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In Vivo Conditioning of Tissue-engineered Heart Muscle Improves Contractile Performance

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Abstract: The ability to engineer cardiac tissue in vitro is limited by the absence of a vasculature. In this study we describe an in vivo model which allows neovascularization of engineered cardiac tissue. Three-dimensional cardiac tissue, termed "cardioids," was engineered in vitro from the spontaneous delamination of a confluent monolayer of cardiac cells. Cardioids were sutured onto a support framework and then implanted in a subcutaneous pocket in syngeneic recipient rats. Three weeks after implantation, cardioids were recovered for in vitro force testing and histological evaluation. Staining for hematoxylin and eosin demonstrated the presence of viable cells within explanted cardioids. Immunostaining with von Willebrand factor showed the presence of vascularization. Electron micrographs revealed the presence of large amounts of aligned contractile proteins and a high degree of intercellular connectivity. The peak active force increased from an average value of 57 μN for control cardioids to 447 μN for explanted cardioids. There was also a significant increase in the specific force. There was a significant decrease in the time to peak tension and half relaxation time. Explanted cardioids could be electrically paced at frequencies of 1–5 Hz. Explanted cardioids exhibited a sigmoidal response to calcium and positive chronotropy in response to epinephrine. As the field of cardiac tissue engineering progresses, it becomes desirable to engineer larger diameter tissue equivalents and to induce angiogenesis within tissue constructs. This study describes a relatively simple in vivo model, which promotes the neovascularization of tissue-engineered heart muscle and subsequent improvement in contractile performance. **Key Words:** Cell culture—Myocytes—Contractile function—Tissue engineering—Angiogenesis—Epinephrine.

Heart disorders remain a global epidemic with constantly increasing mortality rates (1). There is an unprecedented need to develop new treatment options. Cardiac tissue engineering may provide a new treatment option by generating cardiac tissue in vitro. Tissue-engineered heart muscle can be utilized to treat cases of myocardial infarction and for the repair of congenital heart defects. In addition to potential clinical applicability, a cardiac tissue analog may be used in drug development and as a model to study the process of in vitro self-organization that could provide insight into cardiac developmental biology.

There have been several strategies utilized to engineer cardiac tissue in vitro. Temperature sensitive surfaces have been used to manufacture two-dimensional sheets of cardiac myocytes. These sheets have been stacked together to form three-dimensional cardiac muscle (2–4). Synthetic scaffolds have been fabricated from polyglycolic acid and have been seeded with neonatal cardiac myocytes to engineer cardiac tissue equivalents (5–7). In addition, various biodegradable gel systems consisting of gelatin (8–10), alginate (11), and collagen (12–15) have been utilized.

We have developed a method to engineer three-dimensional cardiac tissue constructs and have termed the resulting tissue analog, a "cardioid" (16). Cardioids result from the spontaneous delamination of a confluent monolayer of neonatal cardiac myocytes plated on a culture surface treated to control cell adhesion. Cardioids generate an active force of up to 125 μN upon electrical stimulation. In addition, cardioids can be electrically paced at physiologically relevant frequencies of 1–5 Hz without notable fatigue. Cardioids also exhibit an increase in force

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production in response to ionic calcium and positive chronotropy in response to epinephrine.

The ability to maintain long-term viability of cardioids is limited by the lack of a vasculature within the three-dimensional tissue construct. The lack of a vasculature limits oxygen diffusion to the cells within the inner core of the cardioid resulting in the loss of cell viability. It therefore becomes imperative to promote neovascularization within the avascular cardioids.

There have been several studies describing angiogenesis models for engineered cardiac tissue (4,10,11,17). Cardiac tissue constructs have been engineered in vitro and then implanted in recipient animals to promote neovascularization. The target site for implantation has varied between studies and can be a subcutaneous pocket (4,17), or the host myocardium (10,11). In both cases, survival of the tissue equivalents has been demonstrated along with a vast amount of neovascularization. Although these studies serve to demonstrate the successful neovascularization of engineered cardiac tissue constructs, there is very little information about the changes in contractility resulting from neovascularization.

The objective of this study was to investigate the effect of an in vivo environment on the contractile performance of tissue-engineered heart muscle. We describe a subcutaneous implantation model which promotes the neovascularization of cardioids over a period of 3 weeks. We obtained significant improvement in the contractile performance of cardioids resulting from implantation.

MATERIALS AND METHODS

Approval for animal use was granted by the University Committee for the Use and Care of Animals (UCUCA) in accordance with the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23, 1986). All materials were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise specified.

Isolation of neonatal cardiac myocytes

Cardiac myocytes were isolated from 2- to 3-day-old F344 rat hearts using an established method (18). Briefly, neonatal pups were decapitated and an incision made along the chest cavity to expose the hearts which were excised and placed on ice. The hearts were cut into fine pieces and suspended in a dissociation solution (DS) which consisted of 0.32 mg/mL collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ, U.S.A.) and 0.6 mg/mL pancreatin dissolved in a buffer consisting of 116 mM NaCl, 20 mM HEPES, 1 mM Na_2HPO_4, 5.5 mM glucose, 5.4 mM KCl, and 0.8 mM $MgSO_4$. Serial digestion was carried out in an orbital shaker for 5 min at 37°C and the supernatant was collected in 5 mL of horse serum (Invitrogen Corporation, Auckland, New Zealand). Cells from all the digests were pooled, centrifuged and then suspended in plating medium (PM) consisting of 335 mL DMEM, 85 mL M199, 25 mL fetal bovine serum, 50 mL horse serum, 5 mL antibiotic-antimycotic (Invitrogen Corporation, Auckland, New Zealand). The final cell concentration was adjusted to 2×10^6 cells/mL.

Preparation of the plates

The detailed method for preparing the culture surface for engineering cardiac muscle has been described in detail (16). Briefly, 35-mm culture plates were coated with 1.5 mL of a polydimethylsiloxane (PDMS) elastomer (Dow Chemical Corporation, Midland, MI, U.S.A.). The PDMS surface was subsequently coated with 0.6 μg/cm (2) of natural mouse laminin (Invitrogen Corporation, Carlsbad, CA, U.S.A.). Anchor points were engineered on the laminin coated surface by placing 6 mm long segments of size 0 braided silk sutures (Ethicon, Cornelia, GA, U.S.A.) soaked in 50 μg/mL laminin and pinned 12 mm apart in the center of the culture surface. Two milliliters of PM was added to each plate.

Cell plating

Four million cells (cardiomyocytes + fibroblasts) were plated onto each 35-mm culture surface and cultured in an incubator at 37°C and 5% CO_2. After a period of 3 days, PM was replaced with growth medium (GM) consisting of 365 mL DMEM, 100 mL M199, 35 mL FBS, 5 mL antibiotic-antimycotic (Invitrogen Corporation, Carlsbad, CA, U.S.A.). Cardioids were formed within 14 days of initial cell plating.

Implantation and explantation of cardioids

Laser-cut elliptical acrylic frames were fabricated using a 120 W Laser Engraving Machine (Applied Laser Systems, Pasadena, CA, U.S.A.) from cast clear acrylic poly (methyl methacrylate) sheet. Isogeneic adult F344 rats (Charles River Laboratories, Wilmington, MA, U.S.A.) were anesthetized by an intraperitoneal injection of 0.5 mL of sodium pentobarbital (Abbott Laboratories, Chicago, IL, U.S.A.), shaved, prepped, and a pocket was dissected under the panniculus carnosus. Cardioids were secured to the frame with 8-0 nylon sutures (Ethicon). The frame with the cardioids was placed in the subcutaneous pocket and secured to the underlying mus-

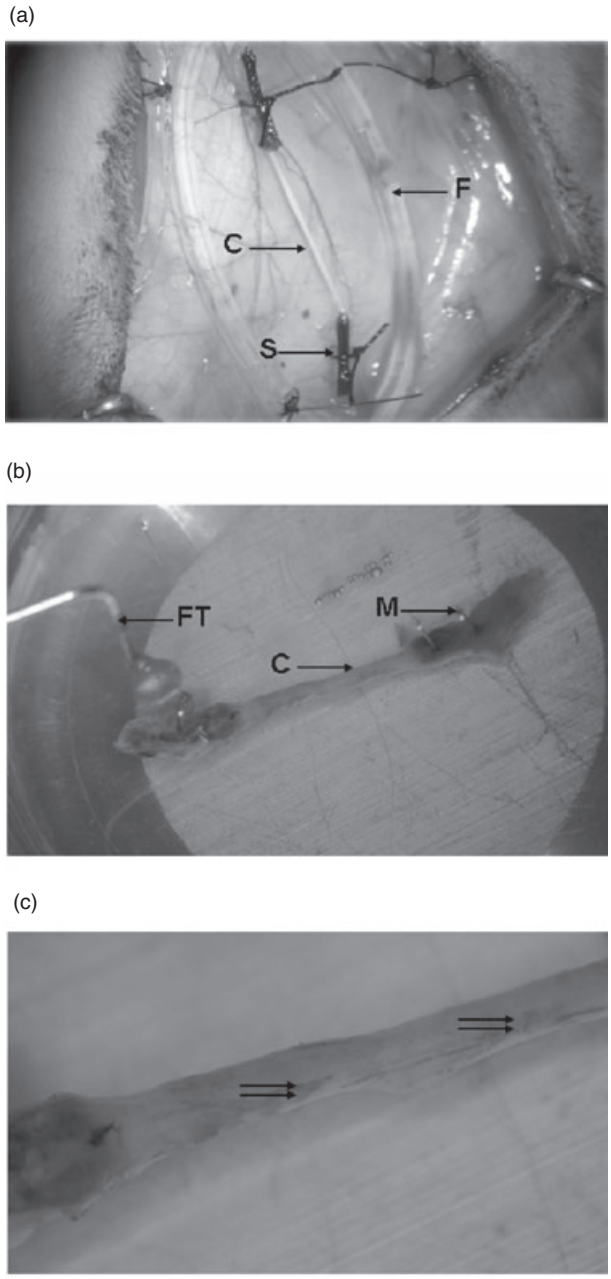

FIG. 1. Implantation model for engineered cardiac tissue. (a) Cardioids (C) were engineered in vitro and were attached to acrylic support frame (F) using sutures (S) and then implanted subcutaneously. (b) Three weeks after implantation, the cardioids were recovered and pinned using 0.1-mm minutien pins (M) to a 35-mm tissue culture surface coated with PDMS. The cardioids were electrically stimulated between parallel platinum electrodes (not shown) and the active force was recorded using an optical force transducer (FT). (c) High magnification image of a postimplantation cardioid showing areas neovascularization (double arrows).

cular fascia (Fig. 1A) with 6-0 polypropylene sutures (Ethicon). The incision was then closed with 4-0 nylon sutures (Ethicon). The animals were returned to an approved facility for recovery and were housed at this facility for a period of 3 weeks. A total of six cardioids were implanted in this manner. To recover implanted cardioids, host rats were anesthetized and the previous incision was re-entered. The cardioids were removed from the surrounding tissue. The cardioids were removed from the elliptical frame and pinned to a 35-mm tissue culture plate coated with PDMS. The animals were euthanized by an overdose of sodium pentobarbital and subsequent removal of the heart. The contractility of the explanted cardioids was evaluated immediately upon recovery. As controls, six cardioids were engineered in vitro and were tested 2 days after complete formation. One control cardioid was lost due to contamination. The remaining five control cardioids were processed for in vitro force testing and histological evaluation in the exact same manner as the explanted cardioids.

Evaluation of contractility

The method for evaluating the contractility of engineered cardiac muscle has been described in detail (16). Briefly, the cardioids were stimulated between parallel platinum electrodes and the active force was measured using a custom built optical force transducer (Fig. 1B). An electrical impulse of magnitude 5 V with a pulse width of 10 ms and a frequency of 1 Hz was used. The active force was measured using a custom made optical force transducer and force tracings were digitally recorded using LabVIEW (National Instruments Corporation, Austin, TX, U.S.A.). The length of each cardioid was adjusted using a micromanipulator to optimize the force generated. The peak active force was normalized to the total cross-sectional area of the cardioid to obtain the specific force. The cross-sectional area of the cardioids was calculated from the construct diameter which was determined by a calibrated eyepiece reticle with a resolution of 5 μm.

We electrically paced the explanted and control cardioids by varying the stimulation frequency between 1 and 5 Hz, while maintaining all other stimulation parameters.

The twitch force and the pacing characteristics were determined for all six explanted cardioids and five control cardioids. The six explanted cardioids were then divided into two groups of three cardioids for further testing. The first group was used for the evaluation of contractility, calcium sensitivity behavior, responsiveness to epinephrine and the length–force relationship. The second group was used for detailed histological evaluation. The control cardioids were also divided into two groups with two cardioids being utilized to obtain calcium sensitivity, responsiveness

to epinephrine and length–force data and three cardioids being used for histological evaluation.

To evaluate the effect of external calcium on contractility, known volumes of 100 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) were added to the bath to bind residual stores of calcium in the cardioids. Calcium was gradually added to the testing bath and stimulated contraction forces were re-evaluated with each addition.

The response of the cardioids to epinephrine was studied by adding known concentrations of 1 mg/mL epinephrine (Abbott Laboratories, North Chicago, IL, U.S.A.) to the GM, with the constructs still attached to the force transducer. The bath epinephrine concentration was varied from 0.2 to 1.2 μg/mL.

The length–force characteristics were evaluated by changing the length of the cardioids in increments of 300 μm and evaluating the active force at each increment. Initially, we evaluated the active force of the explanted cardioid by pinning the construct at length. We gradually decreased the length of the cardioids in increments of 300 μm and evaluated the active force at each increment. We continued this process until the active force decreased to a value of 20–30% of the active force obtained at length. The length of the cardioid was then increased in increments of 300 μN and the active force evaluated at each increment. As the length of the cardioid was increased, the active force first increased, then reached a plateau and then decreased. We stopped increasing the length of the cardioids when the active force decreased to about 20–30% of the maximum active force. The length at which the cardioids generated maximum force was termed L_o.

Histology

Cardioids were fixed in a 4% paraformaldehyde solution for 4 h, rinsed in 70% ethanol and stored in 70% ethanol until use. The cardioids were prepared using a graded ethanol process with an automated tissue processor (Shandon Hypercenter XP, Thermo Electron, Waltham, MA, U.S.A.) for 7 h and 36 min. The cardioids were then paraffin-embedded. The paraffin-embedded samples were sectioned at 7 μm on a microtome processor and placed on ProbeOn Plus slides (Fisher Scientific Company, Pittsburgh, PA, U.S.A.) for immunohistochemistry. Hematoxylin and eosin staining (H&E) was used for morphologic analysis. To identify and localize endothelial cells, immunohistochemical staining for von Willebrand factor (vWF) was performed. The slides were heated in an oven at 60°C for 20 min. The heated slides were then placed in three changes of xylene, 100% ethanol, 95% ethanol, and buffer to prepare for staining. Antigen retrieval was performed for the slides designated vWF by applying Proteinase K (DAKO Cytomation, Carpinteria, CA, U.S.A.) for 5 min at room temperature. The primary antibody vWF antibody, polyclonal rabbit anti-human (DAKO Cytomation) was incubated at a dilution of 1:250 at room temperature for 30 min. The Envision + Peroxidase Kit (DAKO Cytomation) was used for detection of the vWF antibody (DAKO Cytomation). Sections were viewed and photographed using a Nikon Axiophot inverted phase contrast microscope.

Electron microscopy

The method used for electron microscopy has been previously described (19). Briefly, cardioids were fixed for 4 h at 4°C in Karnovsky's solution consisting of 0.1 M sodium cacodylate buffer with 3% formaldehyde and 3% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA, U.S.A.) at pH 7.4. Cardioids were rinsed three times (30 min, 30 min and 4 h) with cacodylate buffer, pH 7.4 with 7.5% sucrose. Cardioids were postfixed in 1% osmium tetroxide for 2 h at room temperature, dehydrated in graded concentrations of ethanol and propylene oxide and embedded in EPON, Eponate 12 resin (Ted Pella Inc., Redding, CA, U.S.A.). Embedded specimens were sectioned at 600 nm thick with a diamond knife on a Sorvall MT-5000 ultramicrotome (RMC, Inc., Tuscon, AZ, U.S.A.). Ribbons of sections were mounted on copper grids, stained with uranyl acetate and lead citrate and examined using a Philips CM-100 transmission electron microscope. Photographs were obtained using a Kodak 1.6 Megaplus (Kodak, Rochester, NY, U.S.A.) high-resolution digital camera.

Statistical analysis

We used the two-sample *t*-test to evaluate changes in contractility and responsiveness to epinephrine resulting from implantation. Minitab V13.31 (State College, PA, U.S.A.) was used for statistical analysis.

RESULTS

The gross appearance of the explanted cardioids was evaluated visually upon recovery. The explanted cardioids exhibited a high of spontaneous contractility directly after explantation, which was determined to be at a frequency of 1–2 Hz. Visual examination also revealed the presence of superficial capillaries at multiple sites along the length of the cardioids. This was evident in all six of the explanted cardioids.

Histological evaluation of cardioids consisted of staining with H&E, staining for vWF and transmis-

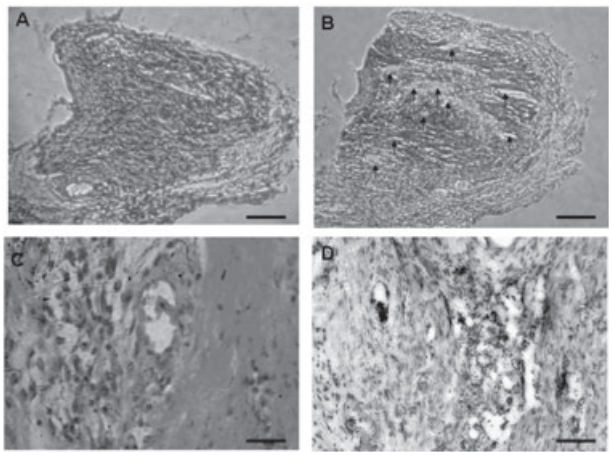

FIG. 2. Immunohistochemistry of explanted cardioids. (A) Staining with hematoxylin and eosin showed the presence of viable cells. (B) von Willebrand staining showed the presence of capillaries (single arrows) throughout the tissue section. (C) and (D) show higher magnification images of hematoxylin and eosin staining and von Willebrand staining. Scale bar represents 75 μm for (A) and (B), 20 μm for (C), and 40 μm for (D).

sion electron microscopy. Neovascularization was macroscopically evident in postimplantation cardioids (Fig. 1C). H&E staining revealed the presence of viable cells throughout the explanted tissue constructs (Fig. 2A). Staining for vWF showed the presence of a significant amount of endothelial cells that had organized to form luminal structures throughout the explanted cardioids (Fig. 2B). Transmission electron micrographs showed the presence of a very large amount of contractile proteins arranged in a hexagonal array. There was also a high degree of intercellular connectivity and the presence of intercalated

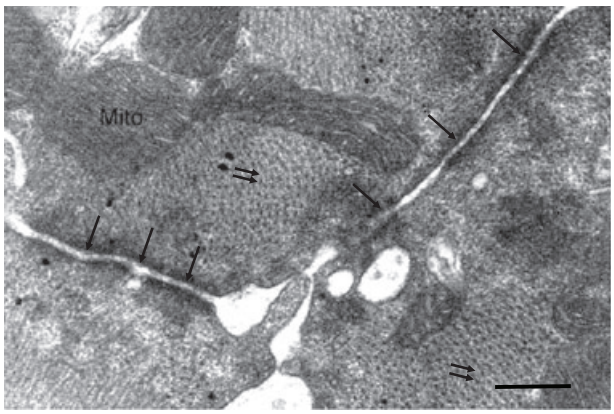

FIG. 3. Electron micrographs (EM) of explanted cardioids. Cross section showing the presence of large amounts of myofilaments (double arrow) with a significant amount of mitochondria (Mito). Intercalated discs between adjacent myocytes are also evident (single arrows). Scale bar represents 500 nm.

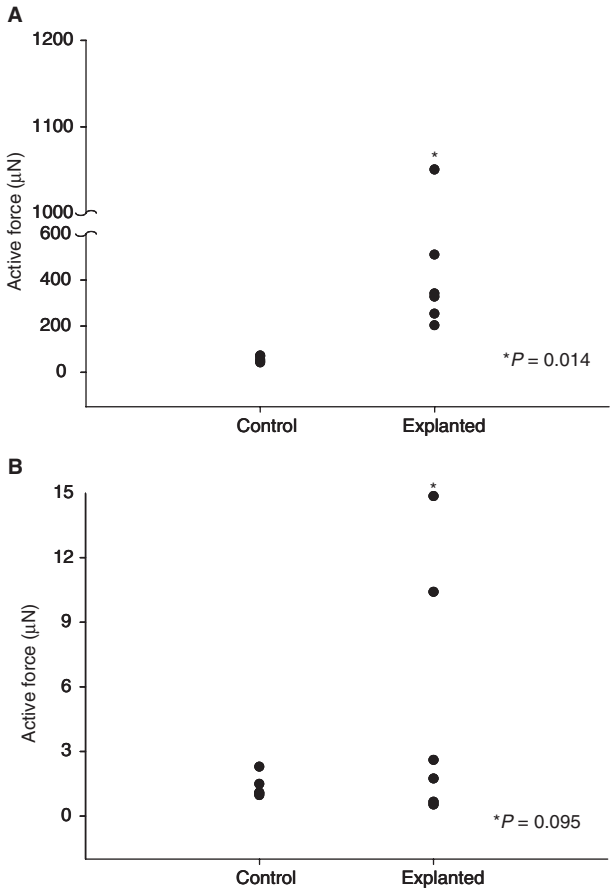

FIG. 4. Contractility measures for experimental cardioids. (A) Peak active force of explanted cardioids was found to be 447.5 ± 323.5 μN (n = 6) and 56.5 ± 13.5 μN (n = 5) for control cardioids. (B) Specific force (active force normalized to total cross-sectional area) was found to be 5.1 ± 6.3 kPa (n = 6) for explanted cardioids and 1.4 ± 0.5 kPa (n = 5) for control cardioids.

discs was evident. A large amount of mitochondria were found to be associated with the myofilaments (Fig. 3).

We evaluated the contractile properties of the cardioids and the active force and specific force were determined (Fig. 4). The peak active force for explanted cardioids was found to be 447.5 ± 323.5 μN (n = 6) and 56.5 ± 13.5 μN (n = 5) for control cardioids (Fig. 4A). The maximum force generated by a single explanted cardioid was found to be 1050 and 71.8 μN for a single control cardioid. The average specific force was found to be 5.1 ± 6.3 kPa (n = 6) with a maximum value of 14.9 kPa for explanted cardioids (Fig. 4B). The average specific force for control cardioids was found to be 1.4 ± 0.5 kPa (n = 5) with a maximum value of 2.3 kPa.

The cardioids were electrically paced at frequencies of 1–5 Hz. The explanted cardioids were able to

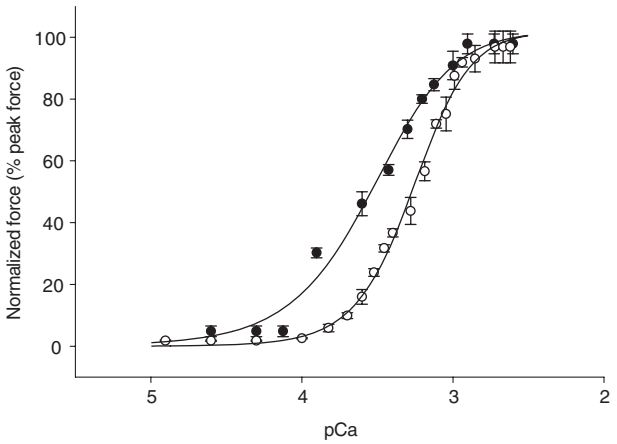

FIG. 5. Calcium sensitivity of experimental cardioids. Cardioids were pinned to a PDMS surface and completely submerged in calcium free DMEM. Residual calcium was removed by adding EGTA to the testing bath and fresh calcium free DMEM re-introduced to the bath. Calcium was incrementally introduced and the active force evaluated at each incremental addition of calcium. The procedure was repeated until there was no increase in active force production upon the addition of calcium. This procedure was repeated for three explanted and two control cardioids. The curves shown represent the calcium sensitivity of a single explanted and a single control cardioid. Values shown represent the mean and standard deviation.

completely relax, that is, produce a force of zero, between active contractions, with the force returning to baseline values when paced at 1 and 2 Hz. This was also the case when explanted cardioids were electrically paced at 5 Hz. This was unlike the case for control cardioids. Control cardioids were able to relax by only 15% of peak force at a frequency of 1 Hz. The ability of control cardioids to relax between active contractions was reduced with increasing pacing frequencies.

We evaluated the responsiveness of cardioids to external calcium and found that the calcium sensitivity characteristics of both explanted and control cardioids were very similar. In the absence of calcium in the testing bath, the active force was not different from baseline noise. However, incremental addition of exogenous calcium produced a sigmodal increase in active force (Fig. 5).

The responsiveness of cardioids to epinephrine was evaluated (Fig. 6). The cardioids exhibited baseline spontaneous contractility upon explantation which persisted for the duration of the force testing. The spontaneous contractility of the cardioids increased with the incremental addition of epinephrine. Addition of 0.2 μg/mL of epinephrine initiated this response and the maximum response was achieved by the addition of 1.2 μg/mL of epinephrine (Fig. 6A–C). A similar response was observed for control cardioids, although the increase in spontaneous contractility was not as significant (Fig. 6D–F). This phenomenon was completely reversible. Complete removal of epinephrine from the bath re-established baseline spontaneous contractility of cardioids, as evaluated by the frequency of spontaneous contractility. A total of three explanted and three control cardioids were tested in this way (Fig. 6G). Epinephrine was found to have an increase in the spontaneous contractility of explanted cardioids.

We evaluated the length–force characteristics of the cardioids. Increasing the length of the cardioids resulted in a proportional increase in active force (Fig. 7). As the length of the cardioid was further increased, a plateau was reached and a further increase in length resulted in a proportional decrease in the active force.

DISCUSSION

The long-term goal of cardiac tissue engineering is to generate functional cardiac muscle in vitro. Tissue-engineered heart muscle may prove to be a valuable tool in drug testing and as a three-dimensional model for basic cardiology research. Various strategies have been utilized to generate three-dimensional cardiac muscle in vitro. Synthetic scaffolds and biodegradable gels have been utilized to promote the formation of cardiac muscle and various self assembly strategies have been successfully implemented as well. One challenge in engineering functional cardiac muscle is to promote the formation of new blood vessels as an intrinsic component of the tissue construct. The blood vessels would act as functional sites for nutrient exchange thereby promoting the long-term survival of tissue-engineered cardiac muscle.

We have previously developed a method to engineer three-dimensional cardiac tissue and have termed the resulting tissue constructs “cardioids” (16). Cardioids are formed from the spontaneous delamination of a confluent monolayer of neonatal cardiac myocytes. Cardioids are electrically excitable and can generate an active force of up to 125 μN upon electrical stimulation. Cardioids can also be electrically paced and exhibit physiological responses to calcium and epinephrine.

An interesting aspect of the cardioid model is the retention of cardiac fibroblast in the initial cell population. The purpose of the cardiac fibroblasts is to generate extracellular matrix (ECM) components which provide mechanical stability during cardioid formation. This ECM is considered to be a suitable substrate for the generation of cardiac muscle and results in physiological functionality of the engi-

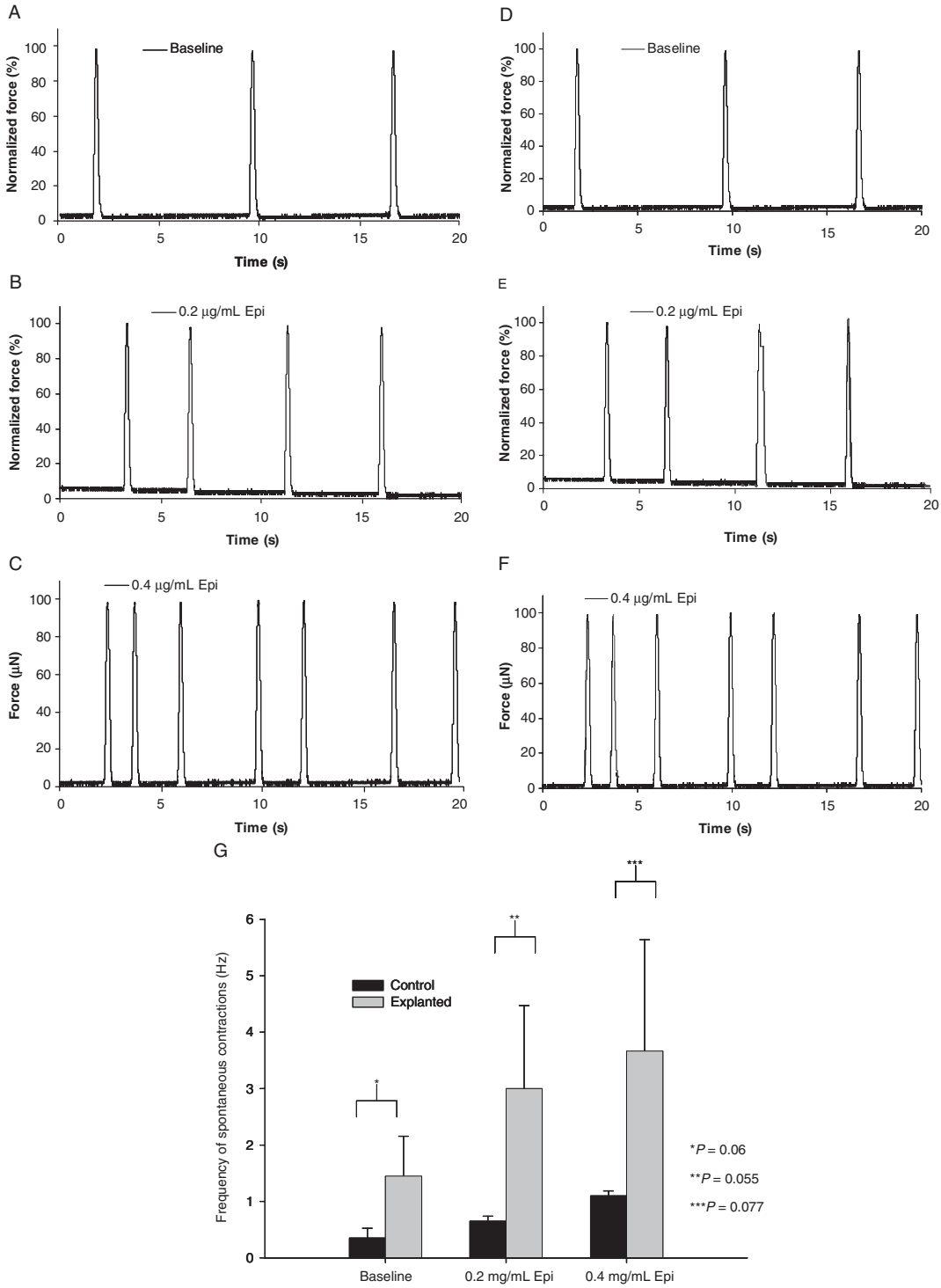

FIG. 6. Responsiveness of experimental cardioids to epinephrine. (A–C) show the behavior of control cardioids and (D–F) show the behavior of explanted cardioids. All force tracings represent spontaneous contractions. In the case of control cardioids, there was a baseline spontaneous contractility with a frequency of 0.15 Hz. Stimulation with 0.2 μg/mL epinephrine increased the frequency to 0.2 Hz while stimulation with 0.4 μg/mL epinephrine further increased the frequency to 0.4 Hz. In comparison, explanted cardioids had a spontaneous contractility of 0.5 Hz, which increases to 1.5 Hz when stimulated with 0.2 μg/mL epinephrine. Increasing the epinephrine concentration to 0.4 μg/mL epinephrine caused no further increase in the number of contractions. (G) The responsiveness of three explanted and three control cardioids was evaluated. The baseline spontaneous contractility increased from 0.35 ± 0.17 Hz (n = 3) for control cardioids to 1.45 ± 0.71 Hz (n = 3) for explanted cardioids. The frequency of spontaneous contractions at an epinephrine concentration of 0.2 mg/mL was found to be 1.1 ± 0.1 Hz (n = 3) for control cardioids and 3.7 ± 2.0 Hz (n = 3) explanted cardioids.

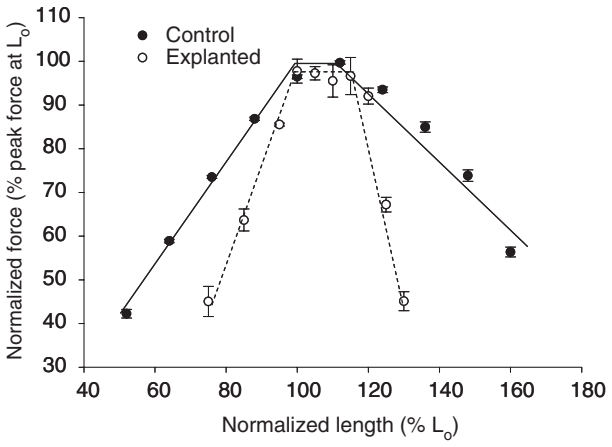

FIG. 7. Length–force relationship of experimental cardioids. The length of each cardioid was optimized for maximum force production. This value was considered L_o and the force measured at every other length was normalized to the force at L_o. In two explanted cardioids and two control cardioids, multiple measurements were recorded over a range of adjusted lengths to fully characterize the length–force relationship. The curve shown is one of two constructs tested in this manner. Values shown represent the mean and standard deviation.

neered construct. An important variable effecting cardioid formation is the ratio of cardiac fibroblasts to myocytes. A low percentage of cardiac fibroblasts will not generate adequate ECM resulting in the formation of mechanically unstable cardioids. A very high percentage of cardiac fibroblasts will result in a lower active force due to the lower percentage of cardiac myocytes. As the cardioid model is more fully characterized, an optimal ratio of cardiac fibroblasts to myocytes will need to be identified.

The purpose of this study was to promote the formation of a vasculature within previously engineered cardioids. Cardioids were implanted in a subcutaneous incision in recipient rats for a period of 3 weeks and then recovered for in vitro force testing and histological evaluation. The model presented in this study is closest to the model presented by Shimizu et al. (4). Shimizu et al. described subcutaneous implantation of engineered cardiac tissue and the constructs were shown to survive for a period of 3 months (4). Significant neovascularization of the implanted constructs was demonstrated (4). However, the effect of neovascularization on the contractility of the tissue-engineered cardiac muscle was not evaluated.

The model presented in this study showed that the cardioids remained viable and maintained a differentiated phenotype during the implantation period. The viability of the cardioids was firstly noted by the spontaneous contractility upon recovery. The viability of the cardioids was further supported by histological and contractility data. Support for differentiated muscle phenotype came from electron micrographs which show the presence of myofilaments that are characteristic of muscle cells. More compelling was the presence of the intercalated discs, which are specific to cardiac myocytes.

The contractility data showed that there was an improvement in function of cardioids resulting from subcutaneous implantation. When considering the contractility metrics of explanted cardioids, it would be interesting to compare the performance of the explanted cardioids to control cardioids, to normal cardiac muscle tissue and to other published models of tissue-engineered cardiac muscle. The maximum active force of explanted cardioids was found to be 1050 μN while the maximum specific force was calculated to be 14.9 kPa. In comparison, the maximum active force generated by control cardioids was found to be 71.8 μN with a maximum specific force of 4.1 kPa. There was a significant increase in the force generating capabilities of the cardioids resulting from implantation. Although the exact mechanism for the increased force production is not clear, vascularization of the cardioids may be a contributing factor. Vascularization could promote the survival of cardiac cells within the tissue-engineered constructs thereby leading to increased cell viability. In addition, cardioids are also exposed to systemic signaling in the host environment which could also modulate contractile properties.

It would be insightful to compare the force generating capacity of explanted cardioids to normal myocardial tissue. One of our goals is to develop models of tissue-engineered cardiac muscle that closely resemble normal myocardium and incorporation of a vasculature is an attempt to achieve this. An adult rat papillary muscle has been shown to generate a peak active of 8130 μN and a specific force of 44.4 kPa (20). Both the active force and the specific force for explanted cardioids are significantly lower than that of an adult papillary muscle. Although there was an improvement in contractility of control cardioids resulting from implantation, the force generating capacity of explanted cardioids was not comparable to normal adult cardiac tissue. This could be due to the early developmental stage of the neonatal cardiac myocytes utilized in this study. Developmentally immature cardiomyocytes are known to contain significantly smaller amounts of contractile proteins per unit cross section of cardiac tissue (21).

It would be interesting to compare the function of explanted cardioids to other published models of tissue-engineered heart muscle. There have been several models described in the literature and the

contractile properties of engineered heart tissue (EHT) have been described in detail. EHTs are generated by casting a suspension of cardiomyocytes in a collagen gel (15). EHTs have been shown to generate an active force of 350 μN with a specific force of 0.4 kPa under basal conditions (15). In comparison, the average specific force of control cardioids was found to be 4.1 kPa and the average specific force of explanted cardioids was found to be 14.9 kPa. Explanted cardioids generate significantly higher forces than EHTs. This could be due to the absence of synthetic scaffolding material in the contractile region of cardioids, thereby permitting uninhibited contractions.

There have not been any published studies detailing the changes in contractile properties of engineered muscle resulting from implantation. Therefore a direct comparison of explanted cardioids is not possible at this point.

In order to further characterize cardiac-specific phenotype, we subjected the cardioids to electrical pacing by varying the stimulation frequency from 1 to 5 Hz. The explanted cardioids were successfully paced at the physiologically relevant frequencies tested. What was most intriguing was the ability of the explanted cardioids to completely relax between active contractions. This behavior was unlike the pacing characteristics of the control cardioids, which were unable to completely return to baseline conditions during active contractions. The ability of the explanted cardioids to relax to baseline conditions between active contractions could be due to changes in calcium handling properties. An increase in the amount of sarco/endoplasmic reticulum Ca++ ATPase (SERCA) or a decrease in the amount of phospholamban could lead to an increase in the rate of calcium handling in the explanted cardioids. During the course of electrical pacing, we observed a decrease in the active force with consecutive contractions for explanted cardioids. This was progressively more significant with increasing pacing frequencies. The decrease in active force is indicative of muscle fatigue and may be due to repetitive contractions of the vascular cardioid without the active flow of nutrients through the newly formed blood vessels.

In order to further characterize the phenotype of the explanted cardioids, we evaluated the responsiveness to external calcium, the responsiveness to the cardio-active drug epinephrine and determined the length–force relationship. We found that the explanted cardioids exhibited a sigmodal response to external calcium. The responsiveness to calcium was very similar to normal cardiac tissue and shows the presence of functional calcium handling properties. The explanted cardioids exhibited an increase in chronotropy in response to epinephrine. The increase in chronotropy could be due to the increased calcium handling properties of the explanted cardioids and provide further support for a cardiac-specific phenotype. The final metric that we evaluated was the length–force curve. Note from Fig. 7 that the active length–tension curve from the explanted cardioids is narrower than the length–tension curve from the cardioids grown in culture. We hypothesize that this is due to improved structural organization both within and between the cardiomyocytes in the explanted cardioids. Specifically, we hypothesize that within the explanted cardioids the sarcomeres are more fully in register and have functional structures for the lateral transmission of force between adjacent cardiomyocytes, further retaining the registration of the contractile apparatus both within and between cells.

CONCLUSIONS

In this study we describe a model for the vascularization of cardioids and subsequent improvement in contractile metrics. The model consists of securing cardioids in a subcutaneous pocket in recipient rats for 3 weeks. Histological data showed the presence of vascularization throughout the cardioids. Electron micrographs provided evidence for the differentiated phenotype of the explanted cardioids. Contractility metrics showed that there was an improvement in functionality of cardioids.

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