Cardiac Tissue Engineering in an In Vivo Vascularized Chamber

Andrew N. Morritt, MBChB, MRCS; Susan K. Bortolotto, PhD; Rodney J. Dilley, PhD; XiaoLian Han, BSc; Andrew R. Kompa, PhD; David McCombe, MD, FRACS; Christine E. Wright, PhD; Silviu Itescu, MBBS; James A. Angus, PhD; Wayne A. Morrison, MD, FRACS

Background—Cardiac tissue engineering offers the prospect of a novel treatment for acquired or congenital heart defects. We have created vascularized pieces of beating cardiac muscle in the rat that are as thick as the adult rat right ventricle wall.

Method and Results—Neonatal rat cardiomyocytes in Matrigel were implanted with an arteriovenous blood vessel loop into a 0.5-mL patented tissue-engineering chamber, located subcutaneously in the groin. Chambers were harvested 1, 4, and 10 weeks after insertion. At 4 and 10 weeks, all constructs that grew in the chambers contracted spontaneously. Immunostaining for \(\alpha\)-sarcomeric actin, troponin, and desmin showed that differentiated cardiomyocytes present in tissue at all time points formed a network of interconnected cells within a collagenous extracellular matrix. Constructs at 4 and 10 weeks were extensively vascularized. The maximum thickness of cardiac tissue generated was 1983 \(\mu\)m. Cardiomyocytes increased in size from 1 to 10 weeks and were positive for the proliferation markers Ki67 and PCNA. Connexin-43 stain indicated that gap junctions were present between cardiomyocytes at 4 and 10 weeks. Echocardiograms performed between 4 and 10 weeks showed that the tissue construct contracted spontaneously in vivo. In vitro organ bath experiments showed a typical cardiac muscle length-tension relationship, the ability to be paced from electrical field pulses up to 3 Hz, positive chronotropy to norepinephrine, and positive inotropy in response to calcium.

Conclusion—In summary, the use of a vascularized tissue-engineering chamber allowed generation of a spontaneously beating 3-dimensional mass of cardiac tissue from neonatal rat cardiomyocytes. Further development of this vascularized model will increase the potential of cardiac tissue engineering to provide suitable replacement tissues for acquired and congenital defects. (Circulation. 2007;115:353-360.)

Key Words: angiogenesis • myocytes • tissue • tissue engineering

Heart failure after myocardial infarction is a major cause of morbidity and mortality worldwide. For end-stage heart failure, heart transplantation is the most effective treatment with good long-term results, but transplantation is limited by inadequate donor organ supply, the complications of immunosuppression, and organ rejection. A possible strategy to restore heart function after myocardial infarction is to replace or augment the damaged tissue with healthy tissue.3 Engineered cardiac tissue could also be used to reconstruct congenital cardiac defects and, in basic cardiology research, could be used to assess the efficacy and safety of new drugs.

Clinical Perspective p 360

The thickness of cardiac tissue engineered in vitro6–10 is limited by the maximum diffusion distances for oxygen and nutrients. Consequently, only thin layers (100 to 200 \(\mu\)m) of tissue could be generated, which leaves the construct interior relatively acellular.6,9,11,12 Although engineered cardiac tissue is quickly vascularized after implantation,6,7,13,14 diffusion alone is unlikely to support thick avascular myocardial constructs. Angiogenesis becomes essential to successfully engineer tissues with a thickness \(>200\ \mu\)m.15

Our group has successfully generated vascularized tissue that incorporates its own supportive extracellular matrix by placement of an arteriovenous blood vessel loop (AV loop) inside a semi-sealed polycarbonate chamber that is implanted into the groin of a rat.16 This encapsulated tissue is supplied by its own vascular pedicle and is transplantable by microsurgical techniques to other parts of the body,16 or possibly to an extracorporeal circulation in vitro. This model supports the survival and growth of adipose tissue,17 implanted skeletal muscle myoblasts,17 and fibroblasts.18

In the present study, we examined the ability of this model to support the survival and growth of implanted neonatal rat
cardiomyocytes. The chamber was seeded with cardiomyocytes and left to develop over several weeks. Tissue harvested between 1 and 10 weeks contained differentiated cardiomyocytes and was profusely vascularized. All tissue constructs harvested after 4 weeks were observed to contract spontaneously and could be paced and pharmacoologically regulated. Cardiac tissue growth was supported by cellular growth and proliferation.

Materials and Methods
All procedures were performed with the approval of the St. Vincent’s Hospital Animal Ethics Committee, under National Health and Medical Research Council guidelines for animal welfare.

Primary Culture of Neonatal Rat Ventricular Myocytes
Neonatal rat cardiomyocytes were prepared by a established method.19 In brief, ventricles from Sprague-Dawley rats (1 to 3 days old; Experimental Medical and Surgical Unit, St Vincent’s Hospital, Melbourne, Australia) were minced into 1 to 3 mm3 fragments and digested in HEPES-buffered 0.1% collagenase/0.1% trypsin/0.1% DNase solution. Cardiomyocytes were separated by Percoll centrifugation, then cultured at 37°C, then for 15 minutes on ice,20 suspended in 150 μL of Matrigel (Becton Dickinson, Bedford, Mass), and then stored on ice until use.

Preparation of Vascularized Tissue-Engineering Chamber and Implantation of Cardiomyocytes
An AV loop was constructed in the right groin region of male CBH/rmu/mu (nuke) rats (Figure 1A) (ARC, Perth, Australia) that weighed 200 to 340 g as previously described.16-18,21-23 The Matrigel cell suspension was placed in the base of the chamber, which had measurements of 0.5 mL internal volume, 1.3 cm internal diameter, 0.5 cm height (Department of Chemical Engineering, The University of Melbourne, Melbourne, Australia), that surrounds the AV loop vessel wall. The tissue was mounted vertically between stainless steel S-shaped anchors and fixed support between 2 parallel platinum field electrodes (5 mm apart and 5 cm long). The signal was amplified (Baker Medical Research Institute Amplifier Model 108, Victoria, Australia) and were embedded in paraffin, and histological sections (5 μm thick) were made and routinely stained with hematoxylin-eosin for evaluation of morphology. Selected slides were nuclear-stained with DAPI (0.1 μg/mL; Molecular Probes) and fluorescence microscopy to identify Dil-labeled cells. Fluorescein-labeled Griffonia simplicifolia lectin 1 (120 μg/mL; Vector Laboratories, Burlingame, Calif) was used to identify endothelial cells. Immunohistochemistry was performed to detect muscle cells with α-sarcomeric actin (clone SC5, 0.5 μg/mL; Sigma, St Louis, Mo), troponin I (2 μg/mL; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), and desmin (clone D33, 2.5 μg/mL; Dako, Glostrup, Denmark).

Morphometry
Desmin-immunostained sections were analyzed by videomicroscopy with a computer-generated 6-point square grid (CAST system, Olympos Denmark, Albertslund, Denmark). Fields were sampled systematically, such that 10% of the specimen was assessed with a minimum of 352 points/specimen. The tissue was categorized into: (1) desmin-stained cardiac muscle, (2) new nonmuscle (connective/granulation) tissue, (3) AV loop, (4) Matrigel, and (5) adipose tissue. Total volume of cardiac muscle in the tissue was calculated by multiplying the percentage of tissue stained as cardiac muscle (determined above) by the total tissue construct volume at harvest.

Echocardiography of the Construct
Construct contraction inside the chamber was evaluated by echocardiography. Rats (n=8) were anesthetized between 3 and 9 weeks with 40 mg/kg ketamine and 5 mg/kg xylazine IP and ECG was used to detect heart rate. A 10-MHz pediatric ultrasound probe (GE Vingmed Ultrasound AS, Horten, Norway) was placed over the chamber to detect construct contraction inside the chamber.

In Vivo Organ Bath Study
A transverse section of tissue 1 to 2 mm thick was cut from the construct, placed immediately in physiological salt solution26 at 37°C, and saturated with 95% O2 and 5% CO2 during the experiment. The tissue was mounted vertically between stainless steel 5-shaped hooks on an acrylic leg in physiological salt solution-filled 20-mL glass-jacketed organ bath heated to 37°C. The upper end was attached to an isometric force transducer (Grass FT03C, Grass Instruments, Quincy, Mass) and the lower end was attached to a fixed support between 2 parallel platinum field electrodes (5 mm apart and 5 mm long). The signal was amplified (Baker Medical Research Institute Amplifier Model 108, Victoria, Australia) and...
used to trigger a data acquisition system (Powerlab Chart v5.4, AD Instruments, Castle Hill, Australia). Tissue period was continuously recorded. The tissue was washed and allowed to equilibrate for 30 minutes.

The tissue section was continuously stimulated with a Grass S88 stimulator (Grass Instruments), with an electrical pulse of 20% suprathreshold, pulse width of 0.3 ms, and frequency of 1 Hz. The tissue was paced by varying the stimulation frequency between 0.2 and 3 Hz while keeping other parameters constant.

The length-force relationship was investigated at 4 (n=3) and 10 (n=6) weeks by raising passive stretch force to 0.2g and adjusting pacing voltage to suprathreshold to stimulate active contraction. When the tissue had stabilized, passive stretch force was raised to 1g in incremental steps every 40 to 60 sec before resetting the passive force to 0.5g.

To investigate the response to epinephrine, the tissue (n=7) was equilibrated with norepinephrine (Sigma), and the organ bath concentration was increased to 10, 100, and 1000 nM at 10-minute intervals before replacing the organ bath with drug-free solution.

To construct calcium concentration response curves in 5 tissues, the calcium concentration was first lowered from the normal level (2.5 mmol/L) to 1 mmol/L by replacing the physiological salt solution with a modified solution for 10 minutes. Subsequently, with pacing at 1 Hz, the bath solution was replaced every 5 minutes with incrementally increased concentrations of calcium (1.5, 2, 2.5, and 3 mmol/L) before it was returned to normal.

Statistics
Data are expressed as mean±SEM. An unpaired Student t test (2-tailed) was performed to compare 2 groups. Multiple group comparison was performed with 1-way ANOVA and subsequent Bonferroni multiple comparison post hoc tests. A value of P<0.05 was considered significant.

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

Results
Spontaneous Contraction
The AV loop remained patent in all chambers. Constructs were observed to contract spontaneously at 4 weeks (online-only Data Supplement, Movie I) and 10 weeks at ~50 to 60 bpm (55±2 bpm) in contrast to the animals’ heart rate of ~200 to 250 bpm (237±5 bpm).

Echocardiograms performed between 3 and 10 weeks (n=8) showed construct contraction within the chamber (n=7) (online Data Supplement, Movie II). In 1 animal, contraction of the tissue construct within the chamber could not be detected by echocardiography; however, when the chamber was opened the tissue was observed to contract spontaneously.

In Vitro Organ Bath Study
Paced at 1 Hz, the construct responded to increases in passive stretch with a length-dependent increase in contractile force (Figure 2A). Active force doubled as the tissue was stretched and declined as the passive force was reduced. Maximum force generated was 1.15±0.37 mN (n=7). The constructs responded to pacing frequencies of 0.25 to 3 Hz (Figure 2B). At higher frequencies (not shown), the tissue contractions were erratic, which indicated some refractoriness to the pacing stimulus. When norepinephrine was applied to the constructs paced at 1 Hz, basal force increased (Figure 2C) in response to the increase of free calcium from 1 mmol/L to 3 mmol/L (Figure 2D). Baseline force did not change as calcium increased, but peak contraction was augmented by ~80% at 3 mmol/L compared with the force at 1 mmol/L calcium.

Histology and Immunohistochemistry
DiI-labeled cardiomyocytes were present throughout the chamber tissue at 1 week, but were predominantly distributed around the AV loop (Figure 3A). Hematoxylin-eosin staining showed small blood vessels and minimal inflammation throughout the tissue (Figure 3B). The construct was composed of muscle cells, Matrigel, and nonmuscle (connective/granulation) tissue. Cardiomyocytes positive for troponin (Figure 4A), α-sarcomeric actin (Figure 4B), and desmin (Figure 4C) around the AV loop had begun to elongate and showed occasional sarcomere formation, whereas those located in the periphery of the construct remained small and...
rounded. Lectin immunostaining (Figure 3C) indicated that profuse angiogenesis had occurred.

By 4 weeks the tissue around the AV loop contained a vascularized interconnected network of elongated and differentiated cardiomyocytes with defined sarcomeres. Two of the 8 tissue constructs contained a small amount of adipose tissue (not shown). By 10 weeks, hematoxylin-eosin (Figure 3E), desmin (Figures 4F and 6), troponin (Figure 4D), and α-sarcomeric actin (Figure 4E) stains showed that the cardiomyocytes, some of which were still DiI-labeled (Figure 3D), had elongated further and had well-defined sarcomeres. Control chambers filled with Matrigel alone (no cells) did not display any staining for cardiomyocytes (not shown). Lectin staining (Figure 3F) of cardiac tissue constructs showed a high density of capillaries in the tissue. Connexin-43 was expressed around the cardiomyocyte periphery at 4 weeks (Figure 3F) of cardiac tissue constructs showed a high degree of vascularization (green). Scale=20 μm.

Tissue Mass and Composition

The tissue mass attained at 4 to 10 weeks was relatively large, with a range of compact cardiac tissue thickness between 300 and 1983 μm (703±108 μm). The composition of constructs was evaluated by counting points (Table). The proportion of cardiac muscle in the constructs increased 3-fold from 1 week (4±2%) to 4 weeks (12±2%), and further increased at 10 weeks (15±4%; 1 versus 4 weeks, P<0.05; 1 versus 10 weeks, P<0.05). The majority of the construct was composed of new nonmuscle tissue: 62±11% at 1 week and 76±3% at 10 weeks. The proportion of the construct occupied by the AV loop progressively decreased from 21±5% at 1 week to 6±2% at 4 weeks, and decreased further to 4±1% at 10 weeks (1 versus 10 weeks, P<0.05). The proportion of Matrigel in the construct decreased from 13±10% at 1 week to 4±2% at 10 weeks, although this was not statistically significant. Because of the progressive resorption of edema fluid and resolution of the inflammatory capsule around the construct, the diminution in the size of the AV loop, and the resorption of the Matrigel, the overall weight and volume of the construct was greatest at 1 week when it almost filled the chamber space. This diminished from 0.3±0.02 mL volume and 0.34±0.03 g weight to 0.13±0.02 mL volume and 0.15±0.01 g weight by 10 weeks (volume at 1 versus 10 weeks, P<0.05; weight at 1 versus 10 weeks, P<0.05).

Discussion

In comprehensive reviews that summarize progress in cardiac tissue engineering as recently as July 2006 and December 2005, strategies of repair are discussed: the classic tissue-engineering protocol of cells seeded into a solid matrix shaped to mimic the organ to be produced; cells seeded into...
a liquid scaffold, which is then implanted; and cells without matrix, which are cultured into sheets and then implanted into the animal. All of these techniques involve initial preparation of implanted cells in vitro, with or without scaffold, and subsequent insertion into living animals with the expectation that the tissue will revascularize spontaneously. The most effective of these methods would appear to be the monolayer or the coil types; however, in both of those methods the maximum thickness of confluent muscle strands that form in vitro is 50 to 100 μm, limited by adequacy of blood supply. High oxygen demand by cardiomyocytes is reflected in capillary density (≈3000 capillaries/mm²) and intercapillary distances of ≈20 μm. The link between angiogenesis and normal cardiac development is well established. Strategies to overcome the problems of ischemic growth limitations and the reconnections once implanted in vivo currently involve in vitro increase in oxygenation, incorporation of perfused vessels in vitro, which are subsequently joined at the time of implantation, cocultures with endothelial cells, and provision of growth factors such as vascular endothelial growth factor. Weaving several constructs in a “chain mail” onlay can maximize revascularization when implanted in vivo.

Our approach is fundamentally different and involves the development of an in vivo 3-dimensional cell culture device where cells are seeded into a protected space with an intensely angiogenic environment and an appropriate liquid matrix. Here the cells grow in parallel with the newly developing capillary bed to form a vascularized interconnected network of cardiac tissue. We have previously shown in both rat and mouse chambers that other implanted tissues and cells, such as muscle, myoblasts, fat, pancreatic islets, fetal tissue, and fibroblasts, can survive with this methodology. By seeding cardiomyocytes into a chamber in the rat, we have grown a living piece of cardiac tissue with a volume of ≈0.2 mL and thickness up to 1983 μm, which easily exceeds the dimensions expected to be supported by diffusion alone (Figure 6). The mean thickness of compact cardiac tissue is similar to that of the adult rat right ventricular wall, is many times larger than all heart tissues engineered in vitro to date, and is comparable to the thickness attained by polysurgery of cell sheet grafts. This approach has recently been adopted in the flow-through pedicle model, which is less angiogenic than the AV loop model, which perhaps explains why up to 20 million cells generated only small amounts of tissue. The concept of tissue engineering in situ with a dedicated blood supply permits the implanted cells, aided by the invasion of inflammatory cells, fibroblasts, and endothelial cells, to orchestrate their own endogenous cascade of appropriate cytokines, chemokines, and matrix production. This occurs in a protected 3-dimensional system that mimics cell culture in vivo. It is now well appreciated that cell behavior, which includes migration, proliferation, and differentiation, is very different in 3-dimensional compared with 2-dimensional environments. The latter is highly artificial and does not reflect in vivo biology. Our method facilitates the development of a

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**Figure 5.** Representative micrographs that show connexin-43, Ki67, and PCNA immunostaining. A, Connexin-43 staining (brown) at 4 weeks showed gap junctions distributed in a scattered, punctate fashion. B, At 10 weeks, gap junctions had increased staining density and concentration at intercalated disc (arrows). C, Ki67/troponin staining of cardiomyocytes at 1 week. D, PCNA/troponin staining of cardiomyocytes at 10 weeks. Scale=10 μm.

**Figure 6.** A representative low-power desmin immunostain at 10 weeks shows the distribution of cardiac muscle tissue (brown) in relation to the original artery (a) and vein (v). Scale=500 μm.
composite tissue, which comprises not only the specialized cardiac muscle fibers but also the essential nonmuscle elements of mature cardiac tissues.

Previous work related to this rat chamber has shown that angiogenesis occurs by 3 days and is maximal at 7 days.23 As tissue forms, the new capillaries progressively extend toward the periphery of the chamber while the earlier vessels mature, which generates an environment that is suitable for survival of implanted cells and generation of new tissue in vivo.23 In the present study, immunostaining demonstrated new blood vessels that sprouted from the AV loop at 1 week and a high density of capillaries throughout the tissue constructs by 4 and 10 weeks (Figure 3F). Tissue harvested at 1 to 10 weeks contained differentiated cardiomyocytes, seen as elongated striated cells, which were found adjacent to newly forming vessels close to the original AV loop. As the chamber tissue contains a defined vascular pedicle, it could be transplanted with standard microsurgical techniques to a distant site, to another animal, or even to an extracorporeal circulation in vitro.

The majority of cardiac muscle in the constructs was derived from implanted cells as evidenced by the presence of DII-labeled cardiomyocytes in tissue harvested at 1 to 10 weeks and the absence of cardiac tissue in control chambers that contain the AV loop alone without implanted cells (either with or without Matrigel).17 The total construct volume consolidates between 1 and 10 weeks, although the absolute volume of cardiac tissue is maintained and the proportion of cardiac tissue increases in association with increased width of the cells. Positive immunostaining with Ki67 and PCNA in tissue constructs harvested at 1 to 10 weeks suggests that a small proportion of cardiomyocytes in the tissue constructs was produced by division of the implanted cardiomyocytes. The present study is therefore the first to show the proliferation of cardiomyocytes in an in vivo system remote from the heart. This progressive growth of the grafted cardiomyocytes over the 10-week study period is consistent with previous work on engraftment of neonatal cardiomyocytes onto adult rat myocardium25 and occurs in normal rat heart development.44

The slow rate of spontaneous beating compared with host heart rate is consistent with the reports in the literature6,8,11,42 and is probably the result of separation of atria from ventricles during cardiomyocyte isolation. To function as a syncytium, it is important that cardiomyocytes express connexin-43 in a proper topographical fashion.45 In the current study, connexin-43 was initially expressed circumferentially, followed by concentration at the intercalated disc, consistent with the reports of others in grafted cardiomyocytes and in normal cardiac development.25

It is striking that the cardiac tissue shows prominent contractile function when observed through the chamber with echocardiography and after opening the chamber at tissue harvest. Some constructs lost spontaneous activity during preparation for the organ bath studies, similar to freshly harvested atrial tissue from small animals, but all responded readily when paced up to 3 Hz. This contractile behavior and the length-tension relationship indicated that the construct behaves like rat cardiac tissue, but there may have been some damage from dissection that destroyed the automatic pacemaker tissue cells that maintained the rhythm in vivo. The positive inotropic response to 1 to 3 mmol/L in these constructs is typical of normal cardiac tissue and shows similar sensitivity to calcium similar to the engineered cardiac tissue of others.9–11,42 Spontaneous tachyarrhythmia in response to a high concentration of norepinephrine while paced at 1 Hz was also noted by others.11,42 The active force produced by our constructs, which is comparable to the force produced by stacked monolayers4 and less than that produced by chronically stretched constructs,46 is greater than that reported by many groups.9–11,42 The constructs may produce less force than adult cardiac muscle47 because of the immaturity of the implanted neonatal rat cardiomyocytes48 and the looser nature of the engineered cardiac tissue. Use of stretch may improve cell orientation and force produced in future constructs, however.

In summary, vascularized, spontaneously beating, 3-dimensional cardiac tissue can be grown by placement of neonatal rat cardiomyocytes in the angiogenic environment of a vascularized tissue-engineering chamber. This tissue arises partly from division of implanted cardiomyocytes, but mostly from growth and organized assembly of these cells. Further development of this vascularized model will increase the potential of cardiac tissue engineering to provide suitable replacement tissues for acquired and congenital defects.

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Disclosures
Professor Morrison is an inventor on the Vascularized Tissue Graft patent and entitled to proceeds derived from commercialization of the patent, and is a board member and employee of the Bernard O’Brien Institute of Microsurgery, which has an interest in the company charged with the commercialization of the Vascularized Tissue Graft patent. The remaining authors report no conflicts.

References
Cardiac tissue engineering offers a potential and novel therapeutic modality for acquired and congenital heart defects. The current work is the first demonstration of the ability to produce a tangibly large amount of new beating heart muscle in an animal. The potential clinical application would most likely be in the context of onlay repair of postischemia damaged heart wall or for cardiac assist in cardiac failure or cardiomyopathy. Human heart tissue grown in immunocompromised animals by this method could be readily applied as a model for cardiotropic drug testing to reduce the need for human trials.
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