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Activated Satellite Cells in Extraocular Muscles of Normal Adult Monkeys and Humans

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Abstract

PURPOSE—Mammalian extraocular muscles (EOMs) are both physiologically and biochemically unique when compared with nonocular skeletal muscles. Recent studies have demonstrated a process of continuous myonuclear addition in normal uninjured myofibers in adult EOMs of rabbits and mice. The current study was conducted to determine whether this process of myonuclear addition is a universal phenomenon in mammalian EOMs.

METHODS—The EOMs from adult uninjured monkeys and humans were examined immunohistochemically for the expression of specific markers of activated satellite cells: hepatocyte growth factor (HGF); the myogenic regulatory factors MyoD, myogenin, and Pax7; and a marker for nuclei in all proliferative phases of the cell cycle, Ki-67. The satellite cell identity of the cells positive for Ki-67, HGF, and Pax7 was determined by colabeling sets of serial sections with either laminin or dystrophin.

RESULTS—In cross sections of monkey and human EOMs, approximately 7% to 8% of the myofiber profiles were associated with Pax7-positive satellite cells and between 2% and 4% were associated with MyoD-positive satellite cells or HGF-positive satellite cells. Similar percentages of satellite cells were positive for myogenin in the orbital layer, but the global layer had few satellite cells that were myogenin positive. An average of 0.72% of the myofibers had Ki-67–positive cells associated with them in the satellite cell position.

CONCLUSIONS—Activated satellite cells were present on myofibers in normal uninjured adult monkey and human EOMs, as visualized with these five distinct markers. The data support the hypothesis that the process of continuous myonuclear addition is most likely active in primate and human EOMs. The presence of continuous myofiber remodeling in EOM suggests new mechanisms that may be responsible for EOM sparing or involvement in skeletal muscle diseases.

Extraocular muscles (EOMs) possess a number of unique properties when compared with limb skeletal muscles. In particular, myofibers in adult EOMs continue to express several molecules that normally are downregulated in mature skeletal muscles, molecules normally associated only with skeletal muscle development and regeneration. These include continued expression of immature myosin heavy-chain isoforms, ¹ neural cell adhesion molecule, ² myogenic growth factors (McLoon LK, Peters E, Wirtschafter JD, ARVO Abstract 2150, 1999), ³ and the immature form of the acetylcholine receptor. ⁴ The factors unique to these muscles that control the upregulation in expression of these molecules is unknown.

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Skeletal muscle myonuclei are known to be postmitotic in adults; however, residing within all skeletal muscles is a population of specialized cells known as satellite cells,⁵ which reside outside the sarcolemma but inside the basal lamina of individual myofibers and are normally quiescent.⁶ After a muscle injury, the satellite cells become activated and divide and are responsible for muscle repair and regeneration. The regenerating muscles reexpress a number of the myogenic growth factors and immature myosin heavy-chain isoforms that remain upregulated in uninjured adult EOMs.

We have recently demonstrated that there is continuous myonuclear addition to normal uninjured myofibers in the EOMs of adult rabbits⁷ and mice.⁸ Bromodeoxyuridine (BrdU) labeling experiments in which daily BrdU injections were administered for 2 and 4 weeks, followed by various BrdU-free periods, identified BrdU-positive myonuclei that were stably integrated into existing EOM myofibers. These BrdU-positive nuclei were not present in the limb skeletal muscles of the same animals. In short-term labeling studies, a BrdU-positive myonucleus was always found in proximity to a BrdU-positive satellite cell.⁹ Thus, a continuous process of renewal occurs in existing adult EOM myofibers in rabbits and mice.

EOMs are known to be preferentially susceptible to certain diseases, such as Graves' ophthalmopathy, ¹⁰ oculopharyngeal muscular dystrophy, progressive external ophthalmoplegias, and myasthenia gravis, ¹¹ and to be preferentially spared in other muscle diseases, such as Duchenne and Becker muscular dystrophies. ^{12–14} The reason for this differential susceptibility is unknown. The process of myonuclear addition in adult EOMs provides a basis for developing a number of hypotheses that may explain the differential involvement of the EOM in these various muscle diseases. However, it is important to determine whether this process of myonuclear addition is a universal phenomenon and not just a peculiarity of rabbits and small rodents. We specifically wanted to determine whether this process occurs in adult primate and human EOMs.

Until relatively recently, there have been no universally accepted methods to distinguish between quiescent and activated satellite cells in muscles. We have identified activated satellite cells using a panel of myogenic lineage-specific markers, including MyoD, myogenin, and Pax7. MyoD is expressed in both activated satellite cells and myoblasts, 15-17 but not in myotubes or mature myofibers. Quiescent satellite cells are MyoD negative.¹⁸ Myogenin is another myogenic regulatory factor that is expressed in activated satellite cells and myoblasts and thus is a good marker for activated satellite cells in our tissue.¹⁵ Pax7 has been identified as a myogenic regulatory factor that labels satellite cells and, in fact, is required for the formation of the satellite cell population in development.¹⁹ Recent work has demonstrated that although both activated and quiescent satellite cells are positive for the hepatocyte growth factor (HGF) receptor, c-Met, only activated satellite cells are positive for HGF.^{20,21} We compared the frequency of expression of these four markers in satellite cells in normal human and primate EOMs. In addition, we examined satellite cells for the expression of the cell proliferation marker Ki-67, which is positive in cells during the proliferative phases of the cell cycle.²² Thus, we were able to confirm, using multiple markers, the presence of activated satellite cells in adult EOMs of both monkeys and humans.

MATERIALS AND METHODS

All normal EOMs, leg muscle, and facial muscle tissues were obtained from monkeys after approval by the Institutional Animal Care and Use Committee, and all human material was obtained after approval from the Institution Review Board for Protection of Human Subjects at the University of Minnesota. EOM, tibialis anterior muscle, and pectoralis major muscle samples were obtained from adult cynomolgus monkeys, all of which were between 5 and 10 years of age. All animal experiments were performed according to the guidelines of the

National Institutes of Health for use of animals in research and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All human EOM tissue and eyelid materials were obtained with consent for use in research from donor eye bank material through the Lions Eye Bank at the University of Minnesota or as surgical waste from normal surgical procedures. The eye donors were 41, 59, 74, and 82 years of age, and none had an identified muscle disease at the time of death. Three of the donors died of cardiac arrest and the fourth of breast cancer. In each case, the specimens were obtained from the midbelly of the EOM, removing more muscle than is normally removed with the donor eye. All human tissue samples were obtained in accordance with the provisions of the Declaration of Helsinki for use of human tissue in research.

All EOM, leg, and eyelid samples were frozen and serially sectioned on a cryostat at 12 m. A number of markers were used to identify both quiescent and activated satellite cells in the monkey and human tissue. For all single-antigen immunohistochemistry protocols, the sections were fixed as required for each specific primary antibody, rinsed in phosphate-buffered saline (PBS), blocked for nonspecific binding with horse serum and avidin-biotin (ABC) blocking reagents (Vector Laboratories, Burlingame, CA), and incubated for 1 hour with the primary antibody. For Pax7 immunohistochemistry, sections were fixed in acetone and incubated with an antibody to Pax7 at a 1:50 dilution (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). For MyoD immunohistochemistry, sections were fixed in 4% paraformaldehyde for 10 minutes and incubated with an antibody to MyoD at a 1:50 dilution (NovoCastra, Newcastle, UK). For HGF, sections were fixed in acetone for 10 minutes and incubated with an antibody to HGF at a 1:20 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For Ki-67 immunohistochemistry, sections were fixed for 10 minutes in acetone and incubated with an antibody to Ki-67 at a 1:25 dilution (Dako Corp., Carpinteria, CA). For myogenin immunohistochemistry, the sections were fixed for 10 minutes in acetone and incubated with an antibody to myogenin at a 1:50 dilution (Dako Corp.).

Sets of serial sections were also prepared using Ki-67, HGF, or Pax7 antibodies. Serial sections were prepared at 12 μ m using a cryostat. The sections were immunostained for the presence of dystrophin or laminin and either Ki-67, Pax7, or HGF, using a procedure previously described.⁸ Briefly, the sections were incubated in an antibody against dystrophin (NovoCastra and Vector Laboratories) at a concentration of 1:20 and reacted with the peroxidase ABC kit (Vectastain; Vector Laboratories). The peroxidase was developed using diaminobenzidine. The sections were incubated in the primary antibodies, as described in the preceding paragraph. The sections were rinsed in PBS, incubated using reagents from the alkaline phosphatase ABC kit, and reacted with the alkaline phosphatase black substrate kit. The staining for dystrophin was brown, and the Ki-67, Pax7, or HGF-positive cells were black. A second set of serial sections was immunostained for laminin (1:100; Sigma Chemical Co., St. Louis, MO) and Ki-67, Pax7, or HGF.

The overall percentages of satellite cells, as identified by location with double staining where appropriate, that expressed Ki-67, HGF, Pax7, MyoD, or myogenin were determined. Cross sections through the EOM and control muscles were analyzed to determine the labeling index for each of the five antigens. This was calculated as percentages of the total number of nuclei in the satellite cell position and total number of myofibers counted. Counts were made in at least four random fields within both the orbital and global layers for each specimen. The number of cells positive for each of the antigens was calculated based on the total number of myofibers in a given microscopic field. At least four fields were counted in the muscle cross sections, and four tissue sections were quantified for each muscle analyzed. All data were analyzed for statistical significance, using either an unpaired, two-tailed *t*-test or using analysis of variance (ANOVA) and the Dunn multiple comparison tests (aided by the 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 if P < 0.05.

RESULTS

The EOMs from adult monkeys contained cells in the satellite cell location positive for Ki-67, Pax7, MyoD, myogenin, and HGF (Figs. 1, 2, 3). Only 0.48% of the orbital layer myofibers and 0.3% of the global layer myofibers in cross section had Ki-67 cells associated with them in the satellite cell location, as demonstrated by labeling of serial sections with Ki-67 and laminin or dystrophin. The percentage of myofibers with satellite cells positive for Pax7 was between 7% and 8% and for MyoD was between 3.4% and 4.3%, in the orbital and global layers, respectively (Fig. 3). Approximately 2.7% of the myofibers in the orbital layer were associated with myogenin-positive satellite cells, but these were far less numerous in the global layer where only 0.4% of the myofibers had myogenin-positive satellite cell associated with them. The percentage of myofibers with HGF-positive satellite cells associated with them was 3% and 2.1% in the orbital and global layers, respectively. The satellite cells positive for each of these markers were randomly located within the total muscle cross-sectional area. Some areas were devoid of satellite cells positive for these markers, whereas other regions had many positive satellite cells in proximity to one another. Limb muscle was essentially negative for all these markers of activated satellite cells. Of the more than 100 sections scanned for each control muscle, each containing thousands of myofibers in cross section, there were no more than two or three MyoD- or Pax7-positive nuclei present. We did not, however, make an attempt to survey specific regions of the control muscles, such as the neuromuscular junction or tendon ends.

The EOMs from adult human orbits also contained both Ki-67- and Pax7-positive cells in the satellite cell position. The position of these cells as satellite cells was verified by double staining serial sections with each marker, together with either laminin and dystrophin. Again, Ki-67positive cells in the satellite cell position were relatively rare compared with those positive for the myogenic regulatory factors and were found associated with 0.66% and 0.4% of the myofibers in the orbital and global layers, respectively (Fig. 4). Similar to the monkey EOMs, approximately 7% of the myofibers were associated with Pax7-positive satellite cells in human EOM. Approximately 2.5% of human EOM myofibers in cross section had a MyoD-positive satellite cell associated with them (Fig. 4). Myogenin-positive satellite cell percentages in human EOM were similar to that seen in monkey—that is, slightly more prevalent in the orbital layer (3.5%) and relatively rare in the global layer of human EOMs, with approximately 0.8% of the myofibers having myogenin-positive satellite cells in association with them. Satellite cells positive for HGF were associated with the human myofiber cross sections, with approximately 2.6% and 1.5% in the orbital and global layers, respectively. As was seen in monkey EOMs, the activated satellite cells were randomly located within the total muscle cross-sectional area. The non-ocular muscles examined were essentially devoid of satellite cells positive for any of these markers, with no more than one to three MyoD- or Pax7-positive cells in the thousands of myofibers in all the muscle cross sections examined.

The fate of the two daughter cells formed after satellite cell division in adult EOMs is unclear. It is possible for both of the daughter cells to form two new satellite cells that both become quiescent; to form two new satellite cells, with one forming a myoblast that fuses into a myofiber and the other becoming a quiescent satellite cell; or to form myoblasts that begin to differentiate. If the last alternative occurred, two satellite cells positive for expression of one or more of the myogenic regulatory factors would be expected. Satellite cells would be found in doublets, with both expressing MyoD, for example, as was seen in the adult rabbit EOM.⁷ The frequency of occurrence of either Pax7- or MyoD-positive satellite cells in doublets on individual myofibers within a single cross section was assessed. Satellite cells positive for these

markers were often in doublets around the myofibers (Figs. 5, 6). Doublets were observed on approximately 11% of the myofibers that had a Pax7- or MyoD–positive satellite cell.

DISCUSSION

Normal uninjured EOMs of both adult monkey and adult humans contain activated satellite cells as identified with these five distinct markers. The cells positive for Ki-67, Pax7, and HGF were demonstrated to be in the satellite cell position by their location between the dystrophin-positive sarcolemma and the laminin-positive basal lamina. The presence of activated satellite cells in the EOMs is in marked contrast to the nonocular control muscles examined, which were essentially devoid of activated satellite cells, as identified by these markers. Multiple markers specific to the myogenic lineage were used to identify activated satellite cells, including Pax7, MyoD, and myogenin. Activated satellite cells were also identified by the presence of HGF-positive immunostaining, a marker previously demonstrated to identify activated satellite cells specifically.^{20,21} Cells positive for Ki-67 were also found in the monkey and human EOMs examined. Although Ki-67 is not a specific marker of the myogenic lineage, positive expression of Ki-67 indicates that these cells located in the satellite cell position were actively in the cell cycle and therefore not quiescent.

Satellite cells are easily located by their position between the sarcolemma and basal lamina using immunohistochemical procedures. However, identification of those satellite cells as quiescent or activated is more problematic. Recent studies have identified a number of molecules that can be used to differentiate between quiescent and activated satellite cells in muscles, including Pax7, MyoD, myogenin, and HGF. Part of the confusion is that much of the work in identification of activated satellite cells has been in vitro, and the expression of these molecules changes markedly over time in culture.²³⁻²⁵ Thus, multiple markers were used to identify activated satellite cells in this study to be sure that these markers would be valid in EOM. MyoD is a well-established marker for activated satellite cells. MyoD is absent in quiescent satellite cells, 18 present in activated satellite cells during regeneration 17 and in myoblasts during development, 16 but absent in myotubes or mature myofibers. The percentage of MyoD-positive satellite cells in both monkey and human EOM resembled that which was seen in rabbit EOM.⁷ The random localization of the activated satellite cells throughout the total cross-sectional area of the EOM mimicked the apparently random position of myonuclear addition in the rabbit EOM myofibers. Although the actual signals that control these processes in EOM are unknown, the random position of these events suggests that they are locally controlled.

Satellite cells positive for myogenin were present in activated satellite cells in the monkey and human EOM as well. We saw a similar number of satellite cells positive for myogenin and MyoD in the orbital layers of both species; however, there were far fewer myogenin-positive satellite cells in the global region of the muscles. There are several possible explanations for this regional difference in the frequency of myogenin-positive satellite cells. Previous studies demonstrated the more transient nature of myogenin expression during muscle regeneration compared with the duration of MyoD expression in activated satellite cells and myoblasts during regeneration.^{18,26–28} The decreased number of myogenin-positive satellite cells in the global region may reflect fusion events. It may be that, when these cells become positive for myogenin, they become more rapidly incorporated into EOM myofibers than those in the orbital region. There is evidence to suggest that there are different populations of satellite cells in skeletal muscles.^{25,29,30} Thus, this difference in the frequency of finding satellite cells positive for myogenin may reflect population difference in the frequency of finding satellite cells

Pax7 labeled the greatest number of satellite cells in both the monkey and human EOM compared with the other markers. Pax7 is a myogenic regulatory factor that is required for the

formation of satellite cells in skeletal muscle.¹⁹ Although the early genes required for craniofacial muscle development are still unknown, Pax7 expression is specifically associated with the development of the lateral rectus muscle.³¹ The higher numbers of Pax7-positive satellite cells compared with those positive for MyoD or myogenin may reflect differences in the length of time Pax7 is expressed during the process of satellite cell activation and division, compared with the other myogenic regulatory factors examined. Pax7 has also been implicated in the formation and maintenance of neuromuscular contacts within muscle spindles throughout life,³² and the increased numbers of satellite cells positive for Pax7 may be partly related to its role in muscle spindle development and maintenance. The presence of large numbers of Pax7-positive satellite cells in these muscles is very intriguing in light of recent studies demonstrating the role of polymorphism in Pax7 gene expression in the control of regeneration in damaged limb skeletal muscle.³³

Ki-67 is a useful marker of the proliferative state, present in 34 and the S, G₂, and M phases of the cell cycle but absent in G₀, is often used to define the growth fraction of neoplastic cell populations. Once cellular differentiation begins, this molecule is downregulated. As predicted, fewer Ki-67–positive cells localized to the satellite cell position were present in EOM compared with those cells expressing specific myogenic regulatory factors. Because expression of this molecule is cell-cycle specific, the number of cells positive for Ki-67 gives some insight into the percentage of satellite cells in the total population that may be in the proliferative state at any given time in adult EOMs.

Recent work has identified HGF as an initiator of satellite cell activation and is considered one of the earliest indicators of the transformation of satellite cells to the activated state.^{20,21} The receptor for HGF, c-Met, is expressed on both quiescent and activated satellite cells.¹⁸ This has been confirmed and extended in recent work.³⁵ HGF expression gives additional support to the concept that there are activated satellite cells in both monkey and human EOMs. It is interesting that there are fewer HGF-positive cells in the satellite cell position than those expressing markers more specific to the myogenic lineage. The duration of expression of these different markers within cells is unknown, and these differences in number may reflect the length of time these molecules are retained in individual cells.

It is particularly interesting that two of the human EOM samples, which came from individuals who were 74 and 82 years of age and thus represent aged muscle, contained activated satellite cells. It appears that the processes that control the activation of satellite cells are still present in aging EOM. Renault et al.³⁶ examined the replicative potential of satellite cells from skeletal muscle samples taken from young and old quadriceps muscle, as well as the replicative potential of quadriceps muscle taken from a person with muscular dystrophy. Contrary to other cell types such as fibroblasts, the muscle satellite cells did not show a regular loss of proliferative potential that was age dependent, but instead showed an overall decrease in both total number of satellite cells and a decrease in the number of satellite cells that responded quickly to growth signals. Although it has long been known that aging skeletal muscles show a decline in mass and function, the intrinsic capacity for muscle regeneration is not impaired with age.³⁷ Localized transgene expression of insulin-like growth factor (IGF) in senescent muscle results in significant increases in both muscle mass and muscle strength.³⁸ It may be that the continued expression of IGF and its receptor in mature EOM³ (McLoon LK, Peters E, Wirtschafter JD, ARVO Abstract 2150, 1999) maintains a population of activated satellite cells in these muscles. We are currently investigating this possibility.

The fate of individual activated satellite cells cannot be directly determined. We know from our previous work that at least some of these cells fuse into existing mature myofibers.^{7,8} There are a number of possible fates for activated satellite cells in the EOM. It is possible that a dividing satellite cell produces (1) two new satellite cells that become quiescent, (2) one

satellite cell that becomes quiescent, and one myoblast that fuses into an existing myofiber, or (3) two myoblasts that fuse into existing myofibers. It is possible that at least some proportion of these satellite cells may express myogenic markers and then downregulate this expression. It is also possible that some percentage of these activated satellite cells may apoptose and never integrate into existing myofibers. Our laboratory and others have recently demonstrated that there a certain amount of apoptosis that occurs in normal, uninjured adult rabbit⁹ and human³⁹ EOMs, and this has been corroborated by the presence of apoptotic markers demonstrated by expression profil-ing.⁴⁰ However, the demonstrated occurrence of myonuclear addition into uninjured myofibers of EOMs in both mature rabbits⁷ and mice⁸ certainly supports the notion that at least some, if not most of these activated satellite cells would follow the same pathway in both human and monkey EOM and fuse into existing myofibers.

The presence of pairs of Pax7- and MyoD-positive satellite cells suggests several additional possibilities. By chance it is possible for two satellite cells that are in proximity to one another to become activated in the presence of the proper signal. This would produce doublets, or pairs, of satellite cells in proximity to each other that express markers specific for activated satellite cells. Although it is possible that a given satellite cell, after it has divided, produces two cells that both continue to differentiate along the myogenic lineage, it is also possible that one of the satellite cells will fuse into an existing myofiber, whereas the other eventually will become quiescent and downregulate its expression of Pax7 or MyoD. In a recent study, single EOM myofibers were reconstructed after a 12-hour BrdU-labeling procedure. Every myofiber with a BrdU-positive myonucleus had a BrdU-positive satellite cell associated with it, either in the same section or within 72 μ m of the labeled myonucleus.⁹ Pairs of MyoD-positive cells were also present in rabbit EOM.⁷ Future studies are anticipated that will clarify the fates of the activated satellite cells in the EOM.

The signals that control the maintenance of a population of activated satellite cells and continuous myonuclear addition in EOMs are unknown. It is noteworthy that this signal appears to be maintained over the lifetime of these muscles, as indicated by the presence of activated satellite cells in the EOMs from the 74- and 82-year-old donors. Experiments are ongoing to try to determine what controls this process. Work in our laboratory has shown that the EOMs continue to express several myogenic growth factors in the adult, including IGF and epidermal growth factor (EGF) (McLoon LK, Peters E, Wirtschafter JD, ARVO Abstract 2150, 1999), factors known to play a role in muscle formation during development and regeneration. This has been corroborated in recent gene-profiling studies.³

Although a great deal of work is necessary before the mechanism for this process is elucidated, it is clear that the presence of a population of activated satellite cells in adult EOM is a widespread phenomenon in mammals. The universality of these findings could have great potential for increasing our knowledge and ability to treat human muscle disorders that either involve or spare the EOM. For example, the EOMs are known to be spared in Duchenne muscular dystrophy, 12-14 and the process of myonuclear addition may play a critical role in the survival of this muscle in that genetic condition. Conversely, the progression of oculopharyngeal muscular dystrophy may be the result of two linked cascades of myonuclear loss and dysfunction, in which programmed myonuclear death within individual mature EOM myofibers may lead to a cumulative failure caused by the replacement myonuclei, which may be increasingly toxic or inadequate in number or function. Understanding the control of this process in the EOM may provide new insights into the development of new strategies for the treatment of diseases of both the EOMs and skeletal muscles in general.

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Figure 1.

EOM from a monkey immunostained for the presence of myogenin in the orbital (A) and the global (B) layers of the muscle. *Arrows*: myogenin-positive satellite cells. Bar, 50 μ m.



Figure 2.

EOM from a human (**A**) and a monkey (**B**) rectus muscle immunostained for the presence of MyoD-positive satellite cells (*arrows*). Bar, 100 μ m.

Satellite Cells from the Extraocular Muscles of Normal Adult Monkey





Percentage of satellite cells from monkey EOM positive for Ki-67, Pax7, MyoD, and myogenin. *Significant difference from orbital layer.



Figure 4.

Percentage of satellite cells from human EOM positive for Ki-67, Pax7, MyoD, and myogenin. *Significant difference from orbital layer.



Figure 5.

Pairs of Pax7-positive nuclei on individual myofibers in a cross section of monkey superior rectus muscle. *Horizontal arrow*: pair of labeled nuclei in proximity to one another. Two Pax7-positive nuclei some distance from one another were often seen on myofiber cross-sections (*vertical arrows*), but these were not counted as doublets. Bar, 50 µm.

Percent of Satellite Cells Found in Pairs on Human EOM Myofibers

Figure 6.

Percentage of satellite cells in doublets that were immunostained for either Pax7 or MyoD on individual myofibers.