Tissue Engineering of Cardiac and Skeletal Muscle: Lessons Learned from Micropatterned Collagen and Microgravity

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Abstract

The need for replacement cardiac and skeletal muscle that is capable of restoring the lost structure and function is very large. Our laboratory is approaching this problem by tissue engineering cardiac and skeletal muscle in vitro, with the goal of achieving sufficient mass to graft the construct to the affected area. We have developed methods to culture muscle cells with an aligned in vivo-like phenotype on a patterned collagen substrate. This technology has been coupled with observation that the high buoyancy and low shear environment of microgravity is necessary to obtain multilayered constructs of muscle. The aligned collagen technology has been adapted to the Rotating Wall Bioreactor to yield constructs with at least 15 layers of muscle cells, indicating that this is a very promising approach to tissue engineering striated muscle.

Introduction

Anomalies of striated muscle (cardiac and skeletal muscle), whether they evolve from a congenital condition, disease (myocardial infarction) or as the result of an accident, or from removal of a tumor in the case of skeletal muscle, can impact the physiological performance as well as the psychological health of a patient. The long-term objective of our research is to develop a striated muscle implant, derived from a patient’s own stem cells, that mimics the architectural organization and physiological function of intact muscle. In this paper, we address our approach to tissue engineering striated muscle which is based on the lessons learned by studying the structure of striated muscle and the insights provided from experiments in microgravity.

Is tissue engineering of a complex tissue like cardiac and skeletal muscle feasible? We believe it is, based on the experience of others who have overcome obstacles similar to those that we face in engineering artificial striated muscle. For example, over the past twenty years great progress has been made in the production of artificial skin (Yannas et al., 1980; Regnier et al., 1990). It is now possible to produce a bioengineered skin in culture that is suitable for the permanent replacement of skin damaged by burns. The key to this technology has been the development of in vitro techniques designed to culture the different cell types of the skin under conditions that approximate the in vivo environment. These experiments have successfully developed techniques to culture the cells that compose skin in a multi-layered pattern. These preparations even express glandular tissue and hair in vitro. The development of prosthetic skin was made possible by a thorough knowledge of the biochemistry of the extracellular matrix and the mechanical, biochemical, and nutritional requirements of the keratinocyte (Bell et al., 1991). More recently, a number of groups have been successful at engineering other organ systems, including urinary bladder (Atala, 2000a, 2000b), blood vessels (Niklason et al., 1999, 2001; Niklason and Langer, 2001), and to a lesser extent myocardium (Bursac et al., 1999, Carrier et al., 1999, and Papadaki et al., 2001) and skeletal muscle (Vandenburgh et al., 1988, 1989). We believe a similar approach can be applied to the production of a bioengineered implant designed to replace or augment damaged cardiac and skeletal muscle.

Since our original microgravity experiments were performed with cardiac muscle, we will present an overview of cardiac muscle and then follow that with data from skeletal muscle. One approach to achieving a successful tissue engineered implant is to pattern the development of the cultured extracellular and cellular components after the intact organ. The logic justifying this approach is that structure and function are intimately associated; therefore, if we are able to recreate the structure, the necessary function should follow. So what can we learn from intact tissue?

Extracellular Matrix and Heart Development:

The intact heart is composed of a series of overlapping cellular layers (Price et al., 1996; Shiraishi et al., 1995). Within any given cell layer the rod-like myocytes of the myocardium are arrayed in parallel with one another along a common axis. This parallel alignment is essential for the efficient distribution of electrical signals throughout the myocardium and the ability of heart to effectively eject blood into the vascular tree. As the individual layers of parallel myocytes develop within the heart during embryogenesis and begin to contract the increased force must be contained and directed to the ventricular chambers (Borg and Caufield, 1979; Markwald et al., 1985). To direct these mechanical forces, a three dimensional, stress-tolerant, connective

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tissue network forms early in development (Markwald et al., 1985). The coordinate elaboration of the ECM during the development of the multilayered myocardium suggests the ECM directs the formation of the complex fiber patterns of the heart by providing positional information to the differentiating myocytes. The composition and arrangement of this connective tissue network, which is composed primarily of interstitial collagens type I and III, continues to be modified as the heart grows into its adult form. The fibrils of this collagen-based network of the heart, composed of interstitial collagens type I and III, continues to be modified as the heart grows into its adult form. The fibrils of this collagen-based network surround the myocytes and anchor adjacent cell layers together. It is now evident that the changes in the ECM that occur in development and disease represent a dynamic adaptation to physiological change. Any perturbation that alters the normal arrangement of the myocytes within the myocardium, either as the result of a congenital defect or a pathological condition, alters the efficiency of electrical conduction and the ability of the heart to generate contractile force. To correct these defects, it is necessary to replace the tissue with a material that mimics the electrical and mechanical properties of the normal tissue. As a consequence of the architecture of the heart any cell-based procedure that is used to repair a segment of damaged myocardium must consist of myocytes that exhibit the functional and structural properties of the surrounding myocardium. In addition to its structural role in the heart, the ECM also functions as a repository for signal molecules such as growth factors, cytokines, and extracellular proteases (Borg et al., 1996, Goldsmith and Borg, 2002). The complex interplay of these biochemical factors and the mechanical events associated with cardiac function direct the formation of the heart and biochemically link the myocytes to the other organ systems, enabling the heart to adapt to a variety of physiological conditions (Nakagawa, et al., 1992; Heldin and Westermark, 1989; Dedhar, 1989). Signals that originate in the ECM play an important role in development as well as in the adaptation to physiological signals throughout life (Carver et al., 1993).

**Functional and Structural Arrangement of the ECM in the Heart:**

The ECM of the heart consists of: 1) collagens; 2) proteoglycans; 3) noncollagenous glycoproteins; and 4) growth factors (Borg et al., 1996, Goldsmith and Borg, 2002). Most of these factors are produced and secreted into the extracellular environment by the fibroblasts of the heart. Of the structural components of the ECM the collagen-based network of the heart, composed of predominantly of type I and III collagen, appears to direct the expression of the rod-like cell shape by cardiac myocytes and the formation of the three dimensional structure of the heart. The chemical identity and arrangement of this network is subject to change during development and in response to different physiological conditions (Rubin et al., 1995). During neonatal development the collagen-based matrix is rapidly elaborated and develops into a stress tolerant network that assists in distributing the mechanical forces associated with cardiac function (Borg et al., 1983). The collagen network of the heart has been demonstrated to control the elastic properties of the ventricular wall (Borg et al., 1981; Weber et al., 1989; Weber et al., 1988; Simpson et al., 1999). In the adult, several factors, including nutritional factors, mechanical stimulation and biochemical feedback, have been shown to regulate the expression and accumulation of collagen (Weber and Brilla, 1991).

**Integrins: Interaction of Collagen and Myocytes:**

In primary cell culture the specific interaction of collagen with receptors of the integrin family controls many aspects of the processes that regulate the shape that a cardiac myocytes expresses under a given set of culture conditions. The integrin family is a large multimember group of transmembrane receptors. These receptors consist of an alpha and a beta chain that are non-covalently linked to one another. Different alpha and beta chains interact with one another to form receptors that bind to components of the ECM. Within the intracellular compartment the cytoplasmic tail of the integrin complex interacts with components of the cytoskeleton, the elaborate internal scaffolding that stabilizes myofibrillar structure and cell shape. In the heart during neonatal life, the alpha 1 beta 1 integrin is the predominate isoform. However, alpha 2, alpha 3, alpha 5, alpha 7 and beta 1 isomers have been identified and are present at varying concentrations under different physiological conditions and states of development (Terracio, et al., 1991).

In myocytes, collagen associates with the cell surface in two distinct ways: 1) perpendicular at a site near the Z band, and 2) lateral associations parallel to the surface of the myocyte. Members of the integrin family mediate the first type of attachment to the surface. These specialized receptors transduce biochemical and mechanical signals from the ECM to the internal milieu of the cell (Hynes, 1992). The integrins are coordinately synthesized with ECM components (Borg et al., 1995), associated with regulation of the cardiac myocyte phenotype (Simpson et al., 1994), and intimately associated with the delivery of contractile force (Simpson et al., 1996; Sharp et al., 1997). Collagen type I and III is also associated and linked to the surface of the cardiac fibroblast through specific interactions mediated by the integrins, both high and low affinity interconnections between the interstitial cells of the heart and collagen have been identified (Tingstrom et al., 1992). These different binding affinities appear to enable the fibroblasts to migrate on collagen (low affinity) as well as to contract and place mechanical tension across the collagen network (high affinity) (Lundgren et al., 1988).

**Regulation of Myocyte Phenotype by the ECM:**

Most *in vitro* cultures of isolated cardiac myocytes have shown that it is necessary to use purified components of the ECM to attach isolated cells and allow them to adapt to the culture environment (Borg and Terracio, 1989).
While it was known that these ECM components are essential to promote the attachment and survival of myocytes *in vitro*, it has only recently been documented that the arrangement of a particular ECM component can dictate the shape that a cardiac myocyte will express *in vitro* (Simpson *et al*., 1994). Specifically, when collagen type I is applied with a defined orientation across a culture dish, a thin gel of collagen with fibrils that are distributed along a common axis can be induced to form (Simpson *et al*., 1994). When fetal or neonatal cardiac myocytes are plated onto this type of surface they spread over the underlying fibrils and express a rod-like cell shape. As a population the cells are distributed along a common axis, the phenotypic information stored in the ECM is actively communicated to the cultured myocyte by specific interactions between the ECM and the cardiac myocyte by the alpha I beta I integrin. An intervention that disrupts the function or alters the total cell surface concentration of this receptor alters the ability of the cell to interact with its environment and alters cell shape and cardiac myofibrillar order (Simpson *et al*., 1994).

**Tissue Culture of Multilayered Aligned Myocytes:**

*Much of the data in support of this work has been derived from experiments conducted by the authors using cultured cardiac myocytes flown in the microgravity of space in collaboration with the National Aeronautical and Space Administration (NASA).*

The first objective of these experiments was to determine if the high buoyancy and reduced shear environment of microgravity would result in multilayered cardiac tissue. Previous studies by others indicated that culture of cardiac myocytes in microgravity would result in multilayered cardiac tissue. The first objective of these experiments was to determine without the organization seen (Simpson *et al*., 1994) that the arrangement of a particular ECM component can dictate the shape that a cardiac myocyte will express *in vitro* (Simpson *et al*., 1994). Specifically, when collagen type I is applied with a defined orientation across a culture dish, a thin gel of collagen with fibrils that are distributed along a common axis can be induced to form (Simpson *et al*., 1994). When fetal or neonatal cardiac myocytes are plated onto this type of surface they spread over the underlying fibrils and express a rod-like cell shape. As a population the cells are distributed along a common axis, the phenotypic information stored in the ECM is actively communicated to the cultured myocyte by specific interactions between the ECM and the cardiac myocyte by the alpha I beta I integrin. An intervention that disrupts the function or alters the total cell surface concentration of this receptor alters the ability of the cell to interact with its environment and alters cell shape and cardiac myofibrillar order (Simpson *et al*., 1994).

**Figure 1** is a phase contrast micrograph of a thin collagen gel painted onto a silicon substrate in a tissue culture dish. The aligned pattern is readily visible and serves as a template for the cells when plated in tissue culture. The cells attach, spread and assume an elongated in vivo-like shape.

Under conventional culture conditions, fetal (Shiraishi *et al*., 1996), neonatal cardiac myocytes (Simpson *et al*., 1994, Simpson *et al*., 1999), and neonatal cardiac fibroblasts (Yost *et al*., 2000) will attach to these collagen gels and express an *in vivo*-like, cell shape. The myocytes are distributed in parallel with one another and as a population; the cells exist as a monolayer in a tissue-like pattern of organization. The aligned phenotype of muscle cells cultured on this substrate is very different from the pattern of organization that is observed when these cells are cultured on native plastic, nonaligned collagen, or laminin (Simpson *et al*., 1994 and figures below for skeletal muscle). The geometry and biochemical nature of this interaction appears to be critical in promoting the expression of the aligned phenotype. Disrupting the aligned structure of the collagen, or altering its chemical composition suppresses the expression of the aligned phenotype (Simpson *et al*., 1994). Blocking the function of either the α1 or β1 integrin (the predominate integrin isoforms expressed by these cells [Terracio, *et al*., 1991]) with antibodies directed against the extracellular domains of these molecules inhibits cell adhesion and disrupts the expression of the aligned phenotype. Similar results were obtained when we used antisense oligonucleotides (ODNs) to inhibit the accumulation of the α1 subunit of the integrin complex (Simpson *et al*., 1998). Blocking the accumulation of vinculin, a cytoskeletal protein of the cardiac costamere and Z-disk, with antisense ODN’s also suppresses the expression of the aligned phenotype (Shiraishi *et al*., 1996). Cells treated with antisense ODN’s against vinculin were unable to assemble normal myofibrils.

The aligned phenotype in these cultures resembles the organization of muscle cells within a single cellular layer of intact heart. Data from our experiments indicate that the phenotypic information that directs the expression of this pattern of organization is stored in the chemical identity and tertiary structure of the extracellular matrix (ECM). This information is translated into signals that control the muscle cell shape. Unfortunately, the
monolayer nature of these cultures makes them unsuitable for reconstructive surgery. In order to be useful for this type of clinical application, the cultures must more closely mimic the three-dimensional structure and mechanical integrity of intact tissue. This requires the prosthesis to be composed of multiple layers of muscle cells arrayed along a common axis. Our attempts to induce the formation of a multi-layered culture under conventional conditions have been unsuccessful. However, large multi-cellular aggregates can be induced to form in the microgravity of space and in devices designed to provide high nutrient delivery in a buoyant, low shear environment on the ground (Akins et al., 1997a, 1997b, Molnar et al., 1997) mimicking the high buoyancy and low shear conditions of space. While these aggregates can be quite large, they are not distributed in a clearly defined, three-dimensional pattern of organization, a hallmark of intact cardiac and skeletal muscle tissue. For this reason, they are also unsuitable for use in reconstructive surgery.

**Shuttle Experiments:**

On three separate space shuttle flights (STS 69, 80 and 95) we have successfully used a confluent monolayer of aligned myocytes as a template for the fabrication of a multi-layered aligned culture. In these experiments, we first established a confluent layer of aligned cardiac myocytes isolated by collagenase digestion of 3-4 day-old rat hearts (Terracio, et al., 1991) on a solid substrate for several days on the ground. The aligned cultures were then placed on the shuttle in chamber one of a device designed to mix different cell populations in the microgravity of space. In a second chamber we placed a suspension culture of freshly isolated myocytes that were preloaded with the membrane-intercalating agent, DiI, prior to loading them into the chamber. When the shuttle reached orbit the suspension of rounded myocytes in chamber two was mixed with the template layer of aligned myocytes in chamber one and maintained for a minimum of 96 hours at 37ºC. The cultures were then fixed in microgravity and preserved for microscopic examination after landing. DiI fluoresces green and allowed us to unambiguously identify the origin of each cell population at the conclusion of the experiment.

The objectives of these experiments were to determine if rounded cells added as a suspension would, a) interact with the template monolayer and form a multi-layered culture and, b) if the rounded cells would attach to the template of aligned cells and adopt an aligned cell shape even though they were not in direct contact with the collagen-coated substrate. These experiments were designed to determine if the pre-existing template of cells could communicate the phenotypic information stored in the underlying collagen gel to a second population of myocytes not in direct contact with the collagen fibrils. In mixing experiments conducted at microgravity, the rounded cells added in suspension attached uniformly over the surface of the template layer (Figure 2A) and spread out in parallel with the long axis of the underlying cells to form multi-layered cultures of aligned myocytes. Microscopic examination of myocytes mixed in ground-based control experiments revealed that cells added in suspension became unevenly distributed and formed aggregates on top of the template culture (Figure 2B). The cells of these aggregates failed to spread on the template layer even after 72 hours of culture. The myocytes of these aggregates also failed to assemble myofibrils (data not shown).

**Figure 2A:** Myocyte cultures 96 hours after mixing in microgravity. Myofibrils have been stained with rhodamine phalloidin and DiI labeled cells are indicated by the arrows and identifies cells originally added in suspension to the template layer of myocytes. Dil redistributes throughout lipid bilayers and are the more fluorescent cells (arrows). This micrograph illustrates the uniform distribution of cells added in suspension to a template layer of aligned myocytes. Confocal microscopic z-series indicated that there were at least 5 layers of cells present.

**Figure 2B:** Is a ground-based control experiment. The cells were added to a template culture consisting of a dense monolayer of aligned myocytes. Cells added in suspension formed aggregates on the surface of the template culture and did not spread out. These are the highly fluorescent round cells seen throughout the micrograph. This image was taken 24 hours after mixing. As in panel A, the cells added in suspension were pre-incubated with the membrane intercalating dye, Dil. During the early phases of culture the Dil is more concentrated and less diffusely distributed than in the cells depicted in panel A. Similar results were seen at 96 hours and in flight experiments in which the cells added in suspension were incubated with antibodies against N-cadherin.
The results of these experiments suggest that phenotypic information in the aligned collagen is communicated by the template layer of myocytes, which are in direct contact with the collagen, to the overlaying myocytes, which are not in direct contact with the collagen. One possible mechanism by which this communication takes place is that the high buoyancy low shear environment facilitates cell-cell interaction and in vivo-like assembly of cells. To determine if the multilayering was conveyed by the template layer by cell-cell communication, we used anti-N cadherin antibodies to block cell-cell communication after mixing. N-cadherin is expressed early in cell and myofibrillar assembly in the heart (Shiraishi et al., 1996). As before, we prepared a template layer of aligned cardiac myocytes in a culture chamber. In a second culture chamber, freshly isolated cardiac myocytes were mixed in suspension with anti-N cadherin antibodies. The two culture vessels were then loaded onto the space shuttle and incubated at 37ºC; upon reaching orbit the two cell populations were mixed as described previously. After 48 and 96 hours, replicate cultures were fixed in paraformaldehyde. Cells treated with irrelevant antibodies served as controls. Microscopic analysis indicated that cells mixed in ground-based experiments in the presence or absence of anti-N cadherin antibodies failed to form multi-layered cultures (similar to Figure 2B). As before, cells added in suspension in ground-based experiments formed a series of aggregates on the surface of the template layer and failed to spread in parallel with the underlying myocytes (similar to Figure 2B). There were no obvious differences in the appearance of these two treatment groups. Control cultures mixed in the microgravity of space formed multi-layered sheets of myocytes that were arrayed along a common axis and in parallel with the underlying collagen fibrils as previously reported. In contrast, cell cultures mixed in microgravity and in the presence of anti-N cadherin antibodies failed to multilayer. These cultures resembled those prepared under conventional ground-based conditions. Rounded cells added under these conditions formed a discontinuous layer of cell aggregates consisting of rounded myocytes on the surface of the template layer, as indicated by staining with rhodamine phalloidin.

We have also adapted the aligned culture methodology to skeletal myocytes in order to develop a tissue engineered skeletal implant. The prototypic skeletal muscle is composed of striated myotubes that are arrayed in parallel with one another along a common axis. Thus, the aligned collagen culture system is ideally suited for skeletal muscle. Each multinucleated myotube functions as a single cell and spans the entire length of the muscle. Adjacent myotubes are tethered together by a connective tissue covering. Congenital defects or pathophysiological conditions may partially disrupt this elaborate pattern of organization within an isolated area of the muscle bed or completely compromise the existence of the entire muscle. To accomplish the goal of reconstructing a dysfunctional muscle, we envision that a patient requiring reconstructive surgery would have several small tissue biopsies taken from donor muscle sites. Satellite cells (precursor muscle cells) would be isolated from this tissue and grown in culture (Blau and Webster 1981, Allen et al., 1998). Once a sufficient mass of satellite cells had been obtained, they would be transferred to our aligned myocyte cell culture system (Simpson et al., 1994). A second option would be the isolation of mesenchymal stem cells from bone marrow or skin biopsy and inducing differentiation into myoblasts (Grigoridis et al., 1988, Saito et al., 1995, Rogers et al., 1995, Young et al., 1998). Myoblasts in the aligned culture system would then be allowed to differentiate under conditions that promote the formation of a continuous sheet of myotubes that are arrayed in multi-layers along a common axis in a bioreactor. These histotypic cultures of myocytes would be transplanted into a muscle bed adjacent to the area of interest and allowed to adapt to the in vivo environment and undergo vascularization. After a period of adaptation the autologous implant would be mobilized, perhaps with a portion of the vascular elements and motor units arising from the original transplant site, and repositioned within the site requiring reconstruction. In the end, the reconstructed area would contain a bioengineered skeletal muscle prosthetic composed of the patient’s own striated muscle cells under the control of the central nervous system.

**Figure 3:** Phase contrast micrographs of skeletal myotubes on aligned (A) and unaligned (B) collagen gels. The cultures on the aligned collagen exhibit parallel arrays of tightly packed skeletal myotubes (arrows). The cultures on unaligned collagen show skeletal myotubes (arrows) in a random or haphazard pattern.
Skeletal Muscle in Aligned Culture

Satellite cells were isolated from the hind limbs of 2-4 days old neonatal rats by digestion with 1.25 mg/ml pronase and separated from fibroblasts by differential attachment. The satellite cells were cultured in DMEM culture medium (Sigma Chemical Co.) containing 25% fetal bovine serum to promote proliferation until they reach confluence. The satellite cells were then transferred to aligned collagen coated culture dishes formed by streaking a solution of neutral collagen on the surface using a sterile nylon paintbrush as described above. This resulted in dense cultures of parallel aligned myoblasts that when fed with DMEM culture medium containing 2-10% horse serum, fused and differentiated into multinucleate skeletal myotubes. The myotubes formed with a parallel alignment in the same direction as the aligned collagen (Figure 3A). Over time the myotubes further differentiated and increased in size. When the same cells were plated on non-aligned collagen under identical conditions they fused and differentiated but the myotubes were randomly arranged and there also appeared to be more contaminant cells (Figure 3B).

Figure 4: Transmission (A) and Scanning (B and C) electron micrographs of skeletal muscle cells grown in the RCCS bioreactor. Panel A demonstrates that the skeletal muscle cells do multilayer, as at least 15 layers of cells can be seen in this cross section. Panel B is an SEM demonstrating that the skeletal muscle cells in the bioreactor have a more robust tubular, in vivo-like appearance than when cultured under standard conditions (C).

To expand on the “Proof of Concept” experiments on the Space Shuttle and demonstrate the feasibility of developing multi-layered cultures of myocytes we elected to use the Rotating Wall Bioreactor system (RCCS model STLV) sold by Synthecon (Houston, TX). Similar to the flight studies, a template layer of aligned myocytes on solid substrate (silicone or collagen sheets) were placed in the bioreactor on the outer wall and cultured at a rotation speed of 20 rpm for 3-7 days before additional cells were added to the cultures. The results were very similar for both cardiac and skeletal muscle cells. Both cell types multilayered (Figure 4A) and resulted in alignment of cells that had a robust tubular appearance (Figure 4B), compared to cells grown under standard culture conditions (Figure 4C). Although it is difficult to determine the exact number of layers of cells from a cross section, as it is difficult to be sure the section is perpendicular and not tangential, figure 4A demonstrates that at least 15 layers of cells existed in these cultures.

The results presented here demonstrate that the combined use of micropatterned collagen substrates and the high buoyancy and low shear of the RCCS bioreactor results in the formation of a multilayered construct of striated muscle. These constructs are the first step toward the development of a tissue engineered muscle construct suitable for transplantation. Future work is planned to further differentiate the muscle cells and to pre-adapt...
these constructs to the mechanical environment by stretching them in vitro prior to transplantation.

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