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CONTINUOUS MYOFIBER REMODELING IN UNINJURED EXTRAOCULAR MYOFIBERS: MYONUCLEAR TURNOVER AND EVIDENCE FOR APOPTOSIS

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Abstract

Unlike normal mature limb skeletal muscles, in which satellite cells are quiescent unless the muscle is injured, satellite cells in mammalian adult extraocular muscles (EOM) are chronically activated. This is evidenced by hepatocyte growth factor, the myogenic regulatory factor, Pax-7, and the cell-cycle marker, Ki-67, localized to the satellite cell position using serial sections and the positional markers laminin and dystrophin. Bromodeoxyuridine (brdU) labeling combined with dystrophin immunostaining showed brdU-positive myonuclei, presumably the result of fusion of activated satellite cells into existing myofibers. One new myonucleus was added to every 1000 myofibers in cross-section using a 12-hour brdU-labeling paradigm. The EOM thus appear to retain a stable nuclear population by an opposing process of apoptosis that results in myonuclear removal as visualized by terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL). Activated caspase-3 was present in localized cytoplasmic domains extending from 10 to 210 μ m within individual myofibers, suggesting segmental cytoplasmic reorganization. Understanding the cellular mechanisms that maintain this process of continuous myonuclear addition and removal in normal adult EOM may suggest new hypotheses to explain the preferential involvement or sparing of these muscles in skeletal muscle disease.

Keywords

apoptosis; extraocular muscles; mitosis; myofibers; satellite cells

Abbreviations

ANOVA, analysis of variance; brdU, bromodeoxyuridine; EOM, extraocular muscles; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling

The extraocular muscles (EOM), along with other craniofacial muscles, have a number of features that distinguish them from nonocular skeletal muscles. They continue to express a

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number of molecules normally downregulated in adult skeletal muscle, including continued expression of immature isoforms of the myosin heavy chain,⁵² neural cell adhesion molecule,²⁵ and the immature form of the acetylcholine receptor.¹⁷ The EOM continue to express a number of growth factors and their receptors,^{10,23} also normally downregulated in adult skeletal muscle.³³ The most novel of these features is the presence of activated satellite cells within adult, uninjured EOM of rabbits, mice, monkeys, and humans.^{25–27} The early embryological development of the EOM manifests distinct regulatory cascades compared to those active in somitic muscle.⁴⁸ This is supported by the demonstration that head mesoderm transplanted to the trunk somite region does not form muscle.³⁰ These observations support the interpretation that the extraocular muscles represent a unique allotype among skeletal muscles.^{21,35}

Myonuclei within limb skeletal muscle fibers are postmitotic in adult mammals; the myonuclei do not divide after formation of their associated myofibers. However, contained within skeletal muscles are satellite cells, which have regenerative potential.²² After injury to the muscle, satellite cells become activated and divide, leading to the formation of new myofibers or repair of existing ones. Thus, satellite cells are known to fuse with existing myofibers that are remodeling in response to low-frequency stimulation,³⁶ exercise,⁴⁷ and overloading.^{1,42} In EOM it is possible that continuous satellite cell division^{26,27} and resultant myofiber remodeling accounts for the differences from normal limb skeletal muscle. We have previously shown that normal adult EOM maintains a population of activated satellite cells.^{27,28} This process of satellite cell division and integration was visualized in the EOM of rabbits and mice that had received daily injections of bromodeoxyuridine (brdU), a thymidine analogue that is a marker of cell division, for up to 3 weeks. After various post-labeling survival periods, brdU-labeled myonuclei were identified within existing myofibers, demonstrating continuous myonuclear addition to existing myofibers. The proliferative activity of satellite cells within adult EOM appears to continue throughout life, as activated satellite cells have been noted in EOM specimens from human donors aged 82 years.²⁸

Our previous approach²⁶ did not allow the timetable of myonuclear addition in a 12- or 24-hour period to be determined. We have now examined short-term myonuclear addition by administering brdU every 2 hours over a 12-hour period. After 24 and 72 hours, the muscles were examined morphometrically for brdU-positive myonuclei to determine the time of their first appearance in EOM myofibers. The percentage of myofibers associated with satellite cells positive for markers of activation, hepatocyte growth factor,⁵⁰ and the myogenic regulatory factor Pax-7,⁴⁵ was determined. In addition, cells localized immunohistochemically in the satellite cell position that were positive for the cell-cycle marker Ki-67 were also quantified.⁴⁶

Since individual myofibers in adult EOM maintain a stable size over time, both at the cellular and gross anatomical level, we hypothesized that myonuclear addition must be accompanied by myonuclear removal. We examined normal EOM of adult rabbits for evidence of apoptotic processes, specifically looking for terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL)-positive myonuclei and myofibers whose cytoplasm was positive for activated caspase-3.

MATERIALS AND METHODS

Adult New Zealand white rabbits, weighing over 4 kg, were obtained from Birchwood Valley Farms and housed in the animal care facility of the University of Minnesota. All animal studies were approved by the University of Minnesota Animal Care Committee and met the guidelines of the National Institutes of Health for care and use of animals in research.

Bromodeoxyuridine labeling was used to follow DNA synthesis and mitosis in adult EOM. The fate of brdU-labeled cells was determined by demonstrating integration of nuclei from brdU-labeled satellite cells into myofibers by using double-immunolabeling for brdU with either dystrophin, to identify the sarcolemma, or laminin, to identify the muscle basal lamina. Specifically, brdU in sterile isotonic saline was injected intraperitoneally at a dose of 50 mg/kg body weight. Eight rabbits received injections of brdU every 2 hours for a period of 12 hours. Five of these eight rabbits were killed after 24 hours and three were killed after 72 hours with an overdose of barbiturate anesthesia. The superior, lateral, medial, and inferior rectus muscles, tibialis anterior muscles, and pectoralis major muscles were removed, frozen in methylbutane chilled to a slurry in liquid nitrogen, and serially sectioned at 12 μ m. The even-numbered sections were processed for expression of brdU (1:100) (Boehringer Mannheim, Indianapolis, IN) and dystrophin (1:20) (Novocastra, Vector Laboratories, Burlingame, CA) in order to ascertain whether the labeled nuclei were within the muscle sarcolemma and therefore were myonuclei. The odd-numbered sections were immunostained for laminin (1:20) (Sigma, St. Louis, MO) to ascertain whether the brdU-labeled nuclei were within the muscle basal lamina and were satellite cells. The extraocular, tibialis anterior, and pectoralis major muscles were obtained from an additional four normal adult rabbits killed by an overdose of barbiturate anesthesia. The muscles were frozen, sectioned at 12 μ m, and immunostained for one of the following: Ki-67, Pax-7, hepatocyte growth factor (HGF), or cMet (the receptor for HGF). The primary antibody concentrations were as follows: Ki-67 (acetone fixed, 1:50; Dako Corp., Carpinteria, CA); Pax-7 (acetone fixed, 1:50; Hybridoma Bank, Iowa City, IA); HGF (no fixation, 1:100; R&D Systems, Minneapolis, MN); and cMet (paraformaldehyde fixed, 1:50; R&D Systems). Ki-67-positive nuclei were identified as in the satellite cell position by staining every other serial section for either laminin or dystrophin.

For double-labeling experiments, serial sections were prepared, quenched with hydrogen peroxide, and incubated with blocking serum, followed by biotin-avidin-blocking reagent (Vector), and every other section was incubated in primary antibody to either dystrophin or laminin. The sections were rinsed in phosphate-buffered saline (PBS), followed by incubation using the Vectastain peroxidase ABC kit (Vector). The peroxidase was developed using DAB without the heavy metal intensification. The sections were rinsed in PBS. For brdU localization, the sections were incubated in 2N HCl for 1 hour at 37°C, followed by neutralization in borate buffer and a PBS rinse. The sections were incubated in the primary antibody to brdU diluted 1:100 (Boehringer Mannheim). The sections were rinsed in PBS and incubated in reagents from the alkaline phosphatase ABC kit (Vector Laboratories) and reacted with the alkaline phosphatase black substrate kit. All brdU myonuclear counts were performed on brdU-dystrophin-labeled sections. The dystrophin or laminin immunostained brown and the brdU-positive nuclei were black. Both brdU labeling and myofiber number were quantified using the Bioquant morphometry system (R&M Biometrics, Nashville, TN). Counts are expressed as mean $\pm$ SEM.

Single myofibers from the extraocular, tibialis anterior, and pectoralis muscles were reconstructed from serial cross-sections. The locations of brdU-positive myonuclei, brdU-positive satellite cells, and brdU-positive cells in the interstitium were recorded along the length of each reconstructed myofiber using Bioquant Nova software (Bioquant, Nashville, TN). Three-dimensional reconstructions were built using the Bioquant Topographer program.

The overall percentage of cells in the satellite cell position that expressed Ki-67, as identified by location between the dystrophin-positive sarcolemma and the laminin-positive basal lamina in serial sections, was determined as a percentage of all myofibers in cross-section. The percentage of myofibers associated with satellite cells positive for Pax-7, cMet, or HGF was also determined. Cross-sections through the EOM and control muscles from four rabbits were analyzed to determine the labeling index for each of the four antigens. This was calculated as

percentages of the total number of labeled nuclei in the satellite cell position divided by the total number of myofibers counted. Counts were made in a minimum of four random fields within both the orbital and global layers of four extraocular muscles and two control muscles from each rabbit. Between 500 and 1500 myofibers were included in the total number of myofibers in the cross-section, and four sections were counted for each muscle. Sections were chosen at random distances from the insertional tendon to the midbelly region of the muscles analyzed. The percent positive was compared between the orbital and global layers and analyzed for statistical significance using either an unpaired two-tailed *t*-test or an analysis of variance (ANOVA) and Dunn's multiple comparison tests with the aid of Prism and Statmate software (Graphpad, San Diego, CA). An *F*-test was used to verify that the variances were not significantly different. Data were considered significantly different at $P \leq 0.05$.

Apoptosis was detected using two methods: TUNEL, using the TdT DAB Apoptosis Detection Kit, following the instructions of the manufacturer (R&D Systems); and activated caspase-3 immunohistochemical labeling. The rabbits were deeply anesthetized with an intramuscular injection of a ketamine:xylazine mixture (1:1; 10 and 2 mg/kg, respectively) and perfused through the heart with 4% paraformaldehyde in PBS. The lateral, superior, and inferior rectus, tibialis anterior, and pectoralis major muscles were removed and postfixed in fixative overnight. A piece of small intestine was also removed and processed identically to serve as a positive control. The muscles were dehydrated and embedded in paraffin following standard procedures, and serially sectioned at 8 μm . Cross-sections were deparaffinized by heating to 57°C for 30 minutes followed by two rinses in xylene. The sections were incubated for 5 minutes each in 100%, 95%, and 70% ethanol; rinsed in PBS; treated with proteinase K for 30 minutes; and again rinsed in PBS. Endogenous peroxidase activity was quenched by incubation in 0.3% H_2O_2 for 10 minutes, and nonspecific staining was blocked by incubation with avidin-biotin blocking reagents (Vector). The sections were washed with DNAase-free water, incubated in TdT-labeling buffer for 5 minutes, incubated in TdT-labeling mixture for 1 hour, followed by incubation in TdT stop buffer for 5 minutes. For visualization of activated caspase-3, the cross-sections were quenched in H_2O_2 for 10 minutes, washed in PBS, and incubated with 0.5 $\mu\text{g}/\text{ml}$ anti-activated caspase-3 antibody (R&D Systems) overnight at 4°C. The following day, the sections were rinsed in PBS, incubated with anti-rabbit secondary antibody for 30 minutes, rinsed in PBS, incubated with streptavidin-horseradish peroxidase (HRP) for 30 minutes, rinsed in PBS, incubated with AEC chromogen for 10 minutes, again rinsed in PBS, and mounted in aqueous mounting medium (R&D Systems).

The numbers of myonuclei and satellite cell nuclei that were positive for TUNEL staining and activated caspase-3-positive cells were quantified for both the global and orbital layers of the extraocular muscles and in the control leg muscle. Due to our prediction of a low frequency of TUNEL-positive cells, the entire muscle cross-section was analyzed at high power; thus, no random sampling was performed. Once identified in a cross-section, serial sections were analyzed to determine the length of individual muscle fibers positive for activated caspase-3 using Bioquant Nova Prime and Topographer software (R&M Biometrics). All data are presented as mean $\pm$ SEM.

RESULTS

The timetable of new myonuclear addition was determined using a short-term labeling paradigm. In extraocular muscles exposed to brdU every 2 hours for 12 hours followed by a 24-hour brdU-free period, brdU-labeled myonuclei were already present within single myofibers (Figs. 1, 2, and 3). Compared with the global layer, the orbital layer had a greater percentage of myofibers with a brdU-positive nucleus for this short time period of brdU exposure, with $0.18 \pm 0.06\%$ ($P = 0.016$) of the myofiber profiles counted having a brdU-positive myonucleus. These nuclei were randomly located and were always found in a

peripheral location directly under the sarcolemma (Figs. 1 and 2). Very few brdU-positive myonuclei were found in global-layer myofibers by 24 hours after this brdU-labeling period, with only $0.014 \pm 0.014\%$ of the global myofibers in cross-section containing a brdU-positive myonucleus (Fig. 3A). Thus, for the muscle cross-section as a whole, this represents 1 new myonucleus for every 1000 myofibers (Fig. 3A).

In contrast to the brdU-labeled myonuclei, the number of brdU-positive satellite cells per number of myofibers in cross-section was similar when the orbital and global layers were compared; approximately $0.54 \pm 0.08\%$ of the myofiber profiles in cross-section were associated with a brdU-positive satellite cell 24 hours after a 12-hour brdU-labeling regimen (Fig. 3B). There were essentially no labeled myonuclei in any of the myofiber cross-sections examined in the tibialis anterior or pectoralis muscles (not shown). There was an occasional brdU-positive satellite cell in these muscles, as well as an occasional brdU-labeled cell in the connective tissue. The limb muscles were not studied in their entirety, and brdU-labeled cells may have been missed, particularly at the tendon ends of the muscles.

In three-dimensional reconstructions, every myofiber with an identified brdU-positive myonucleus had a brdU-positive satellite cell associated with it, either in the same section or within $72 \mu\text{m}$ of the labeled myonucleus (Fig. 2). In the myofibers reconstructed thus far from the muscles examined 24 hours after the 12-hour labeling procedure, only one brdU-positive nucleus was found per individual myofiber. Most reconstructed myofibers had only a single brdU-positive satellite cell associated with them, but two reconstructed myofibers had two brdU-positive satellite cells.

After 72 hours, the number of brdU-labeled myonuclei per total number of myofibers in cross-section was $0.186 \pm 0.05\%$, similar to that at 24 hours in the orbital layer, but had increased to $0.365 \pm 0.09\%$ in the global layer, a significant increase compared to the global-layer labeling at 24 hours ($P = 0.048$; Fig. 3A). Again, these brdU-labeled myonuclei were randomly located along the myofiber length and peripherally located directly under the sarcolemma within the myofiber cross-section. There were essentially no brdU-positive myonuclei and only an occasional brdU-positive satellite cell in the cross-sections of tibialis anterior and pectoralis muscles. Approximately $0.34 \pm 0.07\%$ and $0.56 \pm 0.14\%$ in the orbital and global layers, respectively, of the myofiber profiles in cross-section had an associated brdU-positive satellite cell after 72 hours (Fig. 3A).

Approximately $1 \pm 0.3\%$ of the myofiber cross-sections in the extraocular rectus muscles had cells associated with them in the satellite cell position that were positive for the cell-cycle marker Ki-67, as determined by positional labeling with dystrophin and laminin. Morphometric analysis showed that an average of $4.2 \pm 2.4\%$ of the rectus myofibers in cross-section had a Pax-7-positive satellite cell in association with them. Approximately $16.7 \pm 4.3\%$ of the myofibers in any given cross-section had cMet-positive satellite cells associated with them; $5.1 \pm 1.3\%$ of the myofibers had satellite cells associated with them that were positive for HGF, and thus were in the activated state.

Approximately 3 or 4 myofibers positive for activated caspase-3 were present in a total muscle cross-section of 7000 myofibers (Figs. 4 and 5A). A low level of immunoreactivity was evident in many EOM myofibers, but was not seen in control muscles stained under the same conditions; the cause of this is unknown. Analysis of serial sections demonstrated a distinct segmental localization of the immunostaining, extending for $10\text{--}210 \mu\text{m}$ (Fig. 5B). Individual myofiber length varied from between 2 and 9 mm, although an occasional extremely small or extremely long myofiber was encountered. Apoptotic myonuclei as visualized by TUNEL staining were extremely rare. A TUNEL-positive myonucleus was identified within only $0.035 \pm 0.005\%$ of the orbital-layer myofibers and $0.007 \pm 0.005\%$ of the global-layer myofibers

(Figs. 5A and 6). TUNEL-positive nuclei were found in the satellite cell position within the rectus cross-sections or in the perimysium. Definitive identification of these TUNEL-positive nuclei located outside the myofiber proper is ongoing. TUNEL-positive nuclei were extremely rare in the leg and pectoralis muscle tissue; in thousands of cross-sections an occasional TUNEL-positive nucleus was found in the connective tissue space between myofibers. However, a TUNEL-positive myonucleus was never seen, nor were there activated caspase-3 myofibers in the control muscle sections.

DISCUSSION

These data support the presence of continuous myonuclear addition and removal in single myofibers within uninjured adult EOM in the rabbit. We may have underestimated the number of new myonuclei, because it is possible that satellite cells fuse into an existing myofiber without first dividing. The brdU labeling also may be underestimated as we observed increased myonuclear labeling 3 days after the last brdU injection, particularly in the global layer, suggesting that some satellite cells synthesize new DNA but do not immediately become integrated into an existing myofiber. Differences in myonuclear addition were observed between the orbital and global layers of the EOM at 24 hours. This is interesting in light of the fact that the orbital layer has a much higher percentage of myofibers positive for the immature myosin heavy chain isoforms and other molecules normally expressed only in developing or regenerating skeletal muscle.^{10,23–25,52} Although the mechanism that controls this difference is unknown, anatomic and physiologic evidence suggests these layers subserve different functions.^{32,49}

The presence of large numbers of activated satellite cells in normal EOM, as identified by the expression of Pax-7 and HGF, strongly supports the data from the brdU-labeling experiments. The percentage of myofiber cross-sections with either Pax-7-positive or HGF-positive satellite cells associated with them is similar to the percentage with MyoD-positive satellite cells previously reported.^{26,28} The frequency of activated satellite cells as identified by MyoD and HGF is also similar to that seen in nonhuman primates and humans.²⁸ These observations support the use of these markers for the identification of activated satellite cells in EOM. If cMet-positive immunostaining represents all satellite cells and HGF-positive immunostaining represents only those satellite cells that are in the activated state,⁵⁰ then between 31% and 40% of all the satellite cells in normal extraocular muscle are activated at any given time. In the present study, we identified more activated satellite cells than predicted by the number of brdU-labeled myonuclei. This may reflect differences in the duration of expression of the different markers within activated satellite cells. It is also possible that satellite cells become activated and either return to the quiescent state without fusion into a myofiber or apoptose, a process known to occur during normal myogenesis.⁸ Apoptosis occurs concomitantly with normal differentiation of skeletal myoblasts. These two processes are distinct; one can be inhibited without affecting the other.⁸ In the present study, some of the TUNEL-labeled nuclei observed outside the sarcolemma were probably satellite cells.

The presence of TUNEL-labeled myonuclei and caspase-3 expression indicates a process of myonuclear removal and cytoplasmic remodeling in apparently normal myofibers within adult EOM, concomitant with the process of myonuclear addition. Expression of a caspase-3-mediated signaling cascade is critical for normal muscle differentiation during myogenesis.⁹ Inhibition of caspase-3 during development severely attenuated myoblast fusion and myotube formation.⁴⁰ Our observation of activated caspase-3 expression in defined segments is consistent with fusion events in these muscles. Loss of individual myonuclei within a mature multinucleated myofiber is not without precedence. Single myonuclei positive for apoptotic markers have been seen in myofibers from dystrophic *mdx* mice,⁵¹ mice with a γ -sarcoglycan deficiency,¹⁵ calpain-3-deficient mice,³⁸ and in hindlimb unweighting.² Our quantification

of apoptotic myofiber nuclei most likely represents an undercount, as there is accumulating evidence that apoptosis can occur without the classic DNA laddering or TUNEL staining of the nuclei⁴³ during muscle development⁴⁴ and after denervation.^{6,39} Apoptotic myonuclei and interstitial cells have been found in normal EOM samples used as controls in another study.¹⁹ The mechanism that initiates these apoptotic processes in normal adult EOM remains undefined.

The percentage of brdU-labeled myonuclei is higher (0.1% per myofiber number) than the frequency of apoptotic nuclei (0.05%) and myofibers positive for activated caspase-3 (0.075%). However, the brdU-labeling findings represent a cumulative uptake of brdU over a period of 12 hours. Nuclei are only positive for TUNEL staining for 1 to 3 hours.¹² Since there is no published report suggesting that the EOM change size during aging, these processes are presumably balanced over time. The labeling differential reflects differences in our ability to follow these various processes.

The segmental nature of myofiber remodeling in apparently uninjured EOM myofibers is similar to that in dystrophic myofibers, where defined regions of myofibers are remodeled and the rest of the myofiber remains unaltered.^{5,20} Repair in dystrophic myofibers results from fusion of labeled donor muscle precursor cells into apparently random locations along the length of individual myofibers,^{5,20} as occurs in the EOM. Segmental muscle fiber lesions and myonuclear addition are also seen after repetitive eccentric contraction injury¹¹ and in other types of diseased muscle.⁴¹

The factors that control the process of myonuclear addition and apoptosis in normal EOM are unknown. Although normal mature skeletal muscle downregulates the expression of myogenic growth factors,³³ insulin-like growth factor-1 (IGF-1) and IGF receptor continue to be expressed in mature EOM.^{10,23} In limb skeletal muscle, reintroduction of IGF by viral vectors or in transgenic mice results in significant muscle hypertrophy.^{3,34} One week after a single intramuscular injection of IGF into adult rabbit EOM, there is an increased number of hypertrophic myofibers and a concomitant increase in total muscle force generation compared to control EOM.²³ The role that IGF plays in this process requires definition. Other factors that might influence EOM myofiber remodeling include stretch as a result of eccentric contractions of the muscles during eye movements in the far range of peripheral gaze; the presence of higher numbers of satellite cells in EOM myofibers compared with other skeletal muscles; the presence of a higher proportion of the more slow-cycling, more multipotent type of satellite cell compared with other skeletal muscles⁴; some mechanism that attracts precursor cells from the circulation¹⁴; and/or their unique electrophysiological properties.¹³

The EOM show distinctly different patterns of involvement or sparing in neuromuscular disease than limb skeletal muscle. The EOM are clinically and pathologically spared in Duchenne muscular dystrophy,¹⁸ and the cranial motor neurons are differentially spared in amyotrophic lateral sclerosis³⁷ for unknown reasons. One hypothesis is that satellite cells from muscular dystrophy patients develop premature senescence.⁷ Satellite cells from patients with Duchenne dystrophy have decreased replicative potential,²⁹ including increased rates of telomere shortening.⁷ The EOM from aged humans retain a population of activated satellite cells.²⁸ Studies of EOM have shown little change with aging in muscle³¹ or motor neuron number,¹⁶ in contrast to the significant changes seen in limb skeletal muscles from elderly individuals. This suggests that there is some mechanism preventing replicative senescence in this dividing satellite cell population in EOM. Thus, it is reasonable to postulate a role for continuous myofiber remodeling in the clinical sparing of the EOM in Duchenne and Becker muscular dystrophies. If this process is indeed involved, it suggests new experimental approaches for developing treatments for these diseases.

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FIGURE 1. Photomicrograph of two serially prepared cross-sections through the orbital layer of a rabbit superior rectus muscle. **(A)** Immunostaining for brdU (black) and laminin (brown). Arrow indicates a brdU-positive satellite cell. **(B)** Immunostaining for brdU (black) and dystrophin (brown). Arrow indicates a brdU-positive satellite cell. Bar = 50 μ m.

FIGURE 2.

Reconstruction of a single myofiber from a serially sectioned superior rectus muscle 24 hours after a series of brdU injections every 2 hours for 12 hours. Every odd-numbered section was immunostained for the presence of brdU and dystrophin, and every even-numbered section was immunostained for the presence of brdU and laminin. Individual myofiber cross-sectional areas are indicated in red, blue indicates a brdU-labeled myonucleus, aqua indicates a brdU-labeled satellite cell, and yellow is a brdU cell in the connective tissue. Vertical arrow points to the unidentified brdU-positive nucleus. Horizontal arrow points to the brdU-labeled myonucleus and satellite cell.

FIGURE 3.

(A) Quantification of brdU-positive myonuclei/myofiber cross-section in rectus muscles at 24 hours and 72 hours after a series of brdU injections every 2 hours for 12 hours in cross-sections immunostained for the presence of brdU and dystrophin calculated for the orbital and global layers, and for the total muscle in cross-section at 24 and 72 hours. Tibialis anterior and pectoralis major muscle controls were essentially negative for brdU-positive myonuclei (not shown). *Significant difference vs. orbital layer. **Significant difference vs. 72 hours. (B) Quantification of brdU-positive satellite cells/myofiber cross-section in rectus muscles 24 and 72 hours after a series of brdU injections every 2 hours for 12 hours in serial cross-sections immunostained for the presence of brdU and either dystrophin or laminin. *Significant difference from global layer at 72 hours. Tibialis anterior and pectoralis major muscle controls were essentially negative for brdU-positive satellite cells, although occasional brdU-positive cells were seen in the connective tissue around the muscle fibers.

FIGURE 4. Photomicrograph of the inferior rectus muscle (**a**) and the tibialis anterior muscle (**b**) immunostained for the presence of activated caspase-3 (arrow).

FIGURE 5.

(**A**) The percent of myofibers positive for the immunohistochemical localization of activated caspase-3 or TUNEL-positive nuclei out of the total number of myofibers in cross-sections of lateral, medial, and superior rectus muscles. *Significant difference vs. global layer. (**B**) The length within individual myofibers that was positive for activated caspase-3 was determined in serially sectioned lateral, medial, and superior rectus muscles. Myofibers from these muscles ranged from 2 to 9 mm in length. *Significant difference vs. global layer.

FIGURE 6. Photomicrograph depicting a myofiber with a TUNEL-positive myonucleus (arrow). Bar = 50 μm .