echocardiography under ketamine (20 mg/kg) and xylazine (3 mg/kg) anesthesia. Echocardiograms were recorded with a commercially available echocardiographic system (Sonos 5500, Agilent) equipped with a 7.5-MHz linear phased array transducer. A conventional second harmonic imaging modus was employed. Cardiac cycle loops of short and long axes were digitized for later retrieval and quantification. For each data point 3 different heart cycles were averaged. Additionally, heart rate was assessed.

Confocal Laser Scanning Microscopy
Hearts were excised and perfused with 4% formaldehyde/1% methanol in phosphate buffered saline (PBS) for confocal laser scanning microscopy (CLSM). After an overnight wash in PBS, samples were cryo-protected (10% sucrose, 4°C, overnight) and frozen in pre-cooled (−80°C) isopentane. Samples were mounted in Tissue Tek\textsuperscript{®}. Sections (10 μm) were blocked and permeabilized 1 hour at room temperature (RT) in Tris-buffered saline solution (TBS), pH 7.4, containing 10% fetal calf serum, 1% bovine serum albumin, and 0.5% Triton-X 100. Antibody labeling was performed overnight at RT (α-sarcomeric actinin 1:800, Sigma; connexin 43 1:250, Translab; pan-cadherin 1:100, Sigma; prolyl-4-hydroxylase β 1:200, Chemicon; ED2 1:100, Serotec; CD 45 full strength, Serotec). Secondary antibodies were applied for 3 hour at RT (anti-mouse IgG Alexa 488 1:800, Molecular Probes; anti-rabbit IgG FITC 1:50, Sigma, St. Louis, MO). Actin labeling was performed with phalloidin-TRITC (1 μmol/L; Sigma, St. Louis, MO). After repeated washes with TBS and mounting in Mowiol 4–88 confocal images were recorded with a Zeiss LSM 5 Pascal system using a Zeiss Axiovert microscope.

Transmission Electron Microscopy
Samples were fixed in 2.5% glutaraldehyde in PBS, pH 7.4 overnight at 4°C. After overnight wash in PBS, EHTs were post fixed in osmiumtetroxide/PBS (1:1) for 2 hour at RT. After an overnight wash in PBS at 4°C samples were dehydrated, epon infiltrated, and epon
embedded according to standard protocols. Ultrathin sections (50 nm) were cut (Ultracut UCT, Leica), contrasted with uranyl acetate and lead citrate, and examined by transmission electron microscopy (TEM) with a Zeiss Leo 906 EM system.

**Statistical Analysis**

Data were calculated as arithmetic means±SEM and analyzed using a two-tailed paired t test. A probability value of less than 0.05 was considered significant. "n" indicates the number of independent experiments.

**Results**

**EHT Design**

To design EHTs that fit around the circumference of adult hearts circular casting molds were varied in size, and the reconstitution mixture was adjusted to 70, 100, and 130% of the basal volume yielding EHTs of different size (inner diameter: 5.6, 8, 10.4 mm; thickness 1 mm; Figure 1). On culture day 7 EHTs were placed on a static stretch device (10%) to continue EHT culture under chronic mechanical load. For all implantation experiments EHTs with an intermediate internal diameter were used.

**Contractile Properties of EHT**

Functional properties of EHTs were determined by isometric force measurements in organ baths before implantation (Figure 2a). Concentration response curves performed for initial characterization of Fischer 344 EHTs (n=9) revealed maximal Ca²⁺-stimulated twitch tension (TT) of 0.71±0.05 mN at 2 mM Ca²⁺ (EC₅₀ 0.2±0.05 mM) and maximal isoprenaline-stimulated TT of 0.41±0.04 mN at 0.3 μM isoprenaline (EC₅₀ 5.2±0.8 nM). At baseline Ca²⁺ (0.2 mM) EHTs (n=33) displayed a mean TT of 0.34±0.03 mN and a twitch duration (TD) of 271±12 ms. Maximal Ca²⁺ (2.8 mM) and isoprenaline concentrations (1 μM) increased force of contraction to 200±12 and 185±10%, respectively (Figure 2b). Carbachol in the presence of isoprenaline reduced TT to 141±5% of baseline values. TD was not affected by Ca²⁺, but shortened by isoprenaline to 70±3% of baseline values ("positive lusitropic effect"). Carbachol reversed the isoprenaline effect on twitch kinetics (Figure 2c).

**Echocardiography**

To investigate whether interventions caused changes in LV-function animals were subjected to echocardiography (echo).

**Table**

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**Figure 2.** Contractile Function of Engineered Heart Tissue. (a) Original tracings of single contractions of EHT. (b, c) Twitch tension and twitch duration in percent of baseline values (0.2 mM/L Ca²⁺).

**Figure 3.** EHT Grafting. Different techniques to fix EHTs on the recipients’ hearts. (a) EHT placed around the circumference of the heart. (b) EHTs sutured onto the heart in apical-valvular direction. (c) Explanted heart with EHT 14 days after implantation. (d) Magnification of implantation site. Note injection of vessels into EHT. Bars: 1 cm (a–c), 1 mm (d).
Following a baseline echo (n=9) before implantation LV-function was assayed before explantation at days 7 (n=3), 14 (n=3), and 28 (n=3) after implantation. This analysis revealed no changes in LV-function and data were pooled (Table 1).

Morphology and CLSM
EHTs were grafted onto the uninjured heart employing different fixation techniques (Figure 3a, b). Fourteen days after implantation and in the absence of immunosuppression EHTs were macroscopically almost completely absorbed, and staining for actin revealed a complete loss of cardiac sarcomeric structures (n=11; Figure 4a). When immunosuppression was administered (n=22) EHTs were attached to the epicardial surface and were strongly injected by newly formed blood vessels (Figure 3c, d). At the time of explantation coherent contractions of EHTs were noted. Confocal analysis of phalloidin-TRITC-labeled cryo-sections showed that the cardiac muscle-like structure of EHTs improved in vivo and displayed thicker sarcomeric structures than before implantation (Figure 4b).21 Staining of α-sarcomeric actinin also indicated the integrity of sarcomere structure in implanted myocytes (Figure 5a). Immunolabeling of connexin 43 and cadherins demonstrated electrical and mechanical intercellular connections inside EHTs (Figure 5b, c). Despite some indications of connexin junctions (arrows, Figure 5b) between EHT and host myocardium further studies need to evaluate whether true electrical coupling occurred. Fibroblasts and macrophages were found throughout implanted EHTs whereas the content of leukocytes was low (Figure 5d–f). This indicated that immune responses were sufficiently suppressed by the immunosuppressive therapy. Note that even before implantation EHTs consist of a mixture of cardiac myocytes and nonmyocytes including fibroblasts, smooth muscle cells, macrophages, and other mononucleated cells of leukocytotic origin.21

Transmission Electron Microscopy
A detailed study of implant differentiation and vascularization was performed by TEM. Implanted cardiac myocytes were preferentially found in clusters around newly formed blood vessels (Figure 6a). Presence of erythrocytes within these vessels indicated functional perfusion of grafted EHT. Besides neovascularization of implanted EHTs growth of nerve bundles consisting of myelinated and nonmyelinated nerve fibers was found throughout the grafted tissue constructs (Figure 6b). We have shown previously that cardiac myocytes within EHTs in vitro display a high degree of differentiation.21 M-bands, a morphological marker of terminal differentiation,23 were clearly less developed or absent in...
EHT reconstituted from neonatal Fischer 344 rats (Figure 7a).
Fourteen days after implantation ultrastructure of grafted myocytes (Figure 7b) was indistinguishable from native myocytes (Figure 7c) and featured well developed M-bands. Yet, implanted cells remained embedded in the EHT matrix and could therefore be clearly distinguished from host myocytes.

**Discussion**
The present study demonstrates that EHTs, designed in a suitable shape for tissue grafting, survive and beat for at least 28 days, are strongly vascularized, and acquire a highly differentiated cardiac phenotype when transplanted on the heart of syngenic rats.

Despite the syngenic approach of our study EHTs were completely degraded in the absence of immunosuppression. Apparently, extended washing during the force measurements in Tyrode’s solution was not sufficient to eliminate immunogenic factors. The following mechanisms could account for the immune response: (1) A response to allogenic nonsoluble matrix components (mainly matrigel); (2) an impregnation of implanted cells with soluble components of the reconstitution mixture and culture medium (chick embryo extract, horse serum, soluble components of matrigel); (3) alterations of the expression of self-antigens induced by the procedures of EHT reconstitution, culture, or implantation. By replacement or omitting allogenic components of the reconstitution mixture immune responses might be reduced. However, growth supplements are essential for EHT development. At present, experiments are underway to identify key components for EHT reconstitution and to substitute them specifically.

As an additional mechanism immunosuppression could have prevented bystander effect destruction of implanted cells or resident stem cells. Recently, the presence of resident stem cells in various organs including the heart and their capacity for tissue regeneration has been proposed (reference 24 for review). Thus, it cannot be excluded that EHTs contain progenitor cells that might be protected by immunosuppressive therapy. Moreover, resident stem cells in the recipients’ heart might also be protected from bystander effect destruction. However, contribution of cardiac progenitor cells remains highly speculative and awaits further elucidation.

Interestingly, EHTs, when implanted on the uninjured heart, not only retained their contractile function, but developed thicker and more mature cardiac muscle structures than those seen before implantation. The higher degree of differentiation was substantiated by the demonstration of regular M-bands, a morphological criterion for terminal cardiac muscle differentiation that is hardly seen early postnatally. After 12 to 14 days in culture EHTs present some M-band development. However, these M-bands were by far less frequent and less mature than in grafted EHTs 14 days after implantation. Our observation of cardiac maturation after implantation on the heart in vivo is in line with observations by Reinecke et al. who found that implanted cardiac myocytes from fetal and neonatal rats survived in vivo and gained in size over time with a concomitant increase in sarcomere development. Other groups have demonstrated that implantation of uncommitted progenitor cells or cells of non-cardiac origin were directed into a cardiac phenotype after injection into the heart. These findings support the notion that the adult heart provides a milieu that facilitates differentiation of cardiac myocytes.

As EHTs were implanted on uninjured hearts, it was important to evaluate whether the implantation procedure and immunosuppression per se affect LV-function. As seen by echocardiography this was not the case. One may expect the grafted, electrically active EHT to induce arrhythmia, and our experiments cannot exclude this possible hazard. However, arrhythmias were not observed and are in fact unlikely for several reasons. First, the spontaneous frequency of EHTs after 12 to 14 days of culture is consistently at or below 2 Hz, that is <40% of the normal heart rate of the rat. Second, action potential recordings on EHTs have shown that the majority of cardiac myocytes inside EHTs have an adult...
ventricular action potential without spontaneous diastolic depolarization. Third, electrical coupling of EHT and host could not be demonstrated clearly. However, this awaits further evaluations.

The latter aspect could indicate an important limitation of our current technique because our underlying hypothesis was that a preformed 3D graft will facilitate electrical contact between graft and host. Yet, we believe that this limitation can be overcome by improved fixation techniques, for example, by injuring the surface of the host myocardium or by true replacement of scarred myocardium in the case of an infarcted heart. These experiments are underway.

An exciting aspect was that EHTs were strongly innervated. This may be important for the functional integration of EHTs into the recipients’ hearts and may allow afferent and efferent control of these tissue constructs.

Yet, many obstacles remain. Most importantly, in our view, is the problem concerning the critical size of 3D constructs. This is a conceptional problem of the entire field of tissue engineering. In the absence of vascularization and perfusion, in vitro engineered cardiac muscle constructs will hardly exceed 50 to 100 μm compact tissue thickness. Indeed, similar limitations have been reported. Therefore, it may come as a surprise that EHTs with a thickness of 800 to 1000 μm can be constructed in vitro, survive, and mature when implanted on the heart. However, EHTs do not resemble a homogenous tissue construct but are comprised of a network of relatively thin cardiac myocyte strands (20 to 50 μm) and only some compact muscle strands of 50 to 100 μm. It appears that myocytes gain in size exclusively after implantation and develop thick muscle structures concomitant with vascularization of grafted EHT. Other reasons may lie in the relatively immature and ischemia-resistant phenotype of the originally neonatal cardiac myocytes inside EHTs. In vitro EHT showed spontaneous contractions of ~2 Hz for 14 days in an environment of unperfused medium and 21% oxygen concentration. Moreover, EHTs continued to contract spontaneously when implanted in the peritoneum or onto the heart as observed in this study despite lack of vascularization at least for the first days after implantation. It is likely that improvement of the culture conditions will allow construction of larger EHTs. In addition, the present observation of rapid and extensive vascularization after implantation and the observation of spontaneous formation of primitive capillaries inside EHTs support the idea that implanting several EHTs is a way to further increase the maximal graft size. Other problems concern the immune response and, most importantly, the question how the EHT technique can be transferred to the human. These questions will remain difficult to solve, but the rapid progress in stem cell technology opens the perspective of constructing EHTs from human stem cells.

Conclusion and Future Aspects

The present study demonstrates that EHTs survive, mature, and beat after implantation on uninjured hearts without apparent signs of harm. The benefit of EHT grafting will have to be elucidated in models of cardiac disease. These studies are currently under investigation. We hypothesize that techniques to engineer preformed 3D cardiac grafts such as EHTs can complement the recent efforts in cell grafting and may, in the long run, also allow replacement of larger tissue defects and possibly congenital malformations.

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References


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