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Engineered Cardiac Tissues for *in vitro* Assessment of Contractile Function and Repair Mechanisms

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***Abstract*- For efficiently assessing the potential for grafted cells to repair infarcted myocardium, a simplified surrogate heart muscle system would offer numerous advantages. Using neonatal rat cardiac myocytes in a collagen matrix, we created thin cylindrical engineered cardiac tissues (ECTs) that exhibit essential aspects of physiologic cardiac muscle function. Furthermore, a novel cryo-injured ECT model of myocardial infarction offers the potential for the longitudinal study of mechanisms of cell-based cardiac repair *in vitro*.**

I. Introduction

For a diversity of candidate cell therapies for post-infarction myocardial repair, functional benefits have been severely limited by very low survival rates of implanted cell grafts [1]. In particular, the implanted cells must target and persist in the injured region in sufficient numbers to have a significant impact on cardiac function, they must endure the aggressive inflammatory response of the injured host tissue, and they must survive in an ischemic environment with compromised vascular supply in which the transport of oxygen and nutrients was insufficient to serve the metabolic demands of the original myocardium. Considering this complicated combination of factors, it is possible that the true healing potential of any grafted cells of interest may be inaccurately underestimated using traditional animal models of myocardial infarction. Unfortunately, standard cell culture techniques do not provide a sufficiently realistic environment in which the effects of a given cell therapy on myocardial contractile function can be readily evaluated.

Engineered tissues, in which cells are cultured in a three-dimensional biopolymer scaffold with the long-term goal of serving as surgical replacements for damaged tissues and organs [2], may in the short-term provide a key technology for efficiently screening candidate therapeutic strategies. Thus, our objective was to develop engineered cardiac tissues that can serve as idealized surrogate heart muscle to simulate the natural myocardial niche environment for high-throughput evaluation of novel cell therapies for cardiac repair.

The principles of tissue engineering were used to create myocardial tissues that offer a number of important advantages for *in vitro* cell screening applications. These engineered cardiac tissues (ECTs) have been designed to have a thin cylindrical geometry that facilitates the evaluation of contractile function using the same kinds of muscle testing approaches that have classically been used to study cardiac physiology in isolated papillary muscles and trabeculae [3]. However, in the best long-term muscle culture systems [4], natural trabeculae remain stable up to 48 hours, which is insufficient to evaluate cell integration and differentiation and the resulting effects on the repair process. We demonstrate that ECTs exhibit several essential physiologic characteristics of cardiac muscle function, yet provide a higher level of control than can be achieved with traditional animal or patient studies, and have longer viability (3 weeks in culture) than isolated muscle or whole heart preparations [4, 5].

II. Materials and Methods

A. Cell Isolation and Culture

Primary neonatal rat cardiac myocytes (NRCM) were isolated from 2-3 day old Sprague-Dawley rats (Taconic Farms) using a modification of a published protocol [6]. Following enzymatic digestions and pre-plating, floating cells were suspended in myocyte media consisting of Dulbecco's Modified Eagle Medium with 10% newborn bovine serum (NBS, Hyclone), 1% penicillin-streptomycin (GIBCO BRL), and 4 g/ml of cytosine arabinofuranoside (ara-C, Sigma) to inhibit fibroblast proliferation. A cell population consisting of approximately 80% myocytes, with a yield of nearly 4.5×10^6 cells per neonatal heart, was obtained.

B. Creation of Cylindrical Engineered Cardiac Tissues (ECTs)

An ice-cold sterile collagen solution (1:1:8 mixture of HEPES:10x MEM:collagen) was prepared using purified type I bovine dermal collagen (PureCol, Inamed) and was combined with NRCM suspension (15×10^6 cells/ml) and Matrigel basement

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membrane matrix (BD Biosciences) in a 8:1:1 ratio. Approximately 120 µ l of cold cell-collagen solution was pipetted into custom polydimethylsiloxane (PDMS) elastomer molds (3x3x13 mm), which were precoated with 2% bovine serum albumin (BSA) for 1 h at 37°C to minimize cell and collagen adhesion. Stainless steel needles were inserted at both ends to prevent axial compaction of the gel. The solution was incubated for 2 hrs at 37°C and 5% CO_2 to initiate polymerization of the gel in the mold. Gelled tissues were floated with culture media, half of which was renewed daily.

Within 24 hours of culture, spontaneous contraction was observed, and by 72 hours the tissues were fully compacted (~10 mm long, ~0.6 mm diameter) with highly aligned cells between the two pins, and spontaneous coordinated beating at 1-2 Hz was readily visible. Such ECTs have remained stable in culture for 3 weeks.

C. Measuring Contractile Function of ECTs

After 7-8 days of culture, individual ECTs were transferred to a physiologic muscle bath testing system (**Fig. 1**). One end was attached to a high-sensitivity force transducer (KG4, Scientific Instruments, Germany) and the other end was connected to a micro-manipulator to control tissue length. During testing, the tissue was maintained at 37°C, and circulating Krebs-Henseleit buffer modified with 1.8mM $CaCl_2$ and NBS was aerated with 95% O_2 and 5% CO_2 to maximize oxygen delivery and maintain physiologic pH. The ECT was electrically stimulated with a 5V pulse wave, and isometric twitch force (**Fig. 1C**) was digitally sampled at 400 Hz (National Instruments) as the tissue was stretched from 0 to 20% of its unloaded length. Developed force was calculated from the difference between the maximum and minimum isometric force for each twitch and was converted to developed stress by dividing by the cross-sectional area of the unloaded tissue (details below).

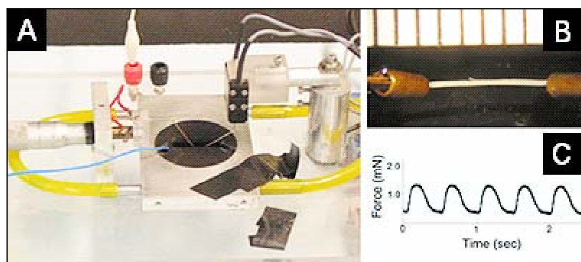

Figure 1: A) Muscle bath system for testing ECTs. B) Close-up view of ECT during testing. Scale, mm. C) 2 Hz isometric twitch tracing of ECT during testing.

D. Histology

Unloaded ECTs were fixed in 3% para-formaldehyde in phosphate buffered saline (PBS). Each tissue's cross-sectional area was calculated from its average major and minor axis dimensions, assuming an elliptical cylinder geometry.

Fixed ECTs were then stained for with rhodamin-conjugated phallodin (1:20 in PBS, Molecular Probes) to visualize f-actin. To visualize gap junctions, samples were treated with 10% non-immune goat-serum to block non-specific binding, exposed to primary monoclonal antibody to connexin-43 (1:100 in 10% blocking solution in PBS, MAB3067, Chemicon) overnight, washed with PBS, and incubated with FITC-labeled anti-mouse IgG (1:100, Molecular Probes). For nuclear staining, tissue samples were labeled with DAPI (1:100 in PBS, Sigma). Laser scanning confocal microscopy (IX-70 with Fluoview software, Olympus) was used for fluorescent imaging, and confocal reflectance microscopy was used to visualize collagen fibrils as previously described [7].

E. Creating a Tissue Engineered Model of Myocardial Infarction

For the purposes of evaluating infarct healing and repair mechanisms, we examined the potential for ECTs to be experimentally cryo-injured using techniques similar to those developed for small animal models of myocardial infarction [8, 9]. Mature ECTs were stained using a live/dead kit (Molecular Probes). The stained tissue was placed on a glass slide on an inverted fluorescent microscope with a few drops of culture media to prevent dehydration. A 1.6 mm diameter stainless steel dowel pin was then removed from liquid nitrogen, immediately laid across the ECT for 5 sec to locally freeze the tissue, and then separated by rapid thawing with additional culture media. The experiment was repeated with a room-temperature pin as a negative control. After 20 min, the tissue was imaged at low magnification (4x-objective) in a sequence of adjacent images spanning the injured region and border zone tissue.

III. RESULTS

Measurements of minimum and maximum twitch force versus axial tissue strain yielded nonlinear "diastolic" and linear "systolic" stress-strain relationships (**Fig. 2A**), characteristic of natural myocardium. The developed (max-min) stress increased monotonically with stretch, consistent with the well known Frank-Starling law of the heart. The

maximum developed stress of approximately 2 kPa represents about 5% of that measured in natural rat trabeculae [3], though the cell density was at least an order of magnitude below physiologic [10]. At 20% strain, the developed stress decreased as stimulation frequency increased from 1 to 4 Hz (**Fig. 2B**), consistent with reports of a negative force-frequency relationship in natural and engineered rat cardiac tissue [11, 12]. A dose-dependent effect of NRCM cell concentration was observed. The developed stress also increased as extracellular calcium concentration varied from 0.5 to 2.0 mM (not shown).

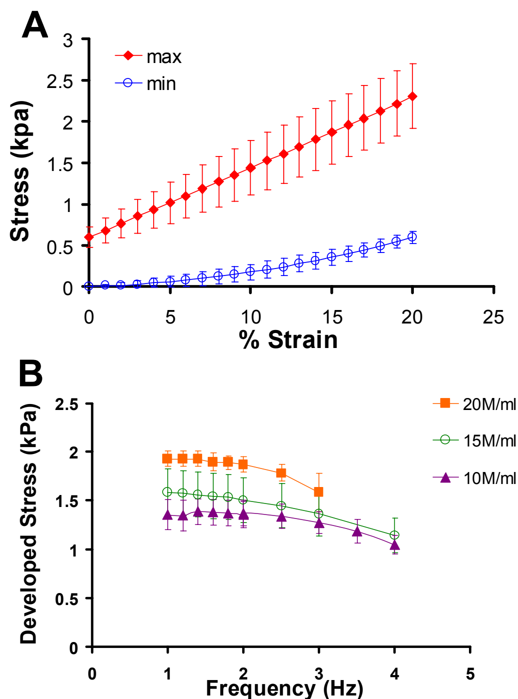

Figure 2: A) Mean (±SD, n=3) maximum and minimum tension of 15 M/ml ECT stimulated at 2 Hz. B) Developed stress vs. frequency at 20% stretch for ECTs with 10-, 15-, and 20- million cells/ml.

Structurally, ECTs exhibited aligned cells with well-developed myofilaments having registered sarcomeres and the gap junction protein connexin-43 distributed along myocyte boundaries (**Fig. 3**). Alignment of extracellular matrix filaments was also observed due to remodeling and compaction of the ECT during tissue culture.

Finally, a method for creating a tissue engineered model of myocardial infarction using cryo-injury was examined. Initially, the ECT was uniformly populated with cells having ~80% viability. Within 5 min after cryo-injury with a frozen steel pin, a clear region devoid of live cells was observed under fluorescence microscopy. A montage of low-power

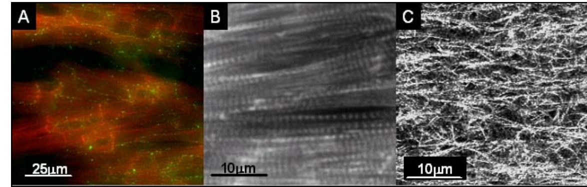

Figure 3: A) Dual stained ECT shows actin in aligned myofibrils (red) and connexin-43 (green) distributed along myocyte boundaries. B) Close-up view of actin stain showing myofibrils with registered sarcomeres. C) Aligned extracellular matrix fibers visualized by confocal reflectance microscopy.

fluorescent images taken 20 min after the injury (**Fig. 4**) clearly illustrates the experimentally infarcted engineered cardiac tissue, which to our knowledge is the first of its kind. No such region of damage was evident after repeating the experiment using a room-temperature steel pin.

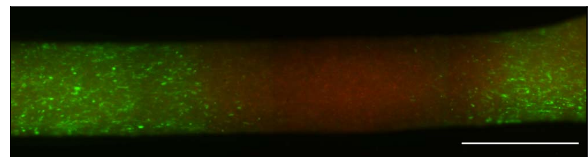

Figure 4: Montage of five digital fluorescent micrographs of an ECT obtained 20 min post cryo-injury. The injury site is clearly identified by dead cells labeled with ethidium homodimer (red) surrounded by living cells stained with calcein (green). Scale bar, 1mm.

IV. Discussion

The objective of this study was to develop functional engineered cardiac tissues that can be used as *in vitro* model systems for efficient evaluation of myocardial contractile function, particularly for applications such as screening novel cell therapies for cardiac repair. Cardiomyocyte-populated collagen gels cast in rectangular wells yielded cylindrical ECTs similar in geometry to natural cardiac trabeculae. The ECTs exhibited functional measures characteristic of natural myocardium including length-, frequency-, and calcium-dependent active tension development.

In addition, we created a novel tissue engineered model of myocardial infarction using a simple cryo-injury method originally developed for small animal studies [8, 9]. In contrast to animal infarct models, ECTs are likely to retain a high percentage of implanted cells since loss to other tissues due to migration or circulation is not possible. Cryo-injured ECTs could provide the paracrine benefits of an injury response for stimulating graft cells without bombarding them with a full intensity inflammatory response. Finally, because ECTs lack a vascular network, functional assessment could be made in an

environment designed to be adequately supplied by the diffusion of oxygen and nutrients so that the benefits of myocardial repair can be clearly distinguished from the confounding effects of neovascularization in the natural heart [13].

Of particular interest are two promising new cell therapies for repair of myocardial infarction pioneered by colleagues at Columbia University. Specifically, these are 1) implantation of highly enriched mesenchymal lineage precursor cells derived from human adult bone marrow to regenerate cardiomyocytes (S. Itescu) [13], and 2) implantation of neonatal mouse cardiac myocytes constitutively expressing normally silent cyclin A2 to stimulate cardiomyocyte mitosis and hyperplasia (H. Chaudhry) [14, 15]. Preliminary studies testing these cells in the above ECTs are currently underway.

V. CONCLUSIONS

In conclusion, we have developed engineered cardiac tissues for *in vitro* assessment of contractile function using standard muscle physiology techniques, and have demonstrated the ability to create a cryo-injury induced infarct. The use of engineered tissues as *in vitro* models of disease represents a paradigm shift in the field of tissue engineering, which has focused almost exclusively on the goal of providing a living surgical replacement for diseased or damaged tissues. Therefore, validation of this tissue engineered cryo-infarct model would provide a novel assay for efficient evaluation of candidate cell therapies for cardiac repair. If this approach can also be generalized to other tissue types, such injury models could represent the next frontier for disciplines in which wound healing has traditionally been studied *in vitro* by scratching a confluent cell culture monolayer.

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