Rapid formation of functional muscle in vitro using fibrin gels

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Innovative Methodology

TWO-DIMENSIONAL cell culture has been essential to the study of muscle function and development, but currently presents major limitations when compared to three-dimensional tissue. This review focuses on the potential of engineered skeletal muscles using fibrin gel casting, providing a novel method to engineer functional Skeletal muscle in vitro. This technique allows the formation of functional muscle tissue that can be used for the study of the functional development of skeletal muscle tissue. The engineered muscles respond to hormonal interventions, demonstrating that fibrin-based gels provide a novel method to engineer three-dimensional functional muscle tissue and that these tissues may be used to model the development of skeletal muscle in vitro.

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We tested the hypothesis that muscle could be engineered using fibrin gel casting and that this muscle would form faster and be functionally superior to other forms of self-organized engineered muscle. Here, we describe the development of an engineered muscle model that forms in 10 days, can be generated from a relatively pure myoblast population, and produces greater force than previous in vitro models. The resulting structures have a diameter of between 100 and 500 μm, produce 805.8 ± 55 μN of tetanic force, and are functional for 6 wk in culture.

MATERIALS AND METHODS

**Myoblast isolation and expansion.** Fischer 344 rats were anesthetized with intraperitoneal injection of pentobarbital sodium (65 mg/kg) and the right and left tibialis anterior muscles were dissected free, trimmed of excess connective tissue and fat, and placed in ice-cold PBS. Muscles were transferred to a new tube and washed three to four times with sterile PBS to remove debris and hair before being cut into small pieces and dissociated in a solution of type II collagenase (0.1%) and dispase (0.05%, diluted in serum-free F12K or DMEM) for 3 h in a 37°C shaking water bath. After digestion, the tissue was filtered through a 100-μm filter, and the flow through was centrifuged for 6 min at 2,500 rpm to pellet cells. The supernatant was aspirated and the cells resuspended in growth medium (10% heat-inactivated FBS and 5 ng/ml FGF-2 in F12K containing 100 U·100 mg·1ml penicillin streptomycin 1 and 2.5 g/ml fungizone). The cells were preplated overnight, and the nonadherent cells were transferred to new plates. This procedure was repeated twice with the second- and third-day plates yielding purer myoblast populations.

**Determination of myoblast enrichment in expanded muscle cultures.** After the second overnight preplating, cells were expanded for 5 days. Select plates from three independent trials were used to determine the purity of the myoblast population by immunostaining. Briefly, the cells were fixed using a 4% solution of paraformaldehyde before permeabilization in ice-cold methanol. The cells were then placed in blocking solution for 1 h before incubation with a primary antibody raised against MyoD (Santa Cruz Biotechnology, Santa Cruz, CA). The plates were washed, incubated with cy3-labeled secondary antibodies (Jackson Immunologicals, West Grove, PA) and 0.1 g/ml 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) in PBS at 25°C for 5 min. Plates were viewed with a Zeiss Axiohot (Thornwood, NY), and images were recorded using Bioquant imaging software. The images were overlaid using National Institutes of Health Image, and the numbers of MyoD /DAPI and MyoD /DAPI cells were determined in four random quadrants per dish.

**3D culture in fibrin gels.** Each 35-mm plate was coated with 1.5 ml of SYLGARD (polydimethylsiloxane) and allowed to cure for 48 h. Two 6-mm-long pieces of silk suture were fixed to the polydimethylsiloxane with 10 × 0.1-mm-diameter stainless-steel minutien pins at each end of the plate with 12 mm in between. The 35-mm plates were sterilized with 70% ethanol for 20 min and then rinsed with 1 ml of PBS. Each plate then received 500 μl of growth media containing 10 U/ml thrombin, and the plate was agitated until the media covered the entire surface. The fibrin gels polymerized ~10 min after the addition.

![Fig. 1. Determination of myoblast enrichment. After preplating, cells from 3 independent isolations were expanded for 5 days, fixed, and stained with both a primary antibody raised against MyoD (A; Santa Cruz Biotechnology, Santa Cruz, CA) and 4,6-diamidino-2-phenylindole (DAPI; B). The MyoD and DAPI images were (C) overlaid using National Institutes of Health Image to determine the percentage of MyoD /DAPI cells. All images were taken at ×20 magnification.](http://jap.physiology.org/)
of 200 µl of 20 mg/ml fibrinogen and, after a further 15 min, were ready for cell seeding.

The preplated cells isolated from the tibialis anterior muscle and expanded in growth media for 5 days were detached from their tissue culture plates by the addition of 1 ml of a 0.25% trypsin and EDTA solution. The cells were collected and pelleted, and the resulting cell pellets were resuspended in growth media and preplated for 15 min. At the end of 15 min, the myoblast-containing medium was collected, total cell number was determined using a hemocytometer, the cell density was adjusted to 10^5 cells/ml with growth media, and 1 ml of this solution was added to the fibrin-coated plates. Beginning 2 days after plating, the growth medium was exchanged every other day until day 7, when the cells were shifted to differentiation media (6% heat-inactivated FBS in DMEM containing 100 U/ml penicillin streptomycin and 2.5 µg/ml fungizone) to promote the formation of myotubes.

Administration of IGF-I. IGF-I was administered at increasing concentrations (25, 50, and 75 ng/plate) by adding the proper amount of IGF-I to the thrombin solution before formation of the fibrin gel. No further IGF-I was administered throughout the course of the study.

Isometric contractile properties test for muscle constructs. All contractile properties were initially measured 14 days after the cells were plated and then repeated at 7-day intervals until the end of the study. The protocol for measuring contractility of engineered muscle constructs was adapted from Dennis and colleagues (9, 11, 15) and Irintchev et al. (14). The variables measured were diameter, passive baseline force, peak twitch force, and peak tetanic force, and the time-dependent variables [time to peak twitch force, one-half relaxation time for a twitch (RT1/2), half-contraction time, and half-relaxation time for a tetanic contraction]. Peak twitch force and peak tetanic force were determined after subtraction of passive baseline force from the total force values. Cross-sectional area was calculated from the measured diameter, assuming a circular cross section. Specific force was calculated as kilonewtons per square meter: the force generated by the construct (kN) divided by its cross-sectional area (m²).

During the 15-min measurement of contractile properties, the temperature of the engineered muscles was maintained at 37 ± 1°C using a heated aluminum platform. To test construct function, one of the artificial tendons was freed from the polydimethylsiloxane substrate, and a force transducer was attached to its minutien pins using canning wax (10). Platinum wire electrodes were positioned on either side of the tissue to electrically stimulate the constructs. Twitches were elicited using a single 1.2-ms pulse at 15 V, whereas maximum tetanic force was determined using a 1-s train of 1.2-ms pulses at 150 Hz and 15 V. Passive baseline force was measured as the average baseline passive force preceding the onset of stimulation. Data files for each peak twitch force and peak tetanic force trace were recorded at 1,000 samples/s and stored for subsequent analysis using LabVIEW data acquisition software.

The length-tension relationship was determined by shortening or lengthening the construct using a calibrated three-axis micromanipulator before a 150-Hz tetanic contraction. The force-frequency relationship for each muscle was determined using a single-twitch or a 1,000-ms tetanic stimulus at 5, 10, 20, 40, 60, 80, 100, and 150 Hz. Peak force in both cases was measured and presented as the percentage of the maximal force obtained at resting length and 150 Hz in that muscle.
The time-dependent twitch parameters were measured directly from the stored data traces for each engineered muscle. Twitch kinetics were assessed by determining the time to peak tension (from the onset of stimulation until peak force is produced) and RT$_{1/2}$ (from peak force production until force is reduced to 50% of the peak) after a single pulse. Kinetics were also assessed during 150-Hz tetanic contractions by measuring the half-contraction time (from the onset of stimulation until 50% of the maximum tetanic force is produced) and the half-relaxation time for a tetanic contraction (from the last stimulation pulse until force is reduced to 50% of the maximum). Each measurement was repeated three times, and the mean value was recorded.

Light microscopy. After determination of contractile properties, constructs were fixed in 2.5% glutaraldehyde and embedded in TissueTec overnight at room temperature. The constructs were then frozen at culture length, and 10-μm sections were made. The sections were then either stained with hematoxylin and eosin or immunostained for myosin heavy chain using the MF20 antibody developed by Dr. Donald A. Fischman and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, at the University of Iowa. Total cell number was determined by DAPI counterstain. Images recorded using bioquant imaging software and the images were overlaid using National Institutes of Health Image.

Transmission electron microscopy. After contractile properties were measured, muscle constructs were pinned at culture length and fixed for 4 h at 4°C in Karnovsky’s solution (0.1 M sodium cacodylate buffer with 3% formaldehyde and 3% glutaraldehyde at pH 7.4). The 3D engineered muscle constructs were then rinsed three times (30 min, 30 min, and 4 h) with cacodylate buffer (pH 7.4) containing 7.5% sucrose. The muscle constructs were then 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, Redding, CA), and 50-nm-thick cross sections were cut for imaging.

Statistics. IGF-1 data is presented as means ± SE for four to six engineered muscle constructs per group. Differences in mean values were compared within groups (e.g., control vs. IGF-I treatment), and significant differences were determined by ANOVA with post hoc Tukey-Kramer honestly significant different test. The level of significance was set at $P < 0.05$.

RESULTS

The myoblast enrichment after preplating was determined using the ratio of MyoD$^+$/DAPI$^+$ to MyoD$^-$/DAPI$^-$ cells in three independently isolated populations (Fig. 1). MyoD$^+$ cells made up 88 ± 4.3% of the total cells counted after 5 days of expansion in growth media containing 5 ng/ml FGF-2.

After expansion, 10$^5$ cells were seeded on top of a loose fibrin gel where they rapidly proliferated to confluence and began to fuse to form myotubes. As fusion began, the force produced by the cells contracted the gel until it formed a cylinder held between two silk sutures pinned to the dish (Fig. 2). During the formation of this cylindrical structure, the myotubes reorganized along the line of force between the two sutures until they had formed a parallel array of myofibrillar proteins (Fig. 3).

Fig. 4. Light and fluorescent micrographs of a 3D engineered muscle. Constructs were fixed, sectioned and stained with hematoxylin and eosin (A), MF20 antibody (recognizing all isoforms of type II myosin heavy chain; B), and DAPI (showing cellular nuclei; C). D: overlay of myosin heavy chain and DAPI shows that the cellular regions of the constructs express myosin heavy chain. Scale bar represents 60 μm.
The ultrastructure of the 3D engineered muscles was determined by light and electron microscopy. Light microscopic images of constructs 3 wk after plating showed a large area of undigested fibrin containing small, dispersed, myosin heavy chain-positive cells (Fig. 4). Electron microscopic images showed a large number of adjacent cells containing the classic hexagonal array of contractile proteins (Fig. 5). Numerous mitochondria were located within the region of contractile protein, suggesting a functional association of the cellular sites of energy production and utilization. Longitudinal sections showed areas of contractile machinery organized within poorly defined but discernable z lines. Although much of the tissue had muscle-like structures, the diameter of the cells was never >10 μm.

When attached to a force transducer and electrically stimulated, the 3D engineered skeletal muscles displayed classical characteristics of muscle physiology including a normal length-tension relationship and positive force frequency (Fig. 6). The engineered muscle constructs had an average diameter of 177 ± 10.5 μm, contracted with a mean maximal twitch force of 329 ± 26.3 μN, and a mean maximal tetanic force of 805.9 ± 55 μN (Fig. 7A). The specific force of the constructs was 36.3 ± 4.23 kN/m² and the tetanic force was 2.6 ± 0.05-fold higher than the twitch force. For a single 1.2-ms twitch, the time-to-peak tension of the constructs was 39.75 ± 0.48 ms, whereas the RT₁/₂ was 35.25 ± 1.65 ms (Fig. 7B). For tetanic stimulation at 150 Hz, half-contraction time and half-relaxation time (RT₁/₂) were 32.75 ± 0.96 and 65 ± 4.08 ms, respectively.

To test the effect of IGF-I on the force production of 3D engineered muscles, increasing concentrations of IGF-I were embedded directly in the fibrin gel. Two weeks after plating, the contractility of the 3D engineered muscle was determined. Embedding 25, 50, or 75 ng of IGF-I in the fibrin gel resulted in 50, 36, and 31% increases in force over untreated 3D engineered muscles (Fig. 8). Treatment with 25 ng of IGF-I also affected construct contractility by slowing time to peak twitch force 26% (control = 43 ± 3.04 vs. 25 ng/ml IGF-I = 57 ± 1.08) and trended to slow RT₁/₂ (control = 44 ± 0.82 vs. 25 ng/ml IGF-I = 51.3 ± 3.33; P = 0.078) but did not reach statistical significance.
DISCUSSION

We have developed a novel system for engineering self-organizing 3D skeletal muscle using a fibrin gel. The fibrin gel-based muscle constructs produce 805 µN of force, display a normal length-tension relationship and positive force frequency, and can be maintained in culture for 6 wk. This new model is technically very easy to produce, is highly reproducible, uses a purer myoblast population, allows the myoblasts to fuse into myotubes before contraction of the gel, forms completely in 10 days, and provides an effective method for long-term culture of primary muscle cells from a variety of species.

The 3D engineered muscle described here is similar to the myooids previously generated in our laboratory and the BAMs generated by Vandenburgh et al. (37), with a few important differences. BAMs are made through a gelation process by mixing myoblasts with a solution containing collagen I and matrigel (17, 18, 20–26, 32). BAMs have proven to be extremely effective as a vehicle for local delivery of growth factors (17, 18, 24), but less is known about their ability to...
generate active force. Without measures of active force, it is difficult to compare the function of BAMs with the model presented here.

The self-organizing engineered muscles, or myoids, previously developed in our laboratory have many of the same properties of the fibrin-based system described here. The primary advantages of the new technique are 1) engineered muscle constructs form in 10 rather than 36 days; 2) by selecting a more pure myoblast population, the fibrin-based constructs produce greater specific force (36.3 vs. 2.9 kN/m²); and 3) the fibrin gel can be mixed with growth factors to provide a slow paracrine-like release of hormones to the muscle cells. The rapid formation of the fibrin-based 3D engineered muscles does not only decrease the overall time required to complete a series of experiments but also allows more effective use of transient transfection techniques to determine the functional role of specific genes within skeletal muscle.

Although the 3D engineered muscles described here have many characteristics of adult skeletal muscle, such as normal length tension, positive force frequency, and a twitch-to-tetanus ratio of 2.5 (adult range is between 3- and 5-fold), it is important to note that there are significant differences. The specific force of these constructs is 36.3 kN/m² compared with the 260 kN/m² of adult skeletal muscle (36). Although this represents a fraction of the adult value, it is similar to the 74 kN/m² in the extensor digitorum longus and 44 kN/m² in the soleus muscle of 1-day-old Wistar rats (5). Furthermore, the 36.3 kN/m² reported here is a 10-fold improvement over previous models of engineered skeletal or cardiac muscle (9, 40).

Another difference between the constructs and adult muscle is that the size of the myotubes in the constructs did not exceed 10 μm, whereas the cross-sectional area of a normal adult fiber is ~100 μm. The small myotube diameter reported here is similar to what has been reported for aneural rat primary myotubes (38, 39). The similarity in myotube size and morphology at the electron microscopic level between aneural rat muscle and the engineered muscle reported here suggests that the myotubes in the engineered muscle constructs may be developmentally arrested in the primary myotube state and do not continue to develop into more adult myofibers. Although this limits the functional capacity of the muscles described here, it also suggests that these tissues may provide an ideal model system for improving our understanding of what factors are required for the transition from primary myotubes to adult myofibers. During development, the transition from primary myotubes to secondary myotubes is dependent on electrical activity. In the absence of electrical activity, secondary myotubes and adult muscle fibers fail to form (38). If the electrical stimulation can be reproduced in vitro, this may promote the transition toward adult myofibers within our engineered muscle model.

Although electrical activity plays a central role in the development of skeletal muscle, hormonal signals, and mechanical inputs (from both bone growth as well as voluntary movements) may be important as well. Although each of these factors may play a role in the development of adult myofibers, it is very difficult to discern the role of each factor in vivo. 3D engineered muscle may provide a powerful tool to begin determining the individual and combined effects of each factor in a controlled environment.

In this study, we have looked at the functional effect of IGF-I on muscle function. IGF-I is a unique trophic factor in muscle because it promotes both the proliferation and differentiation of myoblasts. IGF-I promotes proliferation by activating MAP kinases (7) while it supports terminal myogenic differentiation by inducing a large increase in expression of myogenin (12) and the activation of the phosphoinositide 3-kinase/Akt70-kDa S6 protein kinase pathway (7). In addition, IGF-I plays an important role in determining adult muscle size by increasing amino acid uptake, Akt/S6 protein kinase activity, and DNA and protein synthesis (1, 2, 27). The importance of IGF-I in muscle growth is most strikingly demonstrated by the twofold increase in muscle size in mice carrying a transgene for muscle-specific overexpression of IGF-I (6, 19). IGF-I is not only involved in muscle growth, it also directly effects the expression of important functional proteins (8, 33). Studies from our 3D engineered muscles support these in vivo data. Low levels of IGF-I administered through the fibrin gel produced a 50% increase in force production and a 26% decrease in time to peak twitch force within the 3D engineered muscle. As the concentration of IGF-I was increased, the effect on force and the rate of contraction diminished. The diminished effect of IGF-I at higher concentrations may reflect downregulation of the IGF-I receptor within the myocytes, as has been reported in C2C12 cells (13).

In conclusion, we have developed a fibrin gel-based 3D functional tissue model that allows the long-term culture of skeletal muscle cells. These 3D engineered muscles can be used to determine the effects of specific stimuli on both the functional (force production, endurance capacity, and contractile dynamics) and molecular (level and isofrom of contractile and regulatory proteins) development of skeletal muscle. Thus these tissues may provide an important tool for determining the molecular and cellular mechanisms involved in skeletal muscle development.

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GRANTS

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REFERENCES


