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EXCITABILITY AND ISOMETRIC CONTRACTILE PROPERTIES OF MAMMALIAN SKELETAL MUSCLE CONSTRUCTS ENGINEERED IN VITRO

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Summary

Our purpose was to engineer three-dimensional skeletal muscle tissue constructs from primary cultures of adult rat myogenic precursor cells, and to measure their excitability and isometric contractile properties. The constructs, termed myooids, were muscle-like in appearance, excitability, and contractile function. The myooids were 12 mm long and ranged in diameter from 0.1 to 1 mm. The myooids were engineered with synthetic tendons at each end to permit the measurement of isometric contractile properties. Within each myooid the myotubes and fibroblasts were supported by an extracellular matrix generated by the cells themselves, and did not require a preexisting scaffold to define the size, shape, and general mechanical properties of the resulting structure. Once formed, the myooids contracted spontaneously at approximately 1 Hz, with peak-to-peak force amplitudes ranging from 3 to 30 μN. When stimulated electrically the myooids contracted to produce force. The myooids (n = 14) had the following mean values: diameter of 0.49 mm, rheobase of 1.0 V/mm, chronaxie of 0.45 ms, twitch force of 215 μN, maximum isometric force of 440 μN, resting baseline force of 181 μN, and specific force of 2.9 kN/m^2. The mean specific force was approximately 1% of the specific force generated by control adult rat muscle. Based on the functional data, the myotubes in the myooids appear to remain arrested in an early developmental state due to the absence of signals to promote expression of adult myosin isoforms.

Key words: tissue culture; tissue engineering; organoid; myooid; myogenesis; organogenesis; myocytes; force measurement; muscle contraction; cell culture; myotubes.

Introduction

The spontaneous contractile activity of cultured skeletal muscle cells was first reported in 1915 (Lewis, 1915). Despite the long history, no systematic attempt has been made to quantitatively characterize the contractile properties of cultured skeletal muscles. Passive mechanical strain has been applied to two-dimensional monolayers of cultured myocytes to study the effects of mechanical strain on myotube orientation (Vandenburgh, 1982; Vandenburgh et al., 1989, 1991a), metabolism (Hatfaludy et al., 1989; Vandenburgh et al., 1989, 1991a), and development (Vandenburgh and Kaufman, 1979; Vandenburgh, 1988; Vandenburgh et al., 1989, 1990, 1991a; Perrone et al., 1995). The development of three-dimensional skeletal muscle constructs, previously reported as organoids (Vandenburgh et al., 1991b), has made possible the measurement of the contractile properties of cultured skeletal muscles. Even with this development, measurements of contractile properties have not become widely or systematically employed. The only published data on the contractile properties of skeletal muscle organoids are isometric contractile responses elicited from four avian skeletal muscle organoids at 4° C by elevation of the extracellular potassium (Vandenburgh et al., 1991b). The force developed by the organoids was measured using a lateral loading and displacement arrangement with a calibrated microneedle. This method for the measurement of contractile function of skeletal muscles in vitro is not generally accepted (Faulkner et al., 1997). The lateral loading of the organoid would necessarily change the sarcomere lengths within the organoid, making accurate measurements of isometric force impossible. Smaller lateral deflections could be used to decrease the sarcomere length change, but the experimental error of the measurement would increase due to the reduced deflection angle used in the calculation of the tensile force by vector decomposition. No other data on the contractile function of organoids have been published, the remaining data being limited to biochemical, morphological, or histological assessments (Strohman et al., 1990; Vandenburgh et al., 1991a, 1996; Swasdison and Mayne, 1992; van Wachem et al., 1996; Okano et al., 1997; Shansky et al., 1997; Okano and Matsuda, 1998).

Typically, skeletal muscle organoids have been produced in vitro by the primary culture of embryonic avian skeletal muscle cells (Strohman et al., 1990; Vandenburgh et al., 1991a, 1991b; Swasdison and Mayne, 1991, 1992; Shansky et al., 1997). A synthetic scaffold was not required to define the size and shape of the organoid, though the application of controlled uniaxial mechanical strain was sometimes employed to promote myotube alignment (Vandenburgh et al., 1991b; Vandenburgh, 1992). Organoids have also been produced from mammalian cells by using an established skeletal muscle myoblast cell line (Vandenburgh et al., 1996; van Wachem et al., 1996; Okano et al., 1997; Okano and Matsuda, 1998), such as C2C12, or by the use of primary cells from neonatal rats (Shansky

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et al., 1997). All organoids cultured from mammalian cells have employed an artificial scaffold such as MATRIGEL (Vandenburgh et al., 1996; Shansky et al., 1997) (Collaborative Biomedical, Bedford, MA), or native or modified collagen (van Wachem et al., 1996; Okano et al., 1997; Okano and Matsuda, 1998). The use of synthetic scaffolds may interfere with the ability to measure the contractile properties of organoids due to the large resting tensile force developed over time by the scaffold (Delvoye et al., 1991; Vandenburgh et al., 1996; Okano et al., 1997).

The objectives of the present study were (1) to develop methods to engineer cylindrical muscle-like constructs, using primary myogenic precursor cells from mammals, without the use of a preexisting scaffold, and (2) to develop instrumentation and methods for the measurement of the excitability and isometric contractile properties of these constructs. These structures are termed "myooids," because they are muscle-like in form and function. The term "myooid" is defined as a three-dimensional skeletal muscle construct developed from coculture of mammalian skeletal muscle satellite cells and fibroblasts that employs no artificial scaffold for the contractile region of the structure. The myooid includes anchor materials specifically designed to serve as artificial tendons to allow manual manipulation and attachment of the structure to serve mechanisms and force transducers. In addition, the myooid is electrically excitable, and when activated, generates contractile force, work, and power commensurate with the architecture and developmental state of the muscle cells within the structure.

Materials and Methods

The method involved the culture of mammalian myogenic precursor cells, which included primary satellite cells and fibroblasts. The cells were cultured on a thin SYLGARD substrate layer in 35-mm culture dishes. Two anchor points were provided to establish the longitudinal axis of the myooid, and to act as artificial tendons at each end of the myooid to facilitate attachment to a force transducer for functional evaluation.

Preparation of solutions and media. The culture media were growth medium (GM), consisting of 400 ml HAM F-12 nutrient mixture (GIBCO BRL, Rockville, MD11765-054) with 100 ml fetal bovine serum (FBS, GIBCO BRL 10437-036), and 100 units/ml Penicillin G (Sigma Chemical Co., St. Louis, MO, P-3414), and differentiation medium (DM), consisting of 465 ml Dulbecco modified Eagle medium (DMEM, GIBCO BRL 11995-065) with 35-ml horse serum (HS, GIBCO BRL 16050-114) and 100 units/ml Penicillin G. Preincubation medium (PIM) was prepared with 10% isotonic saline with 0.05% NaN_3 (m/v), added to 90% DM (v/v), and 0.22-μm filter sterilized. The culture and PIM were stored at 4° C until use. Tissues were dissociated in a solution of 4 units/ml dispase (activity 0.85 units/mg, GIBCO BRL 17105-041) and 100 units/ml type IV collagenase (Worthington CLS 4, activity 206 units/mg) (Worthington Biochemical, Lakewood, NJ) in DMEM. For dissociating the surgically removed tissues, the dispase and collagenase solutions were prepared immediately prior to use and 0.22-μm filter sterilized.

The following solutions were used at room temperature in the production of acellularized muscle and tendon fragments for use as anchors: Solution A, 80% glycerol (v/v), 20% isotonic saline, 0.05% sodium azide (w/v), and 25 m*M* ethylenediamine-tetraacetic acid; Solution B, 4% sodium deoxycholate (Sigma D-6750), 0.05% sodium azide (w/v) in distilled water; Solution C, 1% sodium dodecyl sulfate (Fisher S529) (Fisher, Itasca, IL), 0.05% sodium azide in distilled water; Solution D, 0.05% sodium azide in isotonic saline; Solution E, 3% Triton X-100 (Sigma T-9284), 0.05% sodium azide in distilled water. All acellularizing solutions were stored at room temperature until use.

Preparation of culture dishes. The culture dishes were prepared by pouring 1.5 ml SYLGARD (Dow Chemical Corporation, Midland, MI, type 184 silicone elastomer) into each 35-mm diameter culture dish (Falcon 1008). The SYLGARD substrate allowed anchor materials to be pinned in place and provided a surface to which, unless suitably coated, the cells did not adhere. The dishes were capped and allowed to sit on a level surface for 1 wk, then stored for at least 2 wk before use to reduce cell toxicity (Strohman et al., 1990). For experiments to test the effect of laminin density on myooid formation, the SYLGARD substrate was coated with natural mouse laminin (GIBCO BRL 23017-015) by diluting the laminin in 3 ml sterile Dulbecco's Phosphate-Buffered Saline (DPBS) (pH = 7.2, GIBCO BRL 14190-136) per dish, at densities of 0.2, 0.5, 0.75, 1.0, 1.5, and 3.0 μg laminin/cm^2. The dishes were left in an open biological safety hood with the blower running overnight, to facilitate evaporation of the DPBS and deposition of the laminin. Salt crystals remaining from the evaporation of the DPBS were removed by addition of 3 ml DPBS to each culture dish, and subsequently thorough aspiration. Myooids used in the isometric contractile function experiments were cultured on 35-mm diameter culture dishes, with a substrate of SYLGARD coated with 1 μg laminin/cm^2.

Preparation of anchor materials. In the preliminary experiments several anchor materials were evaluated for the ability to support the development of a myooid, using primary cultures of myogenic precursor cells from adult rats. The anchor materials included: stainless steel pins coated with laminin; segments of cellular and acellularized rat tibialis anterior distal tendons; acellularized mouse extensor digitorum longus (EDL), soleus, or tibialis anterior muscle (TBA); and braided silk suture, both laminin-coated and uncoated. Myooids consistently formed only when acellularized muscle or laminin-coated silk-suture anchors were present.

Acellularized muscle anchors were prepared by removal of the soleus, EDL, and TBA muscles from adult male and female C57BL/10 or C3H mice. The mice were anaesthetized with sodium pentobarbital until there was no response to tactile stimuli. At this time the whole muscles were removed from both hind limbs. The mice were subsequently euthanized by the induction of pneumothorax. The muscles were immediately placed in a 60-mm culture dish with 10 ml of DPBS at room temperature (24° C) for 5 to 24 h. The TBA muscles were sliced longitudinally to produce roughly equal sized pieces. The muscles were pinned near each end with 0.10-mm diameter stainless steel minutien pins (Fine Science Tools, Foster City, CA, model 26002-10) into a 100-mm culture dish in which a 2-mm deep layer of SYLGARD had been poured and allowed to cure for at least 2 wk. The muscles were initially soaked in Solution A for 24 to 48 h. The dish was then rinsed with distilled water, and the muscles were soaked in Solution B for 24 h, rinsed, soaked in Solution A for 48 h, rinsed, soaked again in Solution A for 24 h, rinsed, soaked in Solution C for 24 h, rinsed, soaked in Solution E for 24 h, rinsed, soaked in Solution C for 48 h, rinsed, then maintained in Solution D, still pinned in place, until use. All muscle rinsing and solution treatments were performed at room temperature. Immediately before being pinned into the laminin-coated 35-mm culture dishes, each muscle strip was transected at its center to form two anchors.

Silk-suture anchors were prepared using 6-mm long segments of size-0 (Ethicon, metric size 3.5) (Ethicon, Somerville, NJ) braided silk. Each segment was held at the center using a pair of #5 forceps and one end was gently teased by hand to produce a frayed end. The segment was then immersed in a concentrated solution of 50 μg natural mouse laminin/ml DPBS (pH 7.2). The wet suture segments were pinned into each laminin-coated 35-mm culture dish, separated by 12 mm, with the frayed ends facing each other, and were allowed to dry overnight.

Placement of anchors in the prepared culture dishes. One laminin-coated 35-mm culture dish was prepared for the formation of each myooid. Each 35-mm culture dish contained two anchor points separated by 12 mm. Each anchor was held in place by two 0.10-mm minutien pins to prevent rotation. The anchors were pinned into the culture dishes within 1 d of completing the laminin coating process. The acellularized muscle segments were pinned such that the transected surfaces faced each other. The laminin-coated anchor materials were allowed to dry overnight while pinned in the culture dishes. Three milliliters of GM were added to each culture dish, the covers were set in place, and the dishes were exposed to ultraviolet radiation (wavelength = 253.7 nm, bulb G30T8) in a biological safety hood for 60–90 min to sterilize the substrate, culture medium, and anchors. The dishes, with GM and anchors in place, were presoaked in an incubator at 5% CO_2 and 37° C for 5–8 d before plating of cells.

Greater than 95% success in the formation of myooids was achieved by presoaking the culture dishes in GM for 5–8 d in an incubator at 37° C with anchors in place, prior to cell plating. Shorter presoaking times, or no presoaking at all, resulted in a 2–5-mm diameter ring of inhibited cell growth around each anchor. With inadequate presoaking, the cells did not attach to the anchors, and the success rate for myooid formation was less than 5%. Longer presoaking times resulted in a reduced success rate, presumably due to degradation of the laminin coating on the SYLGARD substrate. Cold stor-

age of the laminin-coated dishes at 4° C for 2 wk prior to presoaking also resulted in significant loss of cell monolayer adhesion to the SYLGARD substrate. The presoaking appeared to remove residual toxins from the anchors and may also have deposited adhesion molecules from the serum in the GM onto the substrate surface, which resulted in improved cell adhesion.

Isolation of satellite cells and fibroblasts. Myogenic precursors were harvested from the soleus muscles of adult (5–6 mo old) Sprague Dawley rats by surgical removal of both soleus muscles. The soleus muscle was selected because of the relatively greater number of satellite cells associated with oxidative than with glycolytic fibers (Schultz and McCormick, 1994). Each soleus muscle was sliced longitudinally into six strips, from tendon to tendon. The muscle strips were pinned at slack length into 35-mm culture dishes with SYLGARD, without laminin coating, two per dish, and 3 ml of PIM was added to each dish. Each dish was covered and exposed to ultraviolet light for 60–90 min. Preliminary experiments used preincubation times of 0, 18, 24, 40, 50, 70, and 100 h. Preincubation before enzymatic digestion of muscle tissues to release myogenic precursors resulted in more-dense cell cultures and more-rapid formation of confluent monolayers of cells, presumably due to increased yield of satellite cells. The optimal preincubation time, determined by the minimum time to confluent monolayer formation after cell seeding, was 50 h. For all subsequent experiments, all primary explants were subjected to 50 h of preincubation in PIM at 37° C and 5% CO_2.

After preincubation, the muscle strips were enzymatically dissociated to release the myogenic precursors for primary culture. Under aseptic conditions, pairs of muscle strips were placed in 15 ml of freshly prepared, filter-sterilized dissociation medium, using two pairs of #5 forceps that were dipped in 70% ethanol between each use. The muscle strips were handled by the minutien pins by which they had been pinned into the culture dishes for preincubation, and were dropped directly into the dissociation medium in a conical vial (Falcon 352098). The strips were incubated in a shaker bath at 37° C for approximately 3 h and were occasionally shaken vigorously by hand to promote complete tissue dissociation. The dissociated tissue was centrifuged at 1000 × *g* for 15 min and the supernatant was discarded. No effort was made to isolate myogenic precursor cells from other cells when the tissue was dissociated. Hence myooids developed from coculture of satellite cells and fibroblasts, in the presence of other cell types that would normally be present in adult skeletal muscle and the associated adipose, vascular, and motor nerve tissue.

Culture of myooids. The cells were reconstituted with 2 ml GM for each 35-mm culture dish to be plated. Due to the presence of tissue debris after dissociation, cells could not be counted accurately to determine the plating density. Plating density was recorded as the mass of tissue dissociated for the formation of each myooid. Typically, ~13 mg of dissociated soleus muscle tissue was used to form each myooid. The presoaked GM was aspirated from the culture dishes with prepared anchors, and the dishes were immediately plated with the myogenic precursor cells. The dishes were placed in culture at 37° C and 5% CO_2, and were not handled for 48 h.

Beginning at 48 h after plating, the GM was replaced every 48 h until the cells became confluent, which typically required 3–8 d. The medium was replaced with DM on the next scheduled feeding day after the cells reached confluence and the cells were fed twice per week thereafter. Within a few days of switching to DM the confluent cells fused to form multinucleated myotubes that began to contract spontaneously. Typically, after 2–3 wk in culture the monolayer of myotubes in each culture dish detached from the substrate, while remaining attached to the anchors, and rolled into a cylinder to form the myooid.

Measurement of excitability and contractile properties. Contractile properties were measured using standard in vitro muscle mechanics instrumentation described in detail elsewhere (Faulkner et al., 1997). A force transducer of our own design with a force resolution of 1.4 μ*N* and range of 2.0 m*N* was employed. A force generator (Minns, 1971) was constructed which allowed the force transducer to be calibrated directly while in position on the apparatus. To attach myooids to the force transducer, one of the minutien pins was removed from an anchor and the other minutien pin was sealed with canning wax to a 20-gauge stainless steel wire that projected from the force transducer. The seal was made with a soldering iron at 150° C (300° F). Excessive heating of the myooid was avoided by full immersion of the myooid in an additional 2 ml of fresh DM and restricting the contact time of the soldering iron with the stainless steel wire to less than 2 s. The force transducer position was adjustable on three axes by use of a micromanipulator (Newport MT-XYZ) (Newport, Irvine, CA). Electrical stimulation was applied to the myooids by placement of 36-gauge platinum wire electrodes parallel to the myooid, with a spacing of 5.5 ± 0.5 mm. The electrodes were positioned so that the myooid was parallel to and directly between the electrode wires. The stimulation pulses were square and unipolar. Stimulation was controlled by a Pentium-based computer with a LabVIEW data acquisition system (National Instruments, Austin, TX) and amplified using a Crown DC-300A amplifier. The system permitted control of pulse width, pulse amplitude (voltage), pulse frequency, and duration of each pulse train.

Time in culture for each myooid was defined from the time of cell plating, not from the time of myooid formation. A randomly selected set of myooids was removed from the incubator after 31 ± 4 d in culture for the measurement of contractile properties. Before evaluation of the contractile properties, a period of 3 d was allowed after the complete detachment of the monolayer from the substrate to allow formation of each myooid. During the experiments, the media temperature was maintained at 37° C on a heated aluminum platform. One of the pins in one anchor was affixed to the force transducer and the other pin was removed. The force transducer was raised to allow the remaining pin to clear the SYLGARD substrate below. Based on preliminary experiments, the plateau of the length–force curve occurred at a length ~5% less than the length at which the myooids were held during culture. For this reason, the myooids were shortened by 5% (0.6 mm) from the length at which they were cultured before the measurement of isometric contractile properties.

Myooid diameter was measured using a calibrated eyepiece reticle with a resolution of 5 μm. Baseline force (P_b) was measured before stimulation. Stimulation parameters were determined during a set of preliminary experiments in which the myooids were subjected to a wide range of voltages and pulse durations. In each myooid tested a 70 V, 4-ms wide pulse consistently resulted in a supramaximal stimulation for a single twitch. Peak twitch force (P_t) was therefore determined by applying a single 70 V, 4-ms wide pulse. Rheobase (R_{50}) was defined as the voltage of a 4-ms pulse that elicited a twitch force of 50% of P_t. To normalize the rheobase, the stimulation voltage was divided by the electrode separation and the R_{50} is reported as electric field strength in units of V/mm. To determine the chronaxie (C_{50}), the stimulation voltage was adjusted to twice the rheobase, and the pulse width was reduced until the twitch force once again dropped to 50% of P_t. Excitability curves were generated by subjecting the myooids to single twitches using a matrix of 3, 6, 10, 15, 20, 25, 30, 35, and 40 V crossed with pulse widths of 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, and 2.0 ms.

Maximum isometric tetanic force (P_0) was determined by stimulating the myooid at 40 V and 40 Hz with 1.20-ms pulses for a train duration of 2 s. Specific isometric tetanic force (specific P_0) was calculated by dividing P_0 by the total cross-sectional area (CSA) of the myooid. CSA was calculated from the myooid diameter. Force–frequency data were collected using a pulse width of 1.2 ms, amplitude of 40 V, train duration of 2 s, and frequencies in the range of 3–100 Hz. Length–force curves were generated by applying stimulus trains of 70 Hz at 45 V and 1.2-ms pulse width for 2 s to achieve maximum tetanus for each data point. The length was varied between 50% reduction and 40% increase of length from the initial myooid length of 12 mm, by adjusting the force transducer position using a micromanipulator. The myooid was allowed 30 s to recover to resting baseline force before each subsequent stimulus train. For each myooid, the raw data for the twitch and isometric tension traces were saved to a disk for later analysis. Time to peak twitch force (TPT) and half relaxation time (½RT) for isometric twitches were measured from the raw data traces, using a previously published method (Close, 1964). Briefly, TPT was determined by measuring the time from the end of the latent period until P_t was achieved, and ½RT was determined by measuring the time from P_t until the twitch force decayed to a value equal to ½P_t. The rate of twitch force generation (dP/dt) was determined by measuring the slope between 20 and 80% of P_t.

Histology. The myooids were fixed at 4° C in formaldehyde/glutaraldehyde, 3% each, in 0.1 *M* sodium cacodylate buffer at pH 7.4 (Electron Microscopy Sciences, catalog number 15950) (Electron Microscopy Sciences, Fort Washington, PA) and embedded in EPON (Eponate 12 resin, Ted Pella Inc., Redding, CA) for both light and electron microscopy. The embedded specimens were sectioned ~600-nm thick and stained with Toluidine blue (1%). Sections were viewed and photographed using a Nikon Axiophot inverted phase contrast microscope, with a ×20 objective. Commonly used methods for the identification of myosin isoform expression in muscles, such as gel electrophoresis and adenosine triphosphatase (ATPase) staining of frozen sections (Brooke and Kaiser, 1970), could not be used. After fixing and mounting the myooids in EPON for light microscopic sections, insufficient tissue was available for performing quantitative gel electrophoresis to quantify the myosin

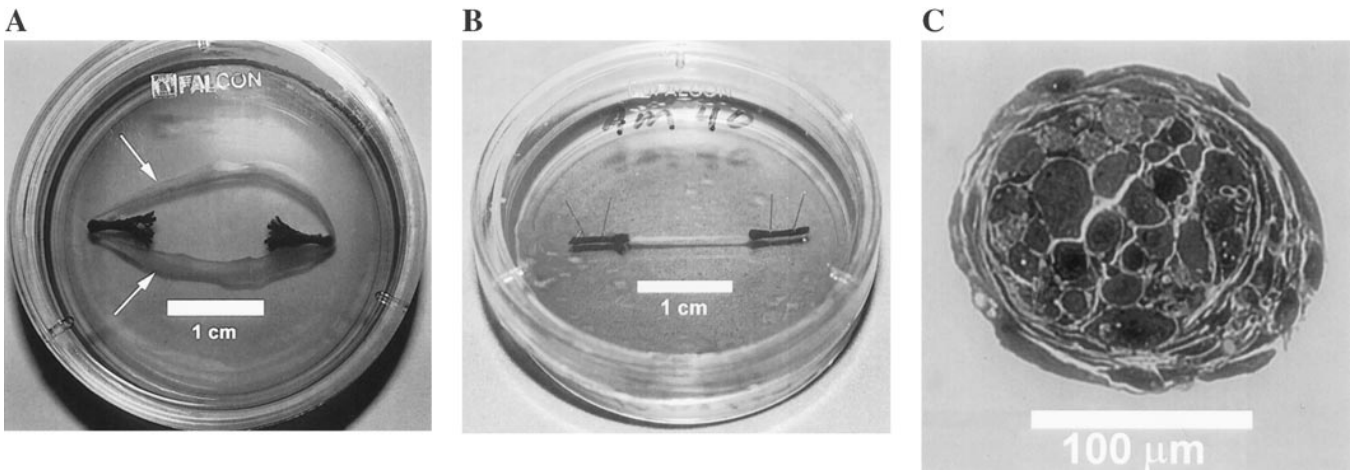

FIG. 1. Formation and morphology of a myooid. (*A*) Myooid formation between laminin-coated suture anchors in a 35-mm diameter culture dish. Myooids form by detaching from the substrate material as a monolayer and rolling into a cylinder between the anchors. The edge and direction of the monolayer as it rolls into a cylinder is indicated by the *arrows*. The detachment time is controlled by the plating density of cells, the density of laminin on the substrate surface prior to plating, and the substrate material. (*B*) The myooid remains attached at each end to anchors consisting of laminin-coated suture. (*C*) A cross-section of a myooid shows several muscle fibers (~20) within the myooid. The flattened cells around the periphery of the myooid are fibroblasts. The section is stained with 1% toluidine blue. (Plate am5)

isoform expression. The use of myosin ATPase staining would be misleading due to the presence of developmental myosin isoforms (Guth and Samaha, 1972).

RESULTS

Formation of myooids in culture. The failure rate due to contamination of the cultures was less than 5%. Contaminated dishes were discarded immediately, and their data were not included in the analysis. In the absence of laminin on the SYLGARD substrate, the primary cells would not form a confluent monolayer on the entire surface, and subsequently detached in small irregular patches in such a way that myooids generally did not form. Myooids readily formed between anchors of laminin-coated suture or acellularized muscle fragments, but did not form between laminin-coated stainless steel pin anchors, or cellular or acellularized tendon segments, or uncoated silk-suture anchors. For all experiments designed to measure contractile function, myooids were grown using laminin-coated silk-suture anchors because the anchors provided an excellent attachment site for the force transducer. In addition, the laminin-coated silk-suture anchors did not require the use of additional animals, as did the preparation of acellularized muscle fragments. Occasionally, when maximally stimulated during evaluation, a myooid broke at some point along its length. The breakage never occurred at the junction between the myooid and the suture anchor, demonstrating that this interface was mechanically sound.

Myooid formation typically began with peripheral delamination of the cell monolayer, which progressed radially inward until the entire monolayer had peeled away from the substrate (Fig. 1*A*). The process of detachment was accelerated by the spontaneous contractions of the myotubes in the monolayer. In some cases, the delamination was rapid enough to be visible under the microscope at low magnification. Once completely detached from the substrate, the monolayer remained attached between the anchors as a sheet of cells rolled into a loose cylindrical shape. Within 3 d, the monolayer had remodeled into a solid cylindrical structure, with myotubes oriented primarily in parallel with the myooid axis as defined by the anchors (Fig. 1*B*). The fibroblasts were generally organized around the periphery of the myooid (Fig. 1*C*). Myotubes adhered to and interdigitated with the strands of the frayed silk-suture anchors. Within 72 h of detachment of the monolayer to form the myooid, most of the void space in the myooid had been removed and the myooid cross-section consisted primarily of myotubes, extracellular matrix, and cellular debris, with no evidence of layering due to rolling up of the monolayer during formation (Fig. 1*C*). When cross-sections from newly formed myooids were compared with those from myooids that had been fully formed for 2 wk or more, a progressive loss of extracellular material was observed. At the time of evaluation of contractile properties, the myooid cross-section occupied by myotubes was in the range of 30–60% of the total CSA.

The cellular disorganization was confirmed by light microscopy of myooid cross-sections and electron microscopy of longitudinal sections (data not shown). The myooid cross-sections indicated a structure different from that of fully differentiated adult mammalian skeletal muscle in that the myooids had many small void spaces, large amounts of disorganized extracellular material, centrally located nuclei in the myotubes, and no vascular structure or innervation. Furthermore, the visible striations in adult muscle fibers, indicative of the highly ordered pattern of sarcomeres, were rarely evident in the myooids in culture. We conclude that the myotubes in the myooids lack the highly regular structural organization of sarcomeres evident in adult muscle fibers.

The laminin concentration on the SYLGARD substrate was used to control cell adhesion and subsequent time of monolayer detachment from the substrate for the formation of the myooid, which also influenced the initial myooid diameter (Fig. 2). The first data point for each laminin density represents the time of initial myooid formation. Laminin densities of 0–3 $\mu g/cm^2$ were evaluated, although Fig. 2 represents data only for laminin densities at which myooids were able to consistently form. Laminin concentrations greater than 1.5 $\mu g/cm^2$ resulted in more-rapid formation of dense monolayers

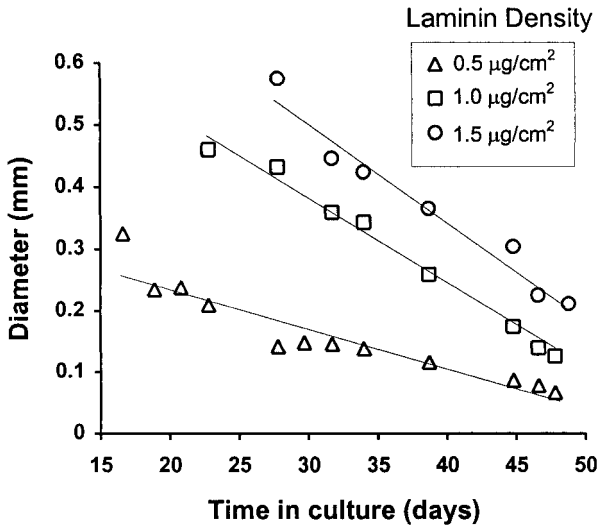

FIG. 2. Effect of laminin-coating density on initial myooid diameter and myooid-formation time from cell seeding. With increasing laminin density, the initial myooid diameter and time to myooid formation is increased. Data for laminin concentrations of 0.0, 0.2, 0.75, and 3.0 μg/cm² are not shown. Each data point represents average values for four myooids. The lines were generated from a first-order linear least-squares interpolation. The r^2 values for laminin densities of 0.5, 1.0, and 1.5 μg/cm² were 0.88, 0.99, and 0.96, respectively. (Plates am21–am44)

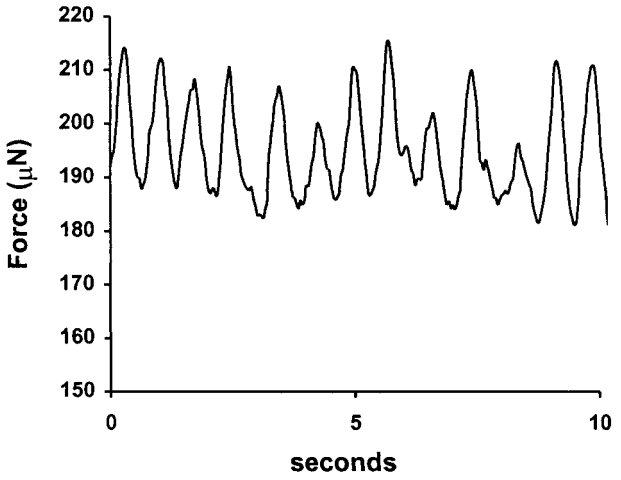

FIG. 3. Spontaneous contractions of a single myooid. The spontaneous contractions begin shortly after myooid formation, typically occur at a regular frequency of 0.8–1.5 Hz, and continue as long as the myooids are maintained in culture. The peak amplitude varies over time to produce periods of more or less spontaneous contractile activity. (Plate a123, 27 d after cell seeding)

of confluent cells and larger initial myooid diameters, but also delayed or sometimes prevented the formation of myooids due to incomplete detachment of the monolayer from the substrate. Cells plated on SYLGARD with only 0.2 μg/cm² of laminin had a myooid-formation success rate of only 25%, whereas cells plated on uncoated SYLGARD had a myooid-formation success rate of less than 5%. For the formation of myooids, the optimal value for laminin coating on SYLGARD was in the range of 0.5–1.5 μg/cm². Myooid diameter decreased steadily during culture, over the period of several weeks (Fig. 2).

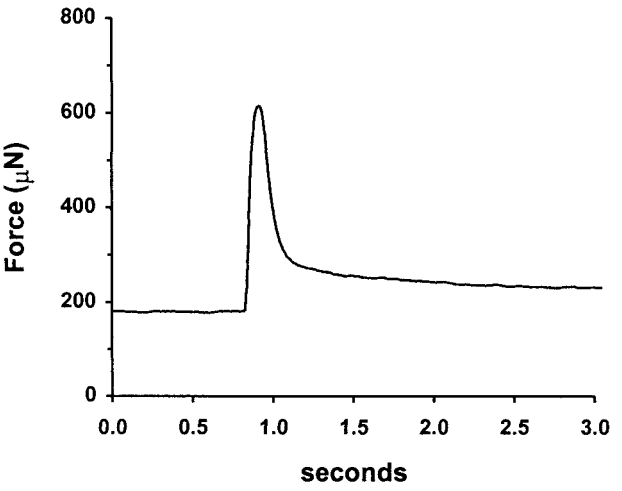

FIG. 4. Peak twitch force of a single myooid. Twitches were elicited using a single 70 V, 4-ms pulse delivered to the myooid via parallel platinum wire electrodes. The latent period was 9–15 ms from the initiation of the stimulation pulse to the initiation of tension generation. For supramaximal twitches, the recovery of baseline tension is delayed for approximately 30 s. Baseline tension is recovered within 4 s for submaximal twitches. (Plate ah15, 28 d after cell seeding)

Within 48 h of formation, the myooids began to contract spontaneously (Fig. 3) and continued to do so for as long as they remained in culture. The peak-to-peak force generated during spontaneous contractions was typically in the range of 3–30 μ*N*, but was occasionally as high as 40 μ*N*. The spontaneous contractions appeared to be more vigorous for several hours after replacement of the culture medium, and occasionally were so vigorous that they were visible with the unaided eye. The mechanical stimulus from the spontaneous contractions themselves, combined with the refractory period of each myotube after a spontaneous twitch, may explain the synchronization of the spontaneous contractions of the myotubes within the myooid. The application of electrical stimulation during isometric contractile force evaluations temporarily attenuated the spontaneous contractions. In general, the peak-to-peak force of the spontaneous contractions was one to two orders of magnitude less than P_t.

Excitability and isometric contractile properties. For the measures of myooid excitability and isometric contractile function, a group of 14 myooids were evaluated 31 ± 4 d after the cells were plated. The myooids were excitable by transverse electrical stimulation between parallel platinum wire electrodes. The general appearance of electrically induced twitches (Fig. 4) and fused tetani (Fig. 6) of myooids was very similar to that of control adult skeletal muscles, though the normalized forces were much lower. When stimulus amplitudes above that required to elicit a peak twitch were applied, the return of force to baseline was delayed for long periods (Fig. 4).

A series of pulses of variable pulse width and voltage were applied to determine the excitability of the myooids. The excitability of a typical myooid is illustrated in Fig. 5. The effect of pulse amplitude (Fig. 5*A*) and pulse duration (Fig. 5*B*) on twitch force were measured to determine the excitability in terms of rheobase and chronaxie. Lower values of both rheobase and chronaxie indicate greater excitability. When measured at the level of 50% of P_t, the myooids had a rheobase (R_{50}) of 1.0 ± 0.02 V/mm (mean ±

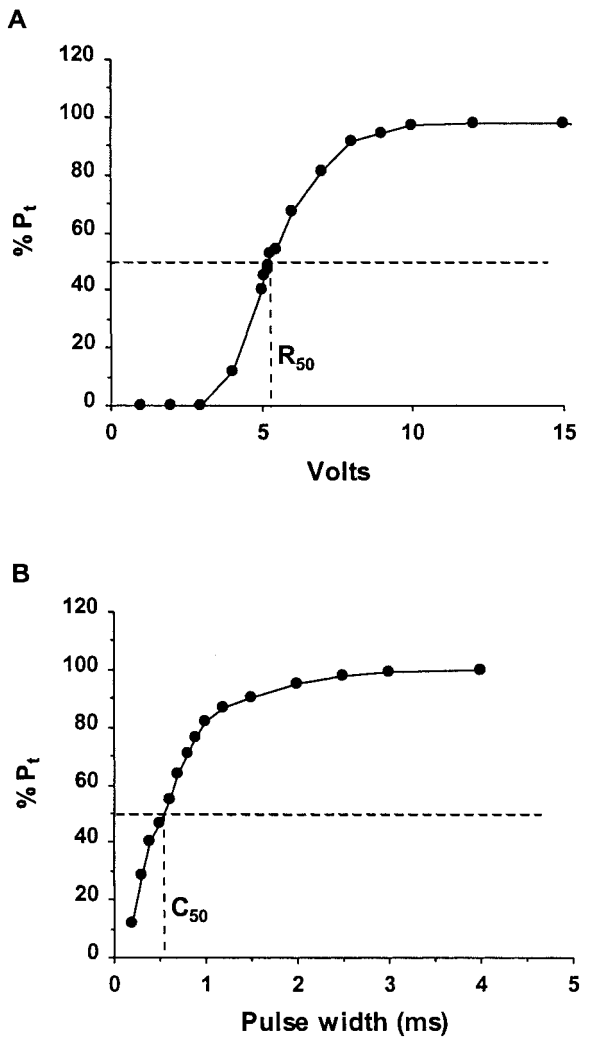

FIG. 5. Excitability curves for a single myooid. For both plates, the twitch tension on the ordinate is expressed as a percentage of peak twitch tension (% P_t), determined with a single pulse at 70 V and 4 ms. (*A*) Using a 4-ms pulse width, voltage was increased incrementally from zero. As voltage is increased, twitch tension increases as a percent of peak tension. The sigmoid shape of the curve results in maximum slope at approximately 50% P_t, the point at which the rheobase (R_{50}) was measured. (*B*) Voltage was set to two times the value determined for the rheobase ($2 \times R_{50}$), and the pulse width was incrementally increased to determine the chronaxie (C_{50}), which was also measured at the 50% P_t threshold. (Plate am44, 34 d after cell seeding)

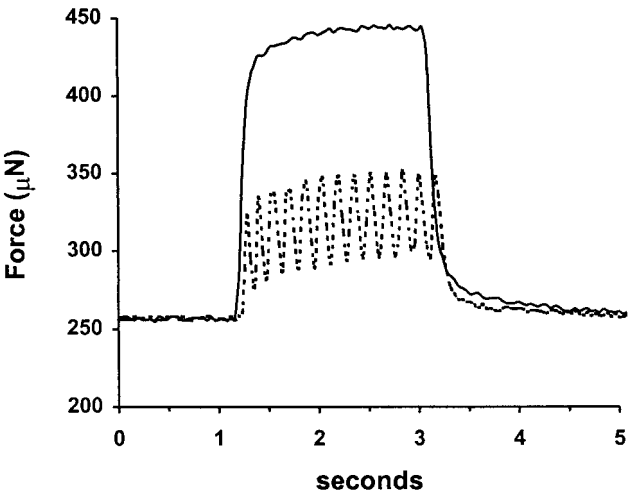

FIG. 6. Fusion of tetanus of a single myooid. Stimulation was at 1.2-ms pulse width, 40 V, for 2 s. The *broken line* represents 6-Hz stimulation frequency, the *solid line* represents 30-Hz stimulation frequency. The force–frequency experiment protocol was applied to the myooids after determination of P_t and P_0. Fusion of tetanus typically occurred at 20 Hz, P_0 was achieved at or below 40 Hz. (Plate am41, 33 d after cell seeding)

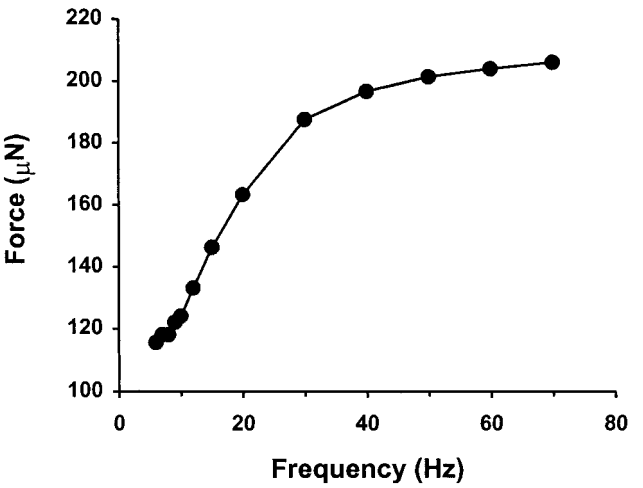

FIG. 7. Force–frequency diagram for a single myooid. Stimulation was at 1.2-ms pulse width, 40 V, for 2 s. Tetanic force increased with increasing frequency up to 100 Hz (the highest frequency employed), but 95% of the apparent asymptotic value was typically achieved at or below 40 Hz. (Plate am41, 33 d after cell seeding)

standard error of mean [SEM]), and a chronaxie (C_{50}) of 0.45 ± 0.02 ms. Generally, pulse widths of 600–1200 μs were required to elicit a maximal twitch, as compared with 60–100 μs for a typical adult rat soleus muscle (unpublished data).

A rapid series of pulses in the frequency range of 3–100 Hz was used to elicit tetanic contractions of the myooids (Fig. 6). Force–frequency data were collected for each myooid, with a representative curve shown in Fig. 7. Typically, the force–frequency plateau began at 30–40 Hz. The myooids had a diameter of 489 ± 36 μm (mean ± SEM), and isometric contractile properties of: P_t = 215 ± 26 μ*N*, P_0 = 440 ± 45 μ*N*, and P_b = 181 ± 16 μ*N*. The specific P_0 was 2.9 ± 0.5 k*N*/m². Myooids with smaller diameters tended to have greater specific P_0-generating capacity (Fig. 8). The dynamic properties of the isometric twitches were TPT = 69 ± 3 ms and ½RT = 116 ± 5 ms (mean ± SEM). The average rate of twitch force development (dP/dt) for all 14 myooids was 4.5 ± 0.6 μ*N*/ms.

Length–force curves of myooids are shown in Fig. 9. For whole muscle, the optimal length for force development (L_o) is a short plateau in the center of the length–force curve, indicating the region of maximal overlap between the thick and thin filaments. The active and passive length–force data shown in Fig. 9 are the raw data from a single myooid. The length–force data for the other myooids were similar, differing principally in the absolute magnitudes of the ac-

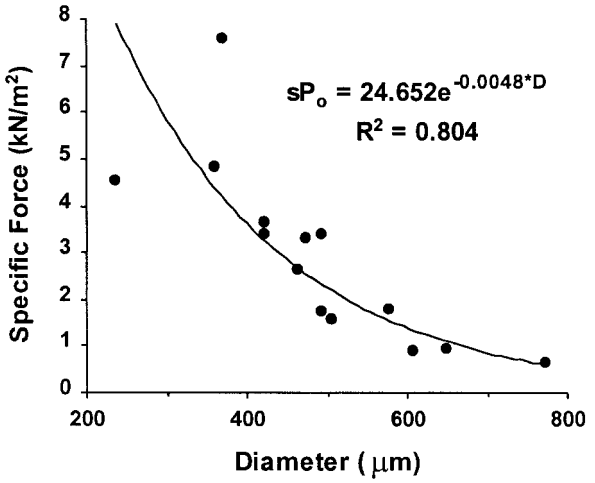

FIG. 8. Relationship between myooid diameter and specific force for 14 myooids. A least-squares approximation was used to fit the data to an exponential decay function, where 'e' is the base of the natural log, and 'D' is the myooid diameter in microns. (Plates x88–x101, 30 d after cell seeding)

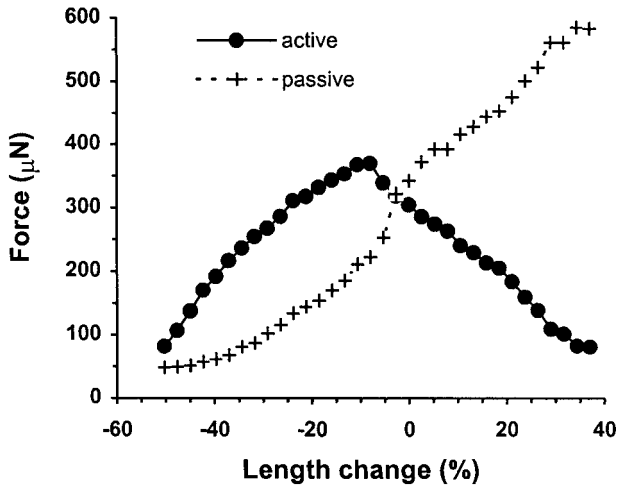

FIG. 9. Length–tension curve for a single myooid. Initial length of the myooid is defined by the distance between the suture anchors, which was set to 12.0 mm at the time the cells were plated. The length was varied using a micromanipulator to which the force transducer was attached. Stimulation for each data point was at 1.2-ms pulse width, 45 V, 70 Hz, for 2 s. (Plate ah85, 27 d after cell seeding)

tive and passive forces at each length. As with whole muscle, the total force of a myooid is the sum of the active and passive forces.

The myooids were cultured at a fixed length (L_c) of 12 mm between suture anchors, which corresponds to a length change of 0% on the abscissa of Fig. 9. The length–force data indicate that the myooids were under passive tensile force at the length at which they were cultured. This is confirmed by the observation that the myooids were suspended above the substrate between the anchors during culture. The passive force at L_c was often as great as the maximum active force at the same length. Typically, the passive force for whole muscle is zero at lengths less than L_o. In contrast, the myooids had significant passive force over the entire range of the length–force curve for which they were able to generate force. Thus the passive component of the length–force curve for myooids is left-shifted from that which would normally be expected for whole adult muscle. The length–force data also indicate that L_o occurs at a length 5–10% shorter than L_c. This was the case for all myooids for which length–force curves were generated. To ensure that the myooids were near L_o for the measurement of the isometric contractile properties, each myooid was shortened by 5% from the initial length of 12 mm prior to the collection of contractile data.

DISCUSSION

The high success rate of >95% for the generation of myooids from primary myogenic precursor cells of mammals was encouraging. The myooids exhibited consistent and repeatable excitability and isometric contractile properties in culture over a period of several months in culture. Despite these encouraging aspects of the experiments, the specific P_0 of the myooids was approximately 1% of the specific P_0 of ~300 k*N*/m² generated by the adult phenotype of control skeletal muscles (Gordon et al., 1966). The P_0 developed by skeletal muscles of adult mammals is a direct function of the proportion of the myofiber CSA composed of sarcomeres containing adult fast and slow isoforms of myosin. Our working hypothesis is that the low specific P_0 of the myooids compared to that of adult muscle fibers results from (1) a large percentage of the myooid cross-section being composed of noncontractile material, (2) a general disorganization at both the cellular and subcellular levels of the myotubes, and (3) myotubes expressing chiefly embryonic and neonatal myosin isoforms.

Little data exists on the specific P_0 of embryonic or neonatal muscles, but for 1-d old Wistar rats, the specific P_0 values were 74 k*N*/m² for the EDL muscles and 44 k*N*/m² for the soleus muscles (Close, 1964). Consequently, myooids composed of myotubes expressing chiefly or exclusively embryonic or neonatal isoforms could account for some, but not all of the difference in specific P_0, since a 10-fold difference still remains. The low specific P_0 of the myooids, even when compared with the specific P_0 of muscles from neonatal rats, indicates that major deficiencies arise in the force development of myooids due, at least in part, to the inclusion of noncontractile material and the disordered structure of the sarcomeres.

The prolonged TPT and ½RT of the twitches, when electrically stimulated, and the plateau of the force–frequency curve, at low frequencies, indicate convincingly that the myotubes in the myooids do not express adult fast isoforms of myosin. The TPT for the myooids, derived from rat soleus muscles, averaged 69.3 ± 9.4 ms (mean ± standard deviation [SD]). By comparison, TPT for whole rat soleus muscles has been reported as 65.0 ± 3.8 ms for neonates and 36.0 ± 2.3 ms for adults (Close, 1964). The ½RT for the myooids was 116.4 ± 19.4 ms (mean ± SD), which was prolonged when compared with previously reported values for soleus muscles from either neonatal (70.0 ± 4.9 ms) or adult (48.0 ± 3.4 ms) rats (Close, 1964). To further test the hypothesis that the myotubes in the myooids were arrested at an early developmental state, the dP/dt was measured from published graphs (Close, 1964), and our results were compared with these values. For soleus muscles from 140-d old rats, the dP/dt was ~24,000 μ*N*/ms, whereas the dP/dt for neonatal rat soleus muscles was only ~140 μ*N*/ms. For the myooids, the dP/dt was 4.5 μ*N*/ms. Because the P_t values differ significantly, comparisons of dP/dt may be more relevant than comparisons of TPT. For a given rate of force development, greater twitch forces

will take longer to develop than lower twitch forces, with correspondingly longer TPT. Taken as a whole, the TPT and dP/dt values for the myooids, when compared with whole soleus muscles from neonatal and adult rats (Close, 1964), support the hypothesis that the myotubes in the myooids remain at a very early developmental state, expressing chiefly embryonic or neonatal isoforms of myosin.

In contrast to the comparison with adult skeletal muscle, the specific P_0 generated by myooids was similar to that reported previously for organoids. In 1991, Vandenburgh et al. (1991) reported the generation of contractile force by organoids of avian origin, following the elevation of extracellular potassium to 75 m*M*. The procedure was performed on four individual organoids at 4° C. The organoids were 1.0–2.0 mm in diameter and 30–35 mm in length. When activated by the extracellular potassium, the force reached a plateau within 30–60 s. The absolute resting force was reported as 149 ± 47 mdyn and the absolute isometric force (sum of passive and active forces) as 285 ± 53 mdyn, or a net increase of 91% over the resting force. Due to a typographical error, forces were reported in mdyn, but were actually measured in dynes (H. Vandenburgh, pers. comm.). The force of the organoids was not normalized by the CSA. Consequently, specific P_0 was estimated based on the diameter and force in dynes (Vandenburgh et al., 1989b). The specific P_0 of the organoids, from 0.9 to 3.6 k*N*/m², were in excellent agreement with our value of 2.9 k*N*/m² for the specific P_0 of the myooids.

In the present study, myooids with a smaller CSA tended to have a higher specific P_0 than those with larger CSA. Cross-sections of the myooids indicated that the percentage of CSA occupied by myotubes in the total myooid CSA is larger for myooids of smaller diameter (data not shown). The negative relationship between the CSA and specific P_0 resulted, at least in part, from a larger percentage of noncontractile tissue in the larger-diameter myooids. For control muscles, specific P_0 is calculated on the basis of the CSA of viable contractile tissue within the muscle (Close, 1964; Faulkner et al., 1997). It was impossible to discriminate between viable and nonviable myotubes within the myooids on the basis of histological sections alone, so the total CSA of each myooid was used for the calculation of specific P_0. This yielded a conservatively low estimate of specific P_0 for the myooids. In the larger myooids, the increased diffusion distance for myotubes near the center of the myooid restricted the diffusion of nutrients and waste products, which might also have contributed to impaired function and a reduced ability of the myotubes near the center of the myooid to generate force. The myotubes within the myooid are typically 10–25 μm in diameter, and therefore myotubes near the center of large-diameter myooids could be separated from the culture medium by a tissue thickness of 4 or 5 myotubes. If diffusion limits the function of myotubes near the center of the myooids, the myooid diameter that we have reported may represent the maximum achievable diameter of these avascular functional in vitro constructs.

In summary, the objective to engineer three-dimensional skeletal muscle tissue constructs using primary cells from adult mammals and measure their excitability and isometric contractile properties was achieved. The constructs, termed myooids, were muscle-like in appearance, excitability, and contractile function. The excitability of the myooids was low when compared with control adult mammalian skeletal muscle, probably due to the lack of innervation of myooids during their development and maintenance in culture. The isometric contractile properties of the myooids were qualitatively similar to those of control muscle, but the normalized forces were considerably lower than values for control muscles from adult animals. The myotubes in the myooids appeared to be arrested in an early developmental state due to the absence of signals to promote cellular and subcellular organization and expression of adult myosin isoforms.

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