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Excitability and contractility of skeletal muscle engineered from primary cultures and cell lines

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Dennis, Robert G., Paul E. Kosnik II, Mark E. Gilbert, and John A. Faulkner. Excitability and contractility of skeletal muscle engineered from primary cultures and cell lines. *Am J Physiol Cell Physiol* 280: C288–C295, 2001.—The purpose of this study was to compare the excitability and contractility of three-dimensional skeletal muscle constructs, termed myooids, engineered from C_2C_{12} myoblast and 10T½ fibroblast cell lines, primary muscle cultures from adult C3H mice, and neonatal and adult Sprague-Dawley rats. Myooids were 12 mm long, with diameters of 0.1–1 mm, were excitable by transverse electrical stimulation, and contracted to produce force. After ~30 days in culture, myooid cross-sectional area, rheobase, chronaxie, resting baseline force, twitch force, time to peak tension, one-half relaxation time, and peak isometric force were measured. Specific force was calculated by dividing peak isometric force by cross-sectional area. The specific force generated by the myooids was 2–8% of that generated by skeletal muscles of control adult rodents. Myooids engineered from C_2C_{12}-10T½ cells exhibited greater rheobase, time to peak tension, and one-half relaxation time than myooids engineered from adult rodent cultures, and myooids from C_2C_{12}-10T½ and neonatal rat cells had greater resting baseline forces than myooids from adult rodent cultures.

tissue engineering; myooid; myogenesis; isometric force; rodent tissue culture

IN VITRO SKELETAL MUSCLE TISSUE engineering involves culturing isolated myoblasts and fibroblasts in an environment conducive to the formation of a three-dimensional tissue construct. Cell sources employed to engineer such constructs include the C_2C_{12} mouse muscle sarcoma cell line (15, 26, 29), embryonic avian muscles (1, 21–23, 25, 28), and skeletal muscles of neonatal (21) and adult (5, 17, 27) mammals. To promote formation of a three-dimensional structure, cells have been cultured in collagen gels (1, 15, 21, 26, 27, 30), on an elastic membrane (1, 21, 28), or between two stationary anchor points to which the cell monolayer remains attached (5, 21, 23). Collagen gels provide a scaffold for the cells and define the size and shape of the construct but may interfere with the force and power production of the myotubes. In the absence of an artificial scaffold, embryonic avian cells were developed into cylindrical organoids when subjected to uniaxial mechanical strain (28) or when cultured on prestrained elastic substrates (21). In one report, the isometric force of organoids was measured by lateral displacement of the organoids with a calibrated microneedle after elevation of the extracellular K^+ concentration (28).

Cylindrical musclelike structures, termed myooids, were engineered from cocultures of primary mammalian myoblasts and fibroblasts (5), without a preexisting scaffold, the application of uniaxial mechanical strain, or prestressed substrates. Myooids were supported under tension between artificial tendons of laminin-coated silk suture; organoids were attached to stainless steel screens. In the case of myooids, the laminin-coated silk suture segments served as flexurally compliant artificial tendons, permitting easy attachment to a force transducer for functional evaluation. The excitability and contractility of myooids were measured with a force transducer by transverse electrical stimulation between parallel electrodes and measurement of axial force development (5). When normalized for cross-sectional area (CSA), the specific force (sP_o) generated by myooids was not different from that of organoids, both being equivalent to ~1% of the sP_o of adult rodent skeletal muscle (5).

The purpose of this study was to compare the CSA, excitability, and contractility of myooids engineered from cocultures of C_2C_{12} myoblast and 10T½ fibroblast cell lines and primary cultures from the skeletal muscles of adult mice and neonatal and adult rats. For simplicity in terminology, myooids engineered from C_2C_{12}-10T½ cells, adult mouse, neonatal rat, and adult rat are referred to as C_2C_{12}-10T½ myooids, adult mouse myooids, neonatal rat myooids, and adult rat myooids, respectively. On the basis of preliminary data and observations, our working hypothesis is that the excitability and contractility of myooids engineered from primary cell sources do not differ, while the excitability and contractility of C_2C_{12}-10T½ myooids are lower than those of myooids engineered from primary cells. Our specific hypotheses are that *1*) the measures of excitability and contractility do not differ among myooids engineered from primary cell sources and *2*)

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C_2C_{12}-10T½ myooids have greater rheobase, chronaxie, time to peak tension, and one-half relaxation time and lower twitch force and peak isometric force than primary rodent myooids.

MATERIALS AND METHODS

The methods for engineering myooids from primary cultures of rat soleus (SOL) muscles and the methods and instrumentation for measuring the excitability and contractility of myooids have been described in detail (5). For the formation of neonatal rat myooids, modifications were made in the surgical techniques for the removal of muscle tissue and in the composition of the cell culture and tissue dissociation media. The formation of myooids from established cell lines was accomplished only with the coculture of C_2C_{12} myoblasts with 10T½ fibroblasts and the use of a reduced-serum culture medium (CM).

Media and solutions. The CM were growth medium (GM) and differentiation medium (DM), which have been described previously (5). For the culture of C_2C_{12} cells, a CM was prepared [465 ml of DMEM (catalog no. 11995-065, GIBCO BRL), 35 ml of fetal bovine serum (FBS; catalog no. 10437-036, GIBCO BRL), and 100 U/ml penicillin G]. For storing neonatal muscle during harvesting, mincing solution (4) was prepared, brought to pH 7.4, and sterilized through 0.22-μm filters. Preincubation medium (PIM) was prepared as described previously (5). The CM and PIM were stored at 4°C until use. Tissues from adult animals were dissociated in an "adult" dispase-and-collagenase (D & C) solution [4 U/ml dispase (catalog no. 17105–041, GIBCO BRL) and 100 U/ml type IV collagenase (CLS 4, Worthington) in DMEM]. The neonatal muscle tissue was dissociated using "neonatal" D & C (ND & C) solution [4 U/ml dispase (catalog no. 17105-041, GIBCO BRL) and 100 U/ml type IV collagenase (CLS 4, Worthington) in mincing solution]. D & C and ND & C solutions were prepared immediately before use and sterilized through 0.22-μm filters.

Isolation and culture of primary cells. All animals were handled in accordance with the *Guide for the Care and Use of Laboratory Animals*. Neonatal Sprague-Dawley rats were delivered in litters of 11–14 within 1 day of birth from Charles River (Wilmington, MA), and muscle tissue was harvested from all the pups in the litter. On arrival, neonates were placed in 15-cm glass petri dishes on ice for 0.75–1.5 h as a general anesthesia. In a biological safety cabinet, the anesthetized pups were completely immersed in 70% ethanol before surgical removal of the muscle tissue. The muscles from the fore- and hindlimbs were removed and placed in 8 ml of mincing solution. ND & C solution (12 ml) was added, and the tissue was dissociated by magnetic stirring at room temperature for 1.5 h. The magnetic stir bar was removed, and the slurry was centrifuged for 15 min at 1,000 *g*. The pellet was resuspended in 20 ml of 0.1% (wt/vol) collagenase type IV (Worthington) in Ham's F-12 nutrient medium (GIBCO BRL) and placed in a shaker bath at 37°C for 1.5 h. The pellet was then centrifuged for 15 min at 1,000 *g* and resuspended in GM. Cells were counted with a hemocytometer, and the solution was adjusted to achieve the desired cell plating density (1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, or 10 cells/cm^2). Two milliliters of the cell suspension were plated onto each 35-mm culture dish with anchors in place, and the culture dishes were placed in a water-saturated incubator with 5% CO_2 at 37°C.

For the harvesting of satellite cells and fibroblasts from adult (5- to 6-mo-old) Sprague-Dawley rats and C3H mice, animals were anesthetized with an intraperitoneal injection of pentobarbital sodium and the whole SOL or extensor digitorum longus (EDL) muscles were surgically removed. Each rat SOL muscle was sliced into six strips longitudinally from tendon to tendon; the mouse SOL and EDL muscles were left whole. The muscles and muscle strips were pinned at slack length in 35-mm culture dishes with Sylgard (Dow Corning, Midland, MI), preincubated for 50 h in PIM in a water-saturated incubator with 5% CO_2 at 37°C, and then dissociated in 7.5 ml of D & C solution per strip or whole muscle for 3–4 h at 37°C. To achieve the greatest possible yield of satellite cells, the dissociated tissue was plated directly into the prepared culture dishes with laminin-coated suture anchors in place, without an attempt to filter the dissociated cells from the tissue debris. The plating density was therefore expressed as the amount of muscle tissue dissociated for each myooid in each 35-mm culture plate. Optimal plating densities for primary cells from rat and mouse muscle had been determined previously, with myooid size and contractility as the parameters to be optimized. The rat SOL muscle was plated at 13 g of tissue per plate, and the adult mouse SOL and EDL muscles were plated at 10 and 13 g of dissociated tissue per plate, respectively. For primary cultures, beginning at 48 h after cell plating, the GM was replaced every 48 h until the cells became confluent, which typically required 3–8 days. One day after the cells reached confluence, the medium was replaced with DM, and the cells were fed three times per week thereafter. Shortly after they were switched to DM, the myocytes fused to form multinucleated myotubes that began to contract spontaneously.

Culture of cells from established lines. Myoblasts of the C_2C_{12} cell line (CRL-1772; American Type Culture Collection, Rockville, MD) formed myooids only when cultured with 10T½ fibroblasts (provided by Dr. G. Salvatori). In preliminary experiments, plating densities of 1×10^6, 1×10^5, 1×10^4, 1×10^3, and 1×10^2 cells/cm^2 were used for cocultured cells and for monocultured C_2C_{12} cells. On the basis of the results from the preliminary experiments, for the cocultures of C_2C_{12} and 10T½ cells the plating density was fixed at 1×10^5 cells/cm^2. The myoblasts and fibroblasts were combined to produce cocultures with 30–80% C_2C_{12} cells in increments of 10%. The C_2C_{12} and 10T½ cells were mixed in CM, plated, and allowed to remain in culture for 48 h before the first media change. Until the myooids were evaluated, regular media changes were carried out every 48–72 h. There were no differences in the excitability or contractility of the myooids formed from different initial ratios of cell types. Consequently, these groups, termed C_2C_{12}-10T½ myooids, were pooled for subsequent analysis.

Myooid formation. For all cell sources, myooids formed by delamination of the monolayer of cultured cells from the Sylgard substrate. Artificial tendons of silk suture with one end frayed and coated with natural mouse laminin were employed, and the Sylgard substrate in each dish was coated with laminin at a density of 1.0 μg/cm^2. The failure rate due to contamination of the culture plates was <5% for each experiment. Contaminated plates were discarded immediately and not included in subsequent analysis. The C_2C_{12}-10T½ myooids frequently did not detach completely from the Sylgard substrate. To aid detachment, the closed tips of a pair of sterile no. 5 forceps (Fine Science Tools) were used to gently tease the myooid from the substrate. The myooids were then allowed to remodel for ≥72 h before evaluation.

Experimental groups. The five experimental groups, based on the source of the cells for the myooids, were C_2C_{12} and 10T½ cells, adult mouse SOL muscle, adult mouse EDL muscle, neonatal rat limb muscle, and adult rat SOL muscle. The values of 13 experimental variables were determined for

each of the 5 experimental groups. Nine variables were measured directly: morphology (diameter), excitability [rheobase (R_{50}) and chronaxie (C_{50})], isometric force measurements [passive baseline (P_b), peak twitch (P_t), and peak isometric force (P_o)], and the time-dependent variables [time to peak twitch force (TPT), rate of force development (dP/d*t*), and one-half relaxation time ($RT_{1/2}$)]. Four additional parameters were calculated from the measured variables: CSA of the myooid from the diameter, sP_o by dividing P_o by the CSA, and the ratios of peak twitch to peak isometric force (P_t/P_o) and passive baseline to peak isometric force (P_b/P_o). Since CSA was calculated directly from diameter, the diameter was not included in the statistical analysis, leaving 12 experimental variables.

Histology. After contractile properties were measured, myooids were pinned at the length at which they were cultured (L_c) 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). Myooids were rinsed three times (30 min, 30 min, and 4 h) with cacodylate buffer (pH 7.4) with 7.5% sucrose. Myooids were 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) for light microscopy. Cross sections were cut 600 nm thick for light microscopy and stained with 1% toluidine blue.

Data collection and analysis. The methods for measuring the excitability and contractility of myooids have been described in detail previously (5). Culture times were defined from the time of cell plating, rather than from the time of myooid formation. The myooid diameter was defined as the minimum diameter of each myooid along the length between the anchors. Myooid diameter was measured using a calibrated eyepiece reticle while each myooid was viewed through a ×10 objective lens on an inverted microscope (Zeiss Axiovert 25, Thornwood, NY). CSA was calculated from the measured diameter, assuming a circular cross section. All contractile properties were measured 32 ± 4 days after the cells were plated and ≥72 h after formation of each myooid. Myooid temperature was maintained at 37 ± 1°C during the measurement of contractile properties by use of a heated aluminum platform.

For measurements of excitability and contractility, one of the artificial tendons of each myooid was affixed to a force transducer. Myooids were excited by electrical stimulation between two parallel platinum wire electrodes. P_b was measured as the average baseline passive force preceding the onset of stimulation. Data files for each P_t and P_o trace were recorded and stored for subsequent analysis. Force data were collected at 200 samples/s. A median filter of rank 2 was applied to all raw force data before storage to minimize digitization noise without causing a phase delay for rapidly changing forces. P_t and P_o were determined after subtraction of P_b from the total force values. Excitability of the myooids was measured by first applying a long-duration (4- to 6-ms) single stimulus pulse while adjusting the stimulus intensity to achieve a twitch of 50% P_t. The resulting stimulus intensity (voltage) was divided by the electrode separation to determine the field strength (V/mm) required to elicit a 50% twitch, which we define as R_{50}. The stimulus intensity was then fixed to twice R_{50}, and the stimulus duration was adjusted until twitch forces of 50% of P_t were once again achieved. The resulting stimulus duration was defined as C_{50}. The time-dependent twitch force parameters were measured directly from the stored data traces for each myooid. TPT was measured from the onset of force development until the peak twitch value was attained. dP/d*t* was determined as the average slope of the rising portion of the twitch force trace, measured between 20 and 80% of P_t. The time from the onset of relaxation to 50% of P_t was defined as $RT_{1/2}$.

For statistical analysis, the data were analyzed using the Tukey-Kramer method for all pairwise comparisons between treatment means, with α set a priori to 0.05. The Tukey-Kramer method is conservative when sample sizes are unequal, as in this analysis, and is more sensitive than the Bonferroni or the Scheffé method, providing narrower confidence intervals for a given α. Each variable was analyzed separately, with calculations of estimators and test statistics performed in Microsoft Excel 97. The *t*-test statistic was calculated using values of the studentized range distribution $q(1 - \alpha, r, \nu)$ (Ref. 14, appendix table). The test, which assumes all independent variables are normally distributed, was applied for each variable when the *F* ratio indicated significant differences between means.

Myooid life span. Myooids from three of the experimental groups (adult mouse SOL and neonatal and adult rat SOL) were maintained in culture beyond 30 days to determine the time over which they remained viable. Myooids continue to contract spontaneously and may be functionally evaluated until the point at which they mechanically fail, typically by breaking at some point between the suture anchors. We define the time from cell plating until the myooid breaks as the "life span" of the myooid. Life spans were not observed for adult mouse EDL myooids, because a culture contamination at the preincubation stage left only enough tissue for the formation of four myooids, all of which were used for measurement of excitability and contractility. C_2C_{12}-10T½ myooids were not included in the life span study, because excitability and contractility were so variable for this group that a large "*n*" was required to achieve statistical significance for comparisons. No contractile function tests were performed on any myooids used for the life span study. Myooids were fed and monitored three times each week until they failed by mechanical breakage between the suture anchors.

RESULTS

Spontaneous activity of myotubes and myooids in culture. When the spontaneous contractile activity of the myotubes before delamination of the monolayer was compared, qualitative and quantitative differences were evident. The C_2C_{12} myotubes contracted singly and sporadically and at a frequency of 3–5 Hz, whereas the sheet of myotubes from the primary explants contracted regularly and vigorously as a syncytium, with a frequency of 0.5–1.5 Hz. Under low-power magnification, spontaneous contractions were observed in all the myooids from primary cells. In contrast, C_2C_{12}-10T[½] myooids often appeared inactive until electrically excited. Occasionally, large-amplitude spontaneous contractions occurred after electrical stimulation of the C_2C_{12}-10T½ myooids.

Myooid formation. Monocultured C_2C_{12} cells never formed a myooid regardless of the plating density, although in all cases they formed a monolayer of myoblasts and myotubes on the substrate. After ~3–10 days in culture, the monolayer of monocultured C_2C_{12} cells delaminated in small (1- to 2-mm-diameter) irregular patches, resulting ultimately in disintegration of the monolayer. In contrast, the cocultures of C_2C_{12} cells and the 10T½ fibroblasts formed myooids ~50% of the time, independent of the mixing ratio of the cells,

Table 1. *CSA, excitability, and time-dependant isometric contractility of myooids*

Experimental Group	*n*	CSA, mm^2	R_{50}, V/mm	C_{50}, μs	TPT, ms	dP/d*t*, μN/ms	$RT_{1/2}$, ms
C_2C_{12}-10T½	19	0.214 ± 0.050	1.93 ± 0.07	416 ± 41	114 ± 5	2.6 ± 0.4	1,343 ± 254
Neonatal rat	11	0.072 ± 0.021	1.01 ± 0.10	603 ± 89	64 ± 6	0.8 ± 0.1	81 ± 4
Adult rat SOL	11	0.153 ± 0.072	1.02 ± 0.06	526 ± 30	65 ± 4	3.2 ± 0.5	100 ± 18
Mouse SOL	7	0.030 ± 0.011	0.87 ± 0.12	530 ± 109	53 ± 5	7.2 ± 3.2	43 ± 4
Mouse EDL	4	0.024 ± 0.009	0.54 ± 0.08	550 ± 96	50 ± 4	5.1 ± 3.8	90 ± 47
Mouse pooled	11	0.027 ± 0.008	0.75 ± 0.09	537 ± 74	52 ± 3	6.3 ± 2.3	62 ± 19

Values are means ± SE. CSA, cross-sectional area of the myooid calculated directly from the minimum myooid diameter; SOL, soleus muscle from adult animals; EDL, extensor digitorum longus muscle from adult animals; R_{50}, rheobase; C_{50}, chronaxie; TPT, time to peak twitch tension (P_t); dP/d*t*, rate of twitch force development from 20 to 80% P_t; $RT_{1/2}$, one-half relaxation time from P_t; mouse pooled data, mouse SOL + mouse EDL data.

in the range of 30–80% C_2C_{12} cells. The inability of the monocultured C_2C_{12} cells to form myooids appeared to be attributable to the absence of fibroblasts. The fibroblasts likely function to provide an extracellular matrix that strengthens the monolayer before delamination, as reported earlier by Vandenburgh et al. (28).

Since no differences were observed between groups of myooids engineered from different proportions of C_2C_{12} cells and fibroblasts, these data were pooled for subsequent analysis. The larger diameters of the C_2C_{12}-10T½ myooids (Table 1) suggest incomplete remodeling of the monolayer of cells into a cylindrical myooid within the allotted 72 h. The success rate for myooid formation using neonatal cells was >95% at plating densities of 1×10^6 cells/cm^2, ~50% at 1×10^5 cells/cm^2, and 0% for lower plating densities. In contrast, the cells from adult rat cultures formed myooids ~95% of the time when plated on 1.0 μg/cm^2 laminin at 13 mg of dissociated tissue per plate.

Excitability and contractility. The mean values for each of the 12 experimental variables in each of the 5 experimental groups are summarized in Tables 1 and 2. Table 1 shows mean values for CSA, excitability (R_{50} and C_{50}), and the time-dependent isometric twitch parameters (TPT, dP/d*t*, and $RT_{1/2}$) of myooids. The mean values for the isometric contractility measures (P_b, P_t, P_o, sP_o, P_b/P_o, and P_t/P_o) are summarized in Table 2. The results of the Tukey-Kramer method for all 120 pairwise comparisons of variables between groups are summarized in Fig. 1 for the five original experimental groups.

None of the 12 variables differed between adult mouse EDL and adult mouse SOL myooids (Fig. 1). Consequently, these data were pooled, and the Tukey-Kramer method was again applied to the data set (Fig. 2). When the pairwise comparisons between the resulting four experimental groups were examined, several trends were apparent. No differences were observed for C_{50} or dP/d*t*. Differences for CSA, P_t, and P_o were each detected in only one of the six comparisons. R_{50}, TPT, $RT_{1/2}$, and sP_o were different in three of the six comparisons, P_b in four comparisons, and P_b/P_o and P_t/P_o in five comparisons (Fig. 2).

The hypothesis that the excitability and contractility of primary rodent myooids are not different was not supported, since *1*) the adult mouse myooids had a mean sP_o of 15.6 kN/m^2, greater than the 4.2 kN/m^2 for the adult rat myooids, and *2*) for neonatal rat myooids, six variables differed compared with the pooled adult mouse myooids and three differed compared with adult rat myooids (Fig. 2). Neonatal rat myooids had greater P_b and P_b/P_o and lower P_t/P_o than either adult rodent myooid group.

Our hypothesis that the excitability and contractility of C_2C_{12}-10T½ myooids differed from those of primary rodent myooids was supported, since C_2C_{12}-10T½ myooids differed from adult mouse myooids in 8 of the 12 variables, from adult rat myooids in 6 of the 12 variables, and from neonatal rat myooids in 5 of the 12 variables (Fig. 2). In all cases, the C_2C_{12}-10T½ myooids were different from primary rodent myooids in terms of R_{50}, TPT, $RT_{1/2}$, P_b/P_o, and P_t/P_o. The C_2C_{12}-10T½ myooids had a greater CSA than the pooled mouse myooids (Fig. 2). In all comparisons, the C_2C_{12}-10T½ myooids had higher R_{50}. The higher R_{50} indicated that the myotubes formed from C_2C_{12} cells required larger stimulus amplitude and, thus, had lower excitability than myotubes formed from any of the

Table 2. *Baseline passive force and contractility of myooids*

Experimental Group	*n*	P_b, μN	P_t, μN	P_o, μN	sP_o, kN/m^2	P_b/P_o	P_t/P_o
C_2C_{12}-10T½	19	371 ± 52	156 ± 27	214 ± 37	4.5 ± 1.6	2.12 ± 0.23	0.74 ± 0.02
Neonatal rat	11	306 ± 32	48 ± 9	115 ± 18	4.0 ± 1.5	3.16 ± 0.52	0.43 ± 0.05
Adult rat SOL	11	124 ± 30	139 ± 24	224 ± 33	4.2 ± 1.2	0.55 ± 0.08	0.59 ± 0.02
Mouse SOL	7	38 ± 14	321 ± 109	576 ± 199	19.2 ± 5.1	0.10 ± 0.03	0.60 ± 0.04
Mouse EDL	4	37 ± 28	162 ± 125	281 ± 218	9.2 ± 4.7	0.17 ± 0.06	0.55 ± 0.03
Mouse pooled	11	38 ± 13	263 ± 83	469 ± 149	15.6 ± 3.8	0.12 ± 0.03	0.58 ± 0.03

Values are means ± SE. P_b, passive baseline force; P_o, peak isometric force; sP_o, specific force; calculated by dividing P_o by CSA, based on the myooid diameter.

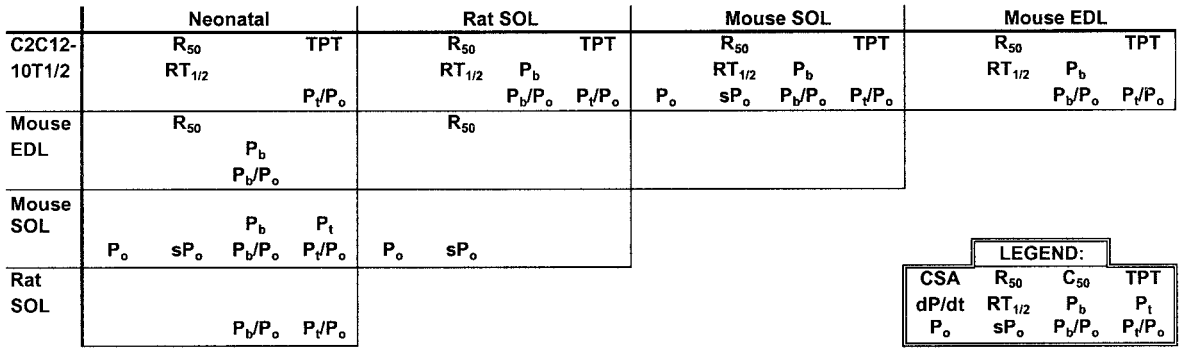

Fig. 1. Summary of paired comparisons, using the Tukey-Kramer method, of myooid excitability and isometric contractile function data. Data were analyzed using the Tukey-Kramer method for all pairwise comparisons between treatment means, with significance threshold set at $\alpha \leq 0.05$. This analysis compared the means of 12 parameters [cross-sectional area (CSA), rheobase (R_{50}), chronaxie (C_{50}), time to peak tension (TPT), rate of force development (dP/d*t*), one-half relaxation time ($RT_{1/2}$), passive baseline force (P_b), peak twitch force (P_t), peak isometric force (P_o), P_b/P_o, P_t/P_o, and specific force (sP_o)] for 5 different experimental groups or "treatments" [C_2C_{12}-10T½, neonatal rat, rat soleus (SOL), mouse SOL, and mouse extensor digitorum longus (EDL)]. Comparisons with significant differences between means are entered. Each of the 10 boxes, for comparisons between treatments, contains all 12 experimental parameters, displayed in the legend. Each parameter always occupies the same position within each box; e.g., TPT is always at *top right*. Within each box, a parameter is displayed only if the difference between the means is significant. With the use of this method to display the results of the Tukey-Kramer analysis of the data, it is possible to readily detect trends in the significant differences between treatments. For example, in no case was the CSA significantly different between treatments, whereas the C_2C_{12}-10T½ treatment differed significantly from all other treatments when R_{50}, TPT, $RT_{1/2}$, and P_t/P_o were considered.

primary cell sources. For C_2C_{12}-10T½ myooids, the $RT_{1/2}$ was an order of magnitude longer than the $RT_{1/2}$ for any of the other myooids.

Life span of myooids in culture. Myooids spontaneously contracted throughout their life span. Typically, the diameter decreased steadily until the myooid broke, usually during a media change after the diameter had decreased to <50 μm. The average and maximum life spans for adult mouse SOL and neonatal and adult rat SOL myooids are presented in Fig. 3. Adult mouse SOL myooids, plated at 10 mg of dissociated tissue per plate, remained viable in culture for an average of 101 ± 12 (SE) days ($n = 8$), with a maximum observed life span of 168 days. Neonatal rat myooids plated at 1×10^6 cells/cm^2 were viable for an average of 52 ± 2.5 days ($n = 25$), with a maximum observed life span of 69 days. Adult rat SOL myooids, plated at 13 mg of dissociated tissue per plate, remained viable for an average of 74 ± 6 days ($n = 11$), with a maximum observed life span of 127 days.

The myooids were suspended above the substrate under continuous tension between the artificial tendons. This observation is consistent with the previous report that myooids were under significant passive tension at the length (L_c) at which they were cultured (5). The myooids in the life span groups were left undisturbed until the myooids broke, so their P_b was not measured. The P_b data from the contractile function groups were used to estimate the initial P_b, at ~ 32 days in culture, of the myooids in the corresponding life span groups (adult mouse SOL and neonatal and adult rat SOL). The life span for each group was then plotted against the estimated P_b. Myooids with higher P_b had correspondingly shorter life spans in culture (Fig. 4).

DISCUSSION

The sP_o for whole muscles from adult rats is ~250 kN/m^2 (3). Few reports exist of the sP_o of mammalian skeletal muscle at early stages of development, but the sP_o for SOL and EDL muscles of newborn Wistar rats were 44 and 74 kN/m^2, respectively (2). The low values of sP_o of skeletal muscles from newborn rats, only 18 and 30% of values for adult rats, have been attributed

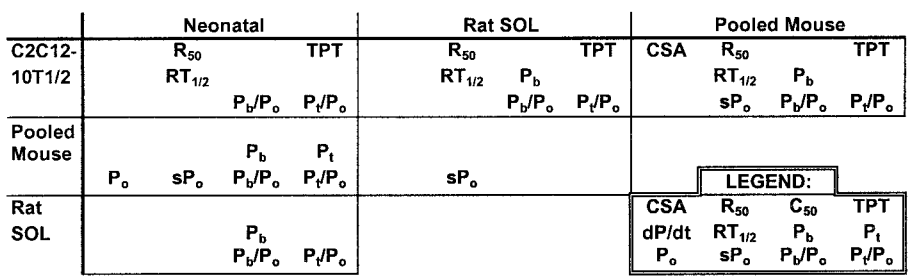

Fig. 2. Paired comparisons of myooid function, with mouse data pooled. For the original 5 experimental groups, no differences were detected between means for any of the 12 experimental parameters when mouse SOL and mouse EDL groups were compared. Mouse SOL and EDL data were pooled, reducing the total to 4 experimental groups or treatments. Data were reanalyzed using the Tukey-Kramer method. As in Fig. 1, each parameter in each box is displayed only if the difference between means is significant at $\alpha \leq 0.05$.

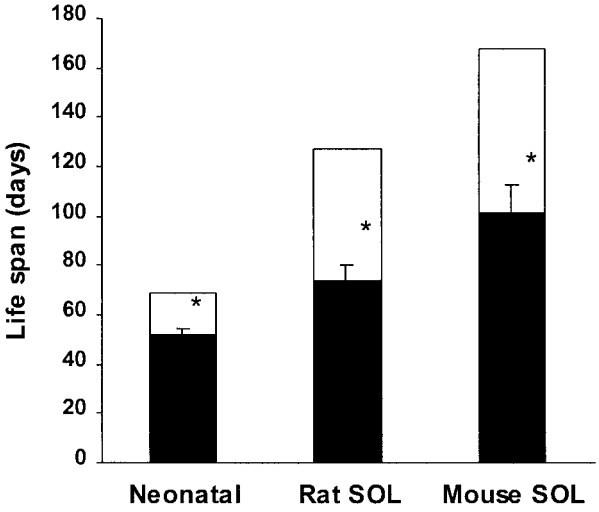

Fig. 3. Life span of myooids in culture. Solid bars, mean life span for each group; error bars, SE. Open bars, maximum observed life span for a myooid within each group. Myooids from neonatal rat muscle, plated at 1×10^6 cells/cm^2 on 1.0 μg/cm^2 laminin with suture anchors, were viable for an average of 52 ± 2.5 days (n = 25), with a maximum observed life span of 69 days. Myooids from adult rat soleus, plated at 13 mg of dissociated tissue per plate on 1.0 μg/cm^2 laminin with suture anchors, continued to remain viable for an average of 74 ± 6 days (n = 11), with a maximum observed life span of 127 days. Myooids from mouse SOL muscle, plated at 10 mg of dissociated tissue per plate on 1.0 μg/cm^2 laminin with suture anchors, remained viable in culture for an average of 101 ± 12 days (n = 8), with a maximum observed life span of 168 days. A one-way ANOVA followed by Bonferroni comparisons when significance was detected ($P \leq 0.05$) indicated that each of the life spans for each of the types of myooids was significantly different from the other two.

to the presence of embryonic and neonatal isoforms of myosin heavy chain (MHC) present in neonatal (11) and developing muscles (18). For each of the five groups of myooids, sP_o of 4–19 kN/m^2 are 5–43% of the values for muscles of newborn rats and 2–8% of the values for muscles of adult rats. Of the myooids, the C_2C_{12}-10T½, neonatal rat and adult rat myooids had the lowest values for sP_o, ~4 kN/m^2, whereas the adult mouse myooids had the highest values, 16 kN/m^2. On the basis of the sP_o, myooids in each of five groups appear to have poorly developed contractile apparatus, in addition to the possibility of the expression of predominantly developmental isoforms of MHC (18).

Although direct measurements of the MHC isoform composition of myooids have not been made, the time-dependent characteristics of the twitch, TPT, $RT_{1/2}$, and dP/dt, as well as P_t/P_o, are consistent with the hypothesis that the predominant myosin isoforms in myooids are developmental, not adult, isoforms. For myooids from each of the primary cultures of muscles from mice or rats, TPT was 50–65 ms, values within the range reported for SOL and EDL muscles of newborn rats (2, 6). Even for the newborn rats, $RT_{1/2}$ varies more widely than the TPT, with a value of 70 ms for the SOL muscles and 50 ms for the EDL muscles. Only the pooled mouse myooid experimental group had an $RT_{1/2}$ in this range, 62 ms, and in the remainder of the myooids from primary cultures $RT_{1/2}$ ranged from 81 to 100 ms. The TPT and $RT_{1/2}$ of the C_2C_{12}-10T½ myooids were considerably more prolonged than the values for other myooids. The time-dependent characteristics of the myooid twitches appear to be dependent on the same mechanisms that impose the differences in the force-velocity relationships of neonatal and adult muscles, namely, the rate of Ca^{2+} release and reuptake in muscle as a function of age from birth (2, 6). The similarity of the TPT and $RT_{1/2}$ of the mouse and rat myooids, regardless of age of the donor, to those of the newborn rats is in contrast to the widely divergent values for the C_2C_{12}-10T$_{1/2}$ myooids. The magnitude of the differences for the C_2C_{12}-10T½ myooids suggests major impairments in development of this cell line in culture.

The maximum rate of rise of force has been used to study the developmental changes in skeletal muscles of neonatal and young rats (2, 6). When normalized as a percentage of the rise in P_o, the maximum rate of rise of force correlates strongly with the maximum velocity of shortening (2). The maximum rate of rise of force in skeletal muscles is low at birth, largely because of the low actomyosin ATPase activity (6), but increases rapidly to achieve adult values after the first month. During this period, the maximum rate of rise of force increases fivefold (6) and the maximum velocity of unloaded shortening of the EDL muscles doubles (2). The dP/dt values of the myooids, expressed in absolute terms of micronewtons per millisecond, were at least four to five orders of magnitude slower than values of 45 and 120 mN/ms reported for SOL and EDL muscles of adult rats (6, 20) and were still two orders of magnitude slower when expressed as a percentage of the P_o. These data suggest that the capacity of the myooids to develop force rapidly and to shorten at high velocity

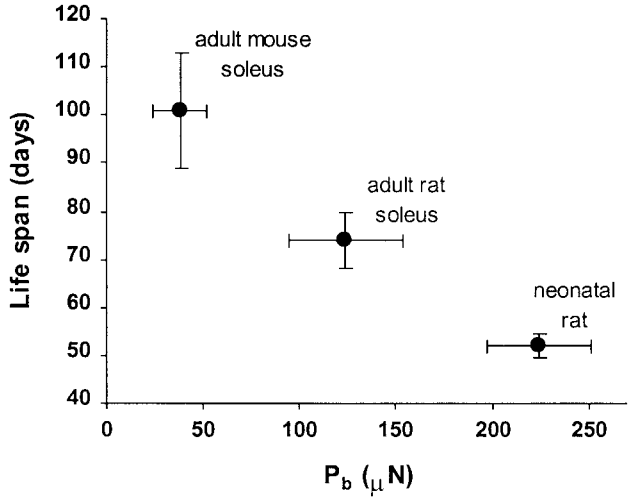

Fig. 4. Effect of P_b on life span of myooids in culture. Life span data are from the 3 life span experimental groups; P_b data are from the 3 corresponding contractile function experimental groups. P_b data could not be collected from the life span groups because the measurement of contractile properties was not carried out in an aseptic environment, so myooids were not replaced in culture after measurement of contractile properties. Thus P_b data represent means at ~32 days in culture. Error bars, SE.

is even more impaired than their ability to generate sP_o.

The P_t/P_o of the four groups of primary rodent myooids ranged from 0.43 to 0.60. These values for P_t/P_o compare favorably with 0.60 ± 0.05 (SD) and 0.53 ± 0.08 reported for SOL and EDL muscles of newborn rats, respectively (2). The ratio decreases rapidly and stabilizes within the 1st mo of life at the adult values of 0.25 for SOL muscles and 0.19 for EDL muscles. As with the other variables, the P_t/P_o of the C_2C_{12}-10T½ myooids was outside the range of the other groups. The similarity of the P_t/P_o data for rodent myooids to that of neonatal and newborn rodents, as well as the time-dependent properties of the twitch, support the conclusion that the myooids are arrested in the state wherein they express predominantly, if not exclusively, embryonic and neonatal isoforms of myosin (2, 11, 18).

The absence of any differences in the excitability or contractility for adult mouse myooids, whether engineered from SOL or EDL muscles, indicates that myooids can be developed from muscles expressing exclusively either slow and intermediate or fast isoforms of MHC (16). Unexpectedly, a fourfold greater sP_o was observed for pooled adult mouse myooids than for adult rat SOL myooids. We have no explanation for such a large difference in such a critical measure of function, particularly in the absence of any other difference between these two groups.

The lower excitability and contractility of C_2C_{12}-10T½ myooids than of the primary cultures of rodent myooids may be attributable to the lack of normal metabolism, phenotype, or growth regulation of C_2C_{12} myoblasts (8, 13, 24). Even when cultured with 10T½ fibroblasts, C_2C_{12} myoblasts formed myooids only one-half as reliably as the 95% success rate for the formation of myooids from primary cultures of skeletal muscles from neonatal and adult rodents. The differences between the C_2C_{12}-10T½ myooids and the primary culture myooids were spread over more than one-half of the 12 variables. Interestingly, one variable that did not differ among the groups was C_{50}, which ranged from 416 to 603 μs. In contrast, R_{50} for the C_2C_{12}-10T½ myooids was two- to fourfold greater than that of any other group. Despite the lack of an explanation for the reduced excitability of the C_2C_{12}-10T½ myooids, the functional data taken as a whole suggest important differences in the cellular organization and chemomechanical coupling of C_2C_{12}-derived myotubes compared with primary myotubes.

Fibroblasts assist in extracellular matrix formation during myogenesis in vitro (7, 12, 19, 21, 22, 28). C_2C_{12} myoblasts did not form myooids when monocultured, yet they formed myooids with some success when cocultured with fibroblasts, substantiating the critical role of fibroblasts in the formation of myooids. As reported earlier (5), fibroblasts generally oriented around the periphery and along the total length of the myooid. Apparently, in primary cultures of skeletal muscles of mice and rats, the simple dissociation of muscle tissue used for this study provided an adequate source of myoblasts and fibroblasts for the formation of the myooids. The P_b of myooids may be generated by the fibroblasts within the myooid, because fibroblasts generate tensile forces in culture in the presence of serum (9, 10). Since larger baseline forces also correlate with shorter myooid life span in culture, further studies should consider the influence of fibroblasts in myooid function and longevity.

The primary limitation with the myooid model as presented is the failure of the myotubes in the myooid to express a fully adult phenotype, in terms of sarcomere structure, excitability, and contractility. We hypothesize that the arrested developmental state of the myotubes involves the expression of chiefly embryonic and neonatal isoforms of myosin, resulting from the culture conditions we imposed, in which there was no application of mechanical strain, either active or passive, no electrical stimulation, and no innervation. As such, the data presented form the basis for future experiments in which experimental interventions can be applied to study the effect of controlled mechanical strain, electrical stimulation, nerve-muscle coculture, and addition of growth factors and trophic agents on muscle development, contractility, and excitability in culture. Thus myooids could prove to be useful in the study of skeletal muscle organogenesis, synaptogenesis, and chemomechanical transduction. The presence of the suture anchors allows the attachment of force transducers and servo motors, so that controlled mechanical strain may be applied while forces are monitored. The three-dimensional, cylindrical structure permits electrical fields to be generated across the tissue more easily and accurately than is possible with a cell monolayer, facilitating chronic exposure of the tissue to electrical fields to influence development as well as activation of the tissue for functional evaluation.

Myooids may also be used for studying the physiology of muscle engineered from cells from animals with neonatal-lethal mutations affecting skeletal muscle, stem cell incorporation into muscle tissue, and plasticity and function of skeletal muscle engineered from the satellite cells of aged mammals. If the myooid model is adapted for use with human tissue, it could be used to test the effectiveness of specific gene therapy approaches to correct such disorders as malignant hyperthermia or muscular dystrophy. Tissue samples or tissue from human fetal tissue banks could be employed, thus eliminating the risks inherent to the introduction of genetic material into living humans until the effectiveness of the therapy in human muscle tissue can be established.

In summary, functional three-dimensional skeletal muscle constructs may be engineered from primary cultures of cells from fast and slow muscles of neonatal and adult rodents. The muscle constructs develop without the use of synthetic scaffolds to support the contractile section of the muscle and can be maintained in culture for several months. The excitability and contractility of myooids is similar to that which would be expected from embryonic or neonatal muscle tissue under conditions of chronic denervation. On the basis

of the consistency, diversity, and magnitude of the differences between the C_2C_{12}-10T½ myooids and primary adult rodent myooids, the C_2C_{12} cells appear to be less suitable than adult rodent primary cultures for engineering functional muscle constructs.

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