Development of a Biological Ventricular Assist Device
Preliminary Data From a Small Animal Model

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Background—Engineered heart tissue (EHT) can be generated from cardiomyocytes and extracellular matrix proteins and used to repair local heart muscle defects in vivo. Here, we hypothesized that pouch-like heart muscle constructs can be generated by using a novel EHT-casting technology and applied as heart-embracing cardiac grafts in vivo.

Methods and Results—Pouch-like EHTs (inner/outer diameter: 10/12 mm) can be generated mainly from neonatal rat heart cells, collagen type I, and serum containing culture medium. They contain a dense network of connexin 43 interconnected cardiomyocytes and an endo-/epicardial surface lining composed of prolylhydroxylase positive cells. Pouch-like EHTs beat spontaneously and show contractile properties of native heart muscle including positive inotropic responses to calcium and isoprenaline. First implantation studies indicate that pouch-like EHTs can be slipped over uninjured adult rat hearts to completely cover the left and right ventricles. Fourteen days after implantation, EHT-grafts stably covered the epicardial surface of the respective hearts. Engrafted EHTs were composed of matrix and differentiated cardiac muscle as well as newly formed vessels which were partly donor-derived.

Conclusions—Pouch-like EHTs can be generated with structural and functional properties of native myocardium. Implantation studies demonstrated their applicability as cardiac muscle grafts, setting the stage for an evaluation of EHT-pouches as biological ventricular assist devices in vivo. (Circulation. 2007;116[suppl I]:I-16–I-23.)

Key Words: tissue engineering ■ myocardium ■ cardiomyocyte ■ regeneration ■ transplantation

Repairing the damaged heart is one of the major challenges in modern medicine. To this end, cardiac tissue engineers attempt to develop technologies that may allow to refurbish failing myocardium with new muscle.1,2 Therapeutically applied artificial myocardium would have to fulfill at least 2 biological functions: (1) it must stabilize the failing heart to prevent further dilation and (2) it must add contractile elements to the heart to improve its systolic function. Passive ventricular restraint devices (eg, CorCap Cardiac Support Device; Acorn Cardiovascular Inc) have been applied to stop adverse left ventricular remodelling and dilation in failing hearts.3 Yet, the widespread use of CorCap Cardiac Support Devices (CSD) has recently been stopped by the US Food and Drug Administration (FDA) despite positive trials in Europe and North America because of safety concerns. Indeed, pericardial constriction may occur in patients with CSD devices necessitating reoperations that are technically challenging.4 Another caveat pertaining to the cardiac restraint approach is the lack of intrinsic contractility in the latter. Thus, development of a CSD that may not only offer restraint but also reintroduce contractile elements, ie, cardiomyocytes, into failing hearts may eventually lead to a novel therapeutic perspective in end-stage heart failure.

Several groups have generated heart muscle constructs with functional and morphological properties of native myocardium in vitro (see overview in Eschenhagen and Zimmermann, 2005).3 Implantation studies in a rat model of myocardial infarction provided first proof-of-concept for a therapeutic application of engineered heart tissue (EHT) in vivo.4 Most tissue engineering studies have focused on the repair of regional myocardial defects, eg, after myocardial infarction, and not on offering passive (restraint) and active (contractility) support to the entire failing heart. We and others have recently reported modifications of established cardiac tissue engineering technologies to generate complex myocardial constructs with different geometries including contractile tubes and muscle networks to support failing hearts.5,7 Here, we propose a novel technology to generate EHT with a pouch-like geometry that may ultimately offer restraint and contractile support as biological ventricular assist device (BioVAD) in vivo. Consequently, our study had 2 objectives: (1) to develop a technology allowing the construction of myocardial pouches and (2) to investigate the applicability of these constructs as cardiac muscle grafts in adult rats.

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Cytes/50% nonmyocytes was immediately subjected to EHT hardening of the reconstitution mixture. Subsequently, culture medium (DMEM, 10% horse serum, 2% chick embryo extract, 100 U/mL penicillin, and 100 g/mL streptomycin) was carefully added to not disturb the reconstitution mixture. The porous agarose mold allowed the agarose to solidify around the spacer. Removal of the glass spacer facilitated free diffusion of culture medium enabling unrestricted supply with nutrients and oxygen. EHTs condensed within 3 to 7 days around the central spacer and started to contract spontaneously. Beating EHTs were transferred onto flexible holders to facilitate auxotonic contractions on culture day 7. Morphological and contractile properties of pouch-like EHTs were studied after 12 to 14 culture days.

**Force Measurements**

Force of contraction (twitch tension [TT]), resting tension (RT), and relaxation time (T2: time to 50% relaxation) of pouch-like EHTs were analyzed under electrical stimulation (2 Hz) in thermostated (37°C) organ baths filled with Tyrode’s solution (mM: NaCl 120, KCl 5.4, MgCl. 1, CaCl. 0.2 to 2.8, NaHPO4 0.4, NaHCO3 22.6, glucose 5, Na2EDTA 0.05, ascorbic acid 0.3) as previously described.8 Inotropic and lusitropic responses to calcium (0.2 to 2.8 mmol/L), isoprenaline (0.1 to 1000 mmol/L), and carbachol (1 μmol/L at maximal isoprenaline) were analyzed as described earlier.9

**Morphological Evaluation of EHT**

Formaldehyde-fixed EHTs were sectioned or processed as whole mounts for light or confocal laser scanning microscopy as described previously.9 Hematoxylin and eosin (H&E) staining was performed as described earlier.9 Pico sirius red staining was performed on deparaffinized sections in saturated picric acid for 1 hour. Dehydrated sections were mounted in Eukitt (Sigma) after washing in acidified water (5 mL/L acetic acid). Antibodies directed against α-sarcemeric actinin (1:1000; clone: EA-53, Sigma) and β-prolyl-4-hydroxylase (1:500; clone: 6-9H6, Chemicon) were used with appropriate secondary antibodies to identify cardiomyocytes and fibroblasts in EHTs, respectively. Phalloidin-Alexa 488 (3.3 U/mL; Sigma) and Bandeiraea simplicifolia lectin-TRITC (10 μg/mL; Sigma) were used to mark f-actin and endothelial cells, respectively. EHTs were labeled with DAPI (1 μg/mL; Molecular Probes) before implantation to facilitate donor cell identification as described previously.9 DRAQ5 (5 μmol/L; Alexis Biochemicals) was applied to label nuclei in EHT sections. Confocal laser scanning microscopy was performed with a Zeiss LSM 510 META system.

**EHT Implantation**

EHTs were implanted in male Wistar rats (n=16; 300 to 350 g; Charles River). Anesthesia was induced in an induction chamber filled with isoflurane (4%) and maintained after intubation and continuous ventilation with isoflurane (1%) supplemented room air throughout the surgery as described earlier.5 The thoracic cavity was opened through a left lateral thoracotomy. The hearts were exposed after excision of the pericardium. Pouch-like EHTs were slipped over the entire left and right ventricles from apex to the base of the hearts and fixed with 2 sutures (6–0 Prolene, Ethicon) at the anterior and lateral base of the beating hearts. Tardomyocel (12500 IU penicillin/kg and 15.5 mg streptomycin/kg, intramuscular injection; Bayer) and buprenorphine hydrochloride (0.1 mg/kg, intraperitoneal injection) were applied during surgery. Cyclosporine A (5 mg/kg), azathioprine (2 mg/kg), and methylprednisolone (2 mg/kg) were administered daily by subcutaneous injection for immune suppression. Apparent signs of immune rejection or inflammatory responses were not observed.

**Statistical Analysis**

Data are presented as mean±SE of the mean. Statistical differences were determined using a repeated ANOVA (concentration response curves) or 1-way ANOVA with Bonferroni post hoc testing (analysis of relaxation times). P<0.05 was considered statistically significant.

**Statement of Responsibility**

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.
Results

Construction of Pouch-Like EHT
EHTs condensed within 3 to 7 days in the spherical casting molds around central glass spacers forming pouch-like constructs with an inner diameter of 10 and an outer diameter of 12 mm (corresponding to a “wall thickness” of \( \frac{1}{10} \) mm). We chose these dimensions to match the size of an adult rat heart (Figure 2). First visible spontaneous contractions of the pouch-like EHTs were noted as early as 2 to 3 days after casting. EHTs maintained their spontaneous contractions (1 to 2 Hz) for at least 14 days (see supplemental video, available online at http://circ.ahajournals.org).

Contractile Properties of Pouch-Like EHT
Pouch-like EHTs could be stimulated electrically in a pulsed field (50 to 100 mA, 2 Hz; Figure 3a) and demonstrated a baseline TT of 0.7 mN at 0.2 mmol/L calcium (n=4). Increasing extracellular calcium to 2.8 mmol/L or addition of 1 \( \mu \)mol/L isoprenaline increased TT to 1.2±0.3 mN (P<0.05) and 0.9±0.3 mN (P<0.05), respectively (n=4; Figure 3b and 3c). RT was 0.4±0.1 mN (n=4) indicating good compliance (TT/RT ratio >1). T2 was 59±4 ms at baseline calcium (0.2 mmol/L). Isoprenaline shortened T2 to 39±3 ms (P<0.05). Addition of the muscarinergic agonist carbachol (1 \( \mu \)mol/L) reversed the inotropic (data not shown) and lusitropic isoprenaline effects (n=4; Figure 3d).

Morphological Properties
Pouch-like EHTs contained a highly interconnected network of differentiated cardiomyocytes (Figure 4). Abundant detection of connexin 43 suggested the formation of an electrical syncytium within EHTs (Figure 4). Notably, the surface of the EHTs was covered by a dense epithelium consisting of prolylhydroxylase-positive cells suggesting that fibroblast-like cells formed a pseudoepi-/endocardium (Figure 5).

Implantation Studies
Pouch-like EHTs could be implanted into immune suppressed rats after mobilizing the heart through a left lateral thoracotomy (Figure 6). All animals survived this procedure (n=16). Fourteen days after implantation, we could clearly identify the engrafted EHTs on the hearts (Figure 7a). H&E (Figure 7b) and pico sirius red (Figure 7c) staining demonstrated the preservation of the grafts on the epicardial surface of the host hearts. Here, it is important to note that EHTs (in vitro and in vivo) consist of collagen (red stain in Figure 7c) and heart cells (yellow stain in Figure 7c). The latter formed clearly distinguishable muscle aggregates in close proximity to the host myocardium (Figure 7d). Confocal laser scanning analyses indicated that engrafted cardiomyocytes formed loose but differentiated muscle networks (Figure 7e). EHT grafts were mostly separated from the host heart by a cell free gap (\( \sim 50 \) to 200 \( \mu \)m; Figure 7f). In addition, the already in vitro observed epithelial surface lining was still present in some areas (Figure 7g). Yet, H&E (Figure 7d) and confocal laser scanning analyses (Figure 7h) demonstrated that engrafted...
muscle also formed intimate contact to the host heart. However, connexin 43 (Cx43) appeared to be less abundant and structured in the border zone comprising host myocardium and graft tissue (Figure 7h) when compared with the remote myocardium (Figure 7i). Importantly, we observed vascularization of implanted EHTs. Many of the newly formed vessels were partially composed of donor cells (DAPI-label; Figure 8).

**Discussion**

The present study demonstrates (1) the development of pouch-like EHT with structural and functional properties of native myocardium and graft tissue (Figure 7h) when compared with the remote myocardium (Figure 7i). Importantly, we observed vascularization of implanted EHTs. Many of the newly formed vessels were partially composed of donor cells (DAPI-label; Figure 8).

In contrast, skeletal myoblast, being the first clinically applied cell species in the heart, cannot electrically couple to host myocytes but still appear to provide some structural support. Recent studies suggested a therapeutic benefit after implantation of bone marrow–derived stem cells in patients with myocardial infarctions. However, these findings were challenged by others.

Myocardial tissue engineering has not entered the clinical scene, but encouraging data have recently been derived from animal experiments. We anticipate that first clinical trials will be started once a scalable cell source has been identified that may be clinically applicable and, secondly, when myocardium can be engineered at a size and with functional properties that may offer significant support to failing hearts. Size certainly matters and clinically applicable myocardial grafts must not only be thick (≈1 to 10 mm) but also cover a large myocardial area to repair a local defect. Yet, many myocardial diseases do not present with a localized dysfunc-
tion but with global ventricular dilation and defects in ventricular systolic shortening. In these patients, implantation of an engineered myocardial pouch may not only stop ventricular enlargement but also offer contractile support to globally failing hearts. Cardiac restraint devices (eg, Acorn CorCap Device) may prevent dilation but cannot offer active contractile support. In contrast, pouch-like EHTs develop contractile force and can be, although technically demanding, slipped over adult rat hearts to cover the entire ventricular myocardium as presented here. This procedure apparently did

**Figure 6.** Implantation of a pouch-like EHT. a, Mobilization of an adult rat heart through a left lateral thoracotomy. b, Implantation of a pouch-like EHT onto the same heart. Bars=10 mm

**Figure 7.** Structure of pouch-like EHTs 14 days after implantation. a, Explanted heart 14 days after implantation of a pouch-like EHT (note that the atria were removed). b, H&E staining of a short axis cross section of a heart with an EHT-graft. c, Pico sirius red staining of a short axis cross section of a heart with an EHT-graft (adjacent to section in b). d, H&E staining of an EHT-graft/host-heart border zone; arrows highlight a cardiomyocyte aggregate within the EHT-graft. e, Cardiomyocyte networks within an engrafted EHT-pouch (actin; green). The DAPI-label (blue nuclei) indicates donor cell origin. DAPI labeled cells were not present in the recipient myocardium (inset). f, Gap (arrows) between EHT-graft and host heart. g, Prolylhydroxylase (P4H; red) positive surface linings of EHT-grafts were partially maintained in vivo. h and i, Connexin 43 (Cx43; red) in the EHT-graft/host heart border zone (h) and within the remote myocardium of the same heart (i). e through i, DAPI label (blue nuclei) indicates EHT-derived cells; f-actin labeled with phalloidin-Alexa 488 (green). Bars=(a) 10 mm, (b and c) 1 mm, (d and e) 100 μm, (f through i) 50 μm
not lead to pericardial constriction and was overall well tolerated (all animals survived the procedure and 14-day follow-up). Pouch-like EHTs did not lose their myocardial structure in vivo and became vascularized within the observation period (14 days). Vascularization was at least partially supported by donor cells (DAPI-label). This finding was not surprising given the presence of primitive capillaries in EHTs in vitro and the strong vascularization of EHT-grafts in a rat model of myocardial infarction. However, whether pouch-like EHTs can be applied on dilated ventricles with contractile dysfunction as BioVADs remains to be evaluated.

How do we envision overcoming problems of cell sourcing and graft dimensions? Human stem cells have recently been identified as a putative source for cardiomyocytes.18,19 Despite these exciting findings, the allocation of cardiomyocytes in a sufficient number to repair a large myocardial infarct (≈1 billion cardiomyocytes will be needed) in a reasonable period of time (days-weeks) remains a paramount task. Genetic selection, induction of cardiomyocyte differentiation by growth factors, and mass culture approaches using bioreactors may allow up-scaling of cardiomyocyte yield, but the feasibility of these approaches has yet to be demonstrated in human stem cell cultures. Moreover, it seems unlikely that cardiomyocytes alone will be sufficient to engineer optimal myocardial surrogate tissue in vitro. We have in fact recently demonstrated that EHTs, being equally composed of myocytes and nonmyocytes, are structurally and functionally superior to EHTs constructed from enriched cardiomyocyte populations. It is very likely that nonmyocytes are necessary for structural and paracrine support in vitro but also facilitate cell engraftment and elicit beneficial effects on the recipient myocardium in vivo. In contrast to cardiomyocytes, human nonmyocytes can be easily derived from cardiac biopsies or other autologous cell sources (eg, bone marrow or fat tissue). Once human cells are available for myocardial tissue engineering, it will remain questionable whether they can be assembled to thick heart muscle. The present bottleneck of suboptimal diffusion in thick tissue structures in the absence of vascularization will have to be overcome. Pure muscle structures in tissue engineered myocardium in vitro generally do not become thicker than 200 μm. However, sequential grafting of thin cell-sheets and implantation of star-shaped EHTs, being composed of a dense network of thin muscle strands, have been applied to generate vascularized myocardium with a diameter of up to 1 mm in vivo. Pouch-like EHTs were similarly vascularized and contained thick muscle aggregates in vivo. However, further improvement of muscle composition is likely to be necessary to confer a significant amount of myocardium to support a failing heart.

Electrical coupling of implanted engineered myocardium to the native myocardium is another important issue. We did previously observe anterograde and retrograde electrical coupling of EHT grafts to infarcted rat hearts. Whether BioVADs couple similarly well to native myocardium will have to be assessed in more detail in further studies. We regularly observed a cell-free gap of 50 to 200 μm between EHT graft and native myocardium as well as a preservation of the nonmyocytes surface lining of the EHT pouches in vivo. Either finding argues against extensive EHT/host myocardium electrical coupling. However, we did also identify areas of intimate graft-host contacts (Figure 7d) which are likely to facilitate undelayed impulse propagation between host and donor myocardium if proper cell-to-cell contacts are established. The principle propensity of cardiomyocyte grafts to couple to native myocardium has been demonstrated by several groups. We could not unequivocally identify gap junction/connexin 43 contacts between EHT grafts and host myocardium. However, coupling through different connexin isoforms or even through electrotonic mechanisms involving myofibroblasts cannot be excluded. Although integration of a tissue graft into the host heart appears to be ideal, it may also go along with conduction abnormalities (eg, ectopic beats, conduction delay leading to reentry tachycardia). This may be controllable by parallel application of antiarrhythmic drugs or cardioverter-defibrillators, as performed in patients with myoblast implants, at least until stable electrical contacts are established. Conversely, one might speculate that electrical integration of EHT pouches would not be desirable at all. Instead, EHTs, functioning as BioVADs, could be electrically stimulated through an integrated biological pacemaker or external stimulation to maintain an electrically separate but functionally synchronized entity. The general feasibility of external electrical stimulation of pouch-like EHT grafts has been demonstrated in the present study (contraction experiments).

Contractile performance of pouch-like EHTs was assessed by contraction experiments in the present study. Similar experiments have been conducted previously with circular EHTs and atrial or papillary heart muscle. We have also performed pressure measurements after inserting a Millar-tip catheter through the aperture of an EHT pouch (data not shown). This was principally feasible but unreliable because...
of technical difficulties (measurement necessitates closure of the EHT aperture around the catheter which was difficult to achieve). Collectively, circular and pouch-like EHTs as well as native myocardium display similar contractile properties (eg, positive inotropic response to increasing concentrations of extracellular calcium; positive inotropic and lusitropic responses to isoprenaline). In fact, contractile force per EHT muscle cell cross-sectional area (≈12% to 30% of 1 mm² total EHT cross section) was 4 to 10 mN/mm² under maximal inotropic stimulation in the present study. This is a bit lower than maximal forces of optimal circular EHT cultures (20 to 40 mN/mm²; unpublished data, 2006). Yet, the difference may stem from the uniform organization of muscle bundles in the same plane in circular EHTs versus the more random organization of muscle in pouch-like EHTs. Importantly, maximal force values in EHTs closely resemble optimal forces of papillary muscle preparations. However, calcium sensitivity in EHTs is markedly higher when compared with papillary muscle. This may be a consequence of an immature calcium handling machinery in EHTs in vitro. Interestingly, Morritt et al demonstrated recently that in vivo conditioned engineered cardiac muscle, being composed mainly of neonatal rat heart cells and Matrigel, apparently regain physiological calcium handling properties. These findings support our own observation that in vitro engineered myocardium is capable of terminal differentiation once the right “growth milieu” is offered.

Conclusion

The present study provides a new technology to generate pouplike EHTs that may eventually be applied as BioVADs in vivo. Structure and function of pouch-like EHTs simulate respective properties in naïve myocardium. Implantation studies demonstrate the applicability of pouch-like EHTs in vivo. However, several important issues will have to be addressed before pouplike EHTs can be considered as a clinically applicable BioVADs concept. These encompass: (1) providing unequivocal evidence for a therapeutic effect of pouplike EHTs in a clinically relevant heart failure model; (2) identification of a scalable cardiomyocyte source and allocation of cardiomyocytes as well as nonmyocytes to BioVAD engineering; (3) achieving muscle tissue dimensions that may offer significant support to large failing ventricles; and (4) providing solutions to safety concerns (eg, arrhythmia induction, unwanted growth, immunologic incompatibilities).

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Disclosures

The University Medical Center Hamburg-Eppendorf has filed a patent application concerning the BioVAD-technology.

References


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