A muscle precursor cell-dependent pathway contributes to muscle growth after atrophy

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Mitchell, Patrick O., and Grace K. Pavlath. A muscle precursor cell-dependent pathway contributes to muscle growth after atrophy. Am J Physiol Cell Physiol 281: C1706–C1715, 2001.—Slow-twitch skeletal muscle atrophies greatly in response to unloading conditions. The cellular mechanisms that contribute to the restoration of muscle mass after atrophy are largely unknown. Here, we show that atrophy of the mouse soleus is associated with a 36% decrease in myonuclear number after 2 wk of hindlimb suspension. Myonuclear number is restored to control values during the 2-wk recovery period in which muscle mass returns to normal, suggesting that muscle precursor cells proliferate and fuse with myofibers. Inhibition of muscle precursor cell proliferation by local γ-irradiation of the hindlimb completely prevents this increase in myonuclear number. Muscle growth occurs normally during the first week in irradiated muscles, but growth during the second week is inhibited, leading to a 50% attenuation in the restoration of muscle mass. Thus early muscle growth occurs independently of an increase in myonuclear number, whereas later growth requires proliferating muscle precursor cells leading to myonuclear accretion. These results suggest that increasing the proliferative capacity of muscle precursor cells may enhance restoration of muscle mass after atrophy.

hindlimb suspension; gamma-irradiation; myonuclear number; satellite cells; soleus

SKELETAL MUSCLE UNDERGOES rapid and profound atrophy in response to a variety of physiological and pathological stimuli. These include hindlimb suspension (HS), spaceflight, bed rest, denervation, and spinal cord transection. Muscles composed of predominantly type I myofibers are affected most during these conditions (for review, see Ref. 6). The slow-twitch soleus muscle is responsible for maintaining posture and counteracting the effects of gravity, and this muscle is preferentially affected in the hindlimb during most forms of atrophy. Ultimately, muscle atrophy is manifested as a decrease in functional capacity and weakness.

The molecular mechanisms responsible for muscle loss are not well understood; however, studies have shown that transcriptional and translational rates are decreased in atrophying muscle (for review, see Ref. 53). Decreased rates of synthesis and increased rates of degradation of myofibrillar proteins are the underlying cause of decrease in muscle mass, since 80% of skeletal muscle is composed of myofibrillar proteins. Several negative regulators of muscle growth have been implicated to play a role in muscle atrophy, including tumor necrosis factor-α, (35) interleukin-6 (19, 55, 56), and myostatin (12, 31, 59); however, their mechanisms of action are not well understood.

The number of nuclei in a myofiber also decreases during muscle atrophy in relation to changes in myofiber volume (for review, see Ref. 3). Such myonuclear loss appears to relate to the concept that each myonucleus has a nuclear domain. A nuclear domain is the volume of cytoplasm within the myofiber regulated by the gene products of a single myonucleus (22, 40). Thus, during atrophy, as the transcriptional and translational demands placed on myonuclei are attenuated, myofibers respond by eliminating myonuclei, possibly to maintain a constant myonuclear domain. A variety of atrophy-inducing stimuli such as denervation (58), spinal cord transection (15), spaceflight (4, 24), and HS (2, 14) induce loss of myonuclei. Atrophying myofibers have been shown to display various nuclear apoptotic markers, suggesting that an apoptotic mechanism contributes to the elimination of myonuclei during atrophy (2, 15, 36, 51).

Muscle growth in mammals during postnatal development or hypertrophy is dependent on addition of nuclei to the myofiber. Because nuclei of the muscle fiber are postmitotic, cells that exist outside of the myofiber must supply these additional nuclei. In some cases muscle growth is totally dependent on these muscle precursor cells (MPCs) (46, 47), whereas in other cases only 50% of the growth is dependent on MPCs (7). These studies used irradiation before the growth stimulus to block the ability of MPCs to proliferate. When juvenile muscles were similarly irradiated (45), increases in fiber size were not blocked totally, indicating that different mechanisms may be responsible for muscle growth, depending on the developmental stage of the myofiber. In avian muscle, increases in muscle size have been observed in the absence of satellite cell proliferation (32, 37).
Two potential sources exist for these new myonuclei in muscle tissue: satellite cells and mesenchymal stem cells. Satellite cells are quiescent MPCs that lie underneath the basal lamina that surrounds each myofiber (for review, see Ref. 49). In response to growth factors, satellite cells are activated to reenter the cell cycle, begin to proliferate, and subsequently fuse with the growing myofiber. Studies on the role of satellite cells in muscle regeneration have suggested that multiple populations of satellite cells exist based on their morphology and differentiation properties (23, 38, 42). Mesenchymal stem cells appear to represent a subset of satellite cells (8, 33) and can give rise to multiple cell lineages including myogenic cells (21, 26). How much these different cell populations contribute to muscle growth is unknown, due both to their small percentage of the MPC pool in some cases and to the lack of specificity of current techniques to modulate the activity of one particular population of MPC over another.

Restoration of muscle mass after cessation of an atrophic stimulus may differ from other forms of postnatal muscle growth for several reasons. First, atrophying myofibers have activated a molecular program, leading to protein degradation and nuclear loss. Presumably this program must be turned off or reduced before muscle growth can commence. Second, de novo formation of new myofibers can occur in some cases during muscle hypertrophy (for review, see Ref. 5), whereas recovery from atrophy is not associated with new myofiber formation, unless muscle degeneration occurs due to the weakened state of the atrophied muscle. Finally, the relative importance of MPCs for regulating muscle growth may differ in atrophied muscle. As MPCs can be negatively affected by conditions that lead to muscle atrophy such as muscle unloading (14, 48) or immobilization (28), possibly atrophied muscles have become less reliant on MPCs for growth as a result.

Understanding the cellular processes by which atrophying myofibers restore mass is important for developing effective pharmacological or genetic therapies to enhance rehabilitation in patients with muscle atrophy. The purpose of this study was to examine the requirement for MPCs in the restoration of muscle mass due to HS-induced atrophy. We show that recovery of the soleus muscle during the first week of recovery appears to be mediated independently of myonuclear addition to myofibers, whereas later muscle growth requires the fusion of MPCs with myofibers, leading to the restoration of myonuclear number.

MATERIALS AND METHODS

Animals

Female Balb/c AnNHsd mice (9–11 wk) were purchased from Harlan Sprague Dawley (Indianapolis, IN). All mice were housed under a 12:12-h light-dark cycle at room temperature, and food and water were provided ad libitum. All procedures were performed in accordance with Emory University’s Institutional Animal Care and Use Committee.

Mice were randomly assigned to one of three groups: 1) HS, 2) HS and recovery, or 3) control. In group 1, mice were hindlimb suspended for 2 wk using a tail suspension device as described previously (25). Hindlimbs were elevated sufficiently so as to prevent the hindlimbs from touching the cage floor or sides. In group 2, HS mice after 2 wk were briefly anesthetized with xylazine (10 mg/kg) and ketamine (65 mg/kg), and the tail harness was removed. Mice were allowed to resume normal cage activity during their period of least activity (light cycle) to minimize damage to hindlimb muscles during the initial recovery period. The length of the recovery period varied depending on the particular experiment. In groups 1 and 2, the mice were monitored twice daily throughout the period of HS. In group 3, mice maintained normal cage activity throughout each experimental procedure. The body weights of mice in group 3 increased by 2 and 3.5% over 14 and 28 days, respectively. In contrast, groups 1 and 2 lost 3.8% body weight over the 14-day HS period, but after the mice resumed normal cage activity for 14 days, body weights in group 2 were not significantly different from mice in group 3 at the same time point.

In some experiments, one hindlimb was γ-irrinated. Anesthetized mice were shielded with a custom made 9-in.-thick lead shield, so that only one of the hindlimbs was exposed to a cobalt-60 source. A dose of 2,500 rads was applied over 15 min, and the dose was verified using a picoamperometer LND ionization probe. This dose of irradiation has been previously shown to inhibit satellite cell proliferation (34, 46), and the effectiveness of the dose was verified in pilot experiments using regenerating muscle (data not shown). HS was started 2 days after the irradiation procedure.

Collection of Soleus and Plantaris Muscles

Mice were euthanized by CO2 inhalation, and both soleus muscles and plantaris muscles were collected using standardized dissection methods, cleaned of excess fat and connective tissue, blotted dry, and weighed on an analytical balance. One muscle was snap frozen in liquid nitrogen for biochemical assays, whereas the contralateral muscle was stretched to resting length (L0) and embedded in Tissue Freezing Medium (TBS, Durham, NC) and frozen in isopentane cooled in liquid nitrogen. These muscles were subsequently used to determine the cross-sectional area (CSA) of type I myofibers. Quantification of myonuclear number and determination of nonspecific myofiber CSA was performed on muscles that were frozen without stretching to L0. For histological assays, 8-µm serial sections were collected from the belly region of the muscle, air-dried, and subsequently used for the immunohistochemical assays described below.

Protein and DNA Quantification

Total protein content of the soleus was determined by homogenizing individual muscles in 350 µl of ice-cold 250 mM sucrose, 100 mM KCl, 20 mM MOPS, and 5 mM EDTA (pH 6.8). Aliquots (2 µl) were measured for protein content using the Bradford assay (9).

Both soleus muscles from individual animals were pooled, and a genomic DNA isolation kit (R&D Systems, Minneapolis, MN) was used to extract DNA from both muscles according to the manufacturer’s protocol. The DNA for each pair of muscles was resuspended in 20 µl of DNase-free water, and a 1-µl aliquot was added to a solution containing 0.05 M NaPO4, 2 M NaCl (pH 7.4), and 0.5 µg/ml Hoechst 33258 (Molecular Probes, Eugene, OR). DNA concentration was determined by measuring the emission at 460 nm after exci-
tation at 365 nm (30) using an Aminco-Bowman luminescence spectrophotometer (Spectronic Instruments, Rochester, NY). Calf thymus DNA was used to construct the standard curve.

**Immunohistochemical Analyses**

**Immunoreagents.** A mouse monoclonal antibody against dystrophin (MANDYS8) was purchased from Sigma Bio-sciences (St. Louis, MO). Mouse monoclonal antibodies against type I (BA-D5) and type II (My32) myosin heavy chain (MHC) were purchased from American Type Culture Collection (Rockville, MD) and Sigma, respectively. Secondary antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Texas Red-conjugated goat anti-mouse IgG was purchased from Cappel (Durham, NC). Vectastain Elite ABC reagent and Vectashield mounting media were purchased from Vector Labs (Burlingame, CA).

**Types I and II MHC immunohistochemistry.** Quadruplicate sections from each muscle were rehydrated in PBS, blocked with 5% horse serum, and incubated in each primary antibody (BA-D5, 1:30; My32, 1:1,000) at room temperature for 1 h. After the sections were washed in PBS + 0.05% Tween-20 (PBST), they were incubated in biotinylated goat anti-mouse IgG (1:250 in PBS + 2% goat serum). After further washes in PBST, sections were incubated in Vectastain Elite ABC reagent, and antibody binding was detected with diaminobenzidine. No staining was observed in control sections incubated with secondary antibody alone. All analyses and photography were performed on an Axiosplan microscope equipped with a video camera and Scion Image and Adobe Photoshop software. Analyses were performed by counting the number of myofibers expressing a particular MHC type in each section two separate times, tagging the stained myofibers to avoid duplicate counting. To calculate the percentage of a particular myofiber type, the average number of positively stained myofibers was divided by the total number of myofibers. The CSA of type I myofibers in the muscle during HS and its subsequent recovery after the 0.05 level of confidence was accepted for statistical significance.

**Statistics**

**Relative RT-PCR**

RNA was isolated from the soleus with TRIZOL reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. RT-PCR was performed in duplicate for each sample using primers specific for MyoD or myogenin. MyoD primers (13): sense, 5′-CCCGCCGCAGAATGGCTCA; anti-sense, 5′-GGTCCTGGTTCCCGTTATGGTG. Myogenin primers: sense, 5′-AGCGGCGTCCCAAAGTGGAGAT; antisense, 5′-GGACGTAAGGGAGTGATGTG. All RT reactions were performed using 2.5 μg of total RNA, and all PCR products were amplified in their linear range. To quantify amplicons, the PCR reactions were spiked with [α-32P]dCTP (3,000 Ci/mmol; ICN Biomedicals, Costa Mesa, CA), and 18S rRNA was used as an internal control in each sample using standard QuantumRNA 18S primers (Ambion, Austin, TX). The PCR products were resolved using 6% nondenaturing PAGE, and individual band intensities in the dried gel were determined using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The density of the amplicons for MyoD or myogenin was obtained using a volume integration protocol in the ImagineQuant program (version 3.3; Molecular Dynamics) and normalized to the density of the 18S rRNA amplicon.

**RESULTS**

**Atrophy and Recovery of the Mouse Soleus**

We first characterized atrophy of the mouse soleus muscle during HS and its subsequent recovery after resuming normal activity. This was done because very few studies to date have used the HS model of atrophy in the mouse.

To analyze muscle atrophy, a combination of biochemical and histological assays were used. Soleus wet weight decreases by 30% and 53% after 1 and 2 wk HS, respectively (Fig. 1A). Similarly, total protein content of the soleus decreases by 40% and 66% after 1 and 2 wk HS, respectively (Fig. 1B). In both assays, the greatest amount of atrophy occurs during the first week of HS. Several studies have demonstrated that type I fibers atrophy the most during HS-induced atrophy of the soleus. Our data show that the CSA of type I myofibers decreases by 51% after 2 wk HS (Fig. 1C). All three assays parallel each other in terms of the degree of atrophy observed. We used 2 wk HS for the remaining of the study because of the large amount of atrophy seen at this time point.

In our studies of the mouse soleus, a decrease in the percentage of type I myofibers does not occur after 2 wk HS (Fig. 1D). To rule out the possibility that HS causes an increase in the number of myofibers coexpressing both type I and type II MHC, we determined the percentage of type II myofibers in the atrophied soleus. This value, as determined by our methods does not change during HS (data not shown). Although most studies using HS have shown that the percentage of

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type I myofibers generally decreases in the soleus with a concomitant increase in the percentage of type II myofibers (for review, see Ref. 53), some studies have reported little or no change in the percentage of type I myofibers (43, 50). Age, species, and strain differences between animals may account for this variability, as well as the methods used to assay for slow vs. fast myofibers.

Next, we analyzed the time course of recovery of the soleus after 2 wk HS. Soleus wet weight, protein content, and CSA of type I myofibers return to control levels by the end of week 2 of recovery (Fig. 2, A–C) and parallel each other in terms of the degree of muscle growth observed. The percentage of type I myofibers does not vary significantly throughout the 2 wk of recovery (data not shown).

Previous studies have shown that some degree of muscle damage occurs when the hindlimb muscles of rats exposed to microgravity are resubjected to weight-bearing conditions (29, 44). The goal of the current study is to examine the cellular processes contributing to the growth of atrophied myofibers and not those involved in the de novo formation of new myofibers that occurs as a result of myofiber degeneration. We therefore analyzed recovering muscles for signs of muscle regeneration by examining hematoxylin and eosin stained sections for the presence of centrally nucleated myofibers. No signs of myofiber degeneration or regeneration are observed at any stage during the 2-wk recovery period (data not shown). This is probably due, at least in part, to allowing the mice to resume normal cage activity during their period of least activity.

Taken together, these data show that the mouse soleus atrophies by ~50% after 2 wk HS, with the majority of the atrophy occurring in the first week. Using the biochemical and histological assays in this study, we found that restoration of muscle mass is complete after 2 wk of resuming normal cage activity.

Changes in Soleus Myonuclear Number During HS and Recovery

We next examined whether the ~50% decrease in myofiber CSA is accompanied by elimination of myonuclei from atrophying myofibers, as shown in most studies with rat muscles. Because a significant decrease in myonuclear number should lead to decreased DNA content of the soleus after 2 wk HS, first the DNA content of the soleus was quantified. Indeed, as shown in Fig. 3A, a 31% decrease in DNA content of the soleus occurs after 2 wk HS. Subsequently, myonuclear number of atrophied and recovered muscle was quantified to prove that this loss of DNA corresponds to a parallel loss of myonuclei. Myonuclei were counted in serial sections of the soleus by counting the number of DAPI-stained nuclei inside the myofiber perimeter as defined by dystrophin staining (Fig. 3B). Our results demonstrate that 2 wk HS results in a 35% loss of myonuclei (Fig. 3C). The magnitude of this loss is in agreement with previously reported data on the loss of myonuclei in the soleus of spinal cord transected rats (16). Myonuclear number was determined for 2-wk-recovered muscles to ascertain whether myonuclei are added back to growing myofibers. As seen in Fig. 3C, myonuclear number after 2 wk of recovery increases significantly compared with 2 wk HS, and returns to control values. These data show that myonuclei are eliminated from myofibers during HS and are subsequently added back during the period in which muscle size is restored to control values.
MyoD and Myogenin mRNA Levels Are Increased During Recovery

The soleus atrophies by ~50% after 2 wk HS and doubles in size during the ensuing 2-wk recovery period. Quantification of myonuclear number during atrophy and recovery suggests that new nuclei are incorporated into recovering soleus myofibers. As MPCs are the only source of additional nuclei for growing myofibers, we analyzed the expression of MyoD and myogenin mRNAs, markers of MPC activation/proliferation and differentiation, respectively, during recovery. Relative RT-PCR analyses revealed that MyoD mRNA is increased fivefold compared with control at day 4 of recovery (Fig. 4A). MyoD mRNA levels then gradually decrease to control values over the remaining 10 days. Myogenin mRNA levels peak at days 2 and 4 of recovery (9-fold increase over control levels) and then decrease to near control values over the ensuing 10 days (Fig. 4B). Interestingly, myogenin mRNA levels peak before an increase in MyoD mRNA. Previous studies have demonstrated a similar increase in myogenin mRNA before MyoD mRNA during muscle regeneration and hypertrophy (1, 42), possibly reflecting the existence of a population of MPCs that does not require a proliferative stage before terminally differentiating.

To rule out the possibility that the increases in MyoD and myogenin mRNA levels during recovery reflect expression by myofibers rather than MPCs, we used γ-irradiation to eliminate proliferating cells in the atrophied hindlimbs. Myofibers are resistant to γ-irradiation (34, 39), whereas mononucleated cells surrounding the myofibers are sensitive to γ-irradiation. Irradiation is currently the only way of locally eliminating MPCs, and several published studies over the last 10 years have successfully used γ-irradiation to address the role of MPCs during muscle growth (7, 23, 46, 47). One hindlimb was γ-irradiated, whereas the contralateral (nonirradiated) hindlimb served as the control. γ-Irradiation inhibits the increases in MyoD (Fig. 4C) and myogenin (Fig. 4D) normally observed at days 2 and 4 of recovery, indicating that MPCs are responsible for the changes in MyoD and myogenin mRNA expression. Note that the magnitude of the increases in MyoD and myogenin mRNA levels in Fig. 4, C and D, for the irradiated muscles are smaller than in Fig. 4, A and B; however, there is little variability between samples within individual groups. This is likely due to the fact that these experiments were performed with different sets of animals and using different lots of PCR reagents. Together, these data suggest that MPCs proliferate and differentiate during the first week of recovery.

γ-Irradiation Attenuates Recovery of the Soleus Muscle

We next examined whether proliferation, differentiation, and fusion of MPCs with muscle fibers are required for recovery of muscle mass after 2 wk HS. Although several populations of MPCs have been alluded to in the literature, we hypothesized that proliferating MPCs would be the main source of additional nuclei for recovering myofibers. To restore the 35% loss of nuclei from myofibers during HS, proliferative MPCs would be required, so that a reserve population of myogenic cells exists within the tissue after the recovery process to play a role in any later muscle growth. One of the hindlimbs received a single dose of γ-irradiation, and the contralateral hindlimb served as the nonirradiated control. Using muscle weight and total protein content as assays of muscle growth, the soleus regains 60% of its weight and 46% of its protein content after 1 wk of recovery. γ-Irradiation does not attenuate...
the recovery of either of these parameters in the soleus after 1 wk (Fig. 5, A and B). These results demonstrate that this dose of γ-irradiation is not toxic to myofibers, as there is no difference in the degree of growth between the irradiated and nonirradiated muscles at 1 wk of recovery. At 2 wk of recovery, however, γ-irradiation significantly attenuates recovery of the soleus. In the irradiated muscles, muscle weight and total protein content at 2 wk of recovery are not different from 1 wk of recovery. Furthermore, myofiber CSA is restored by only 50% after 2 wk of recovery in the presence of γ-irradiation (Fig. 5C). In addition, myonuclear number is not restored to control values in the irradiated muscles (Fig. 5D). In fact, myonuclear number of the irradiated muscles after 2 wk HS is not different from the myonuclear number after 2 wk HS. Thus this dose of γ-irradiation is successful in the elimination of proliferating MPCs. Moreover, these results argue against a role for nonproliferative MPCs or circulating stem cells in the recovery process because there is no difference in myonuclear number between 2 wk HS and 2 wk recovery in the irradiated soleus. Taken together, these data demonstrate that the initial ~50% of muscle growth can occur independently of an increase in myonuclear number, whereas further growth is dependent on proliferative MPCs.

The Atrophied Plantaris Recovers Normally in the Presence of γ-Irradiation

The following experiments were performed to investigate whether muscles with a different contractile phenotype and hence a different degree of atrophy require proliferating MPCs for recovery to occur. Using muscle weight and myofiber CSA as measures of muscle atrophy and growth, we found that the plantaris atrophies by 25 and 23%, respectively, after 2 wk HS. The degree of atrophy observed in this study is consistent with previously reported data on HS-induced atrophy of the plantaris (27, 57). The plantaris recovers to control values after 2 wk of resuming normal activity (Fig. 6, A and B). The plantaris is composed of predominantly type II myofibers, and thus is not expected to atrophy to the same degree as a muscle composed of predominantly type I myofibers, such as the soleus. γ-Irradiation does not attenuate recovery of plantaris myofiber CSA (Fig. 6B). These results suggest that proliferating MPCs are not required for recovery of the plantaris after 2 wk HS. Myonuclear number of plantaris myofibers was quantified to determine whether myonuclei are lost during HS. Myonuclear number decreases by ~10% after 2 wk HS (Fig. 6C), however, this decrease is not statistically significant. No difference in myonuclear number is observed between con-
suggesting that both MPC-dependent and MPC-independent mechanisms contribute to the growth of atrophied muscle.

Both DNA content and myonuclear number of the soleus are decreased ~35% after 2 wk HS. These results are in agreement with several reports showing that myonuclei are eliminated from myofibers during atrophy, possibly to maintain a constant myonuclear domain (for review, see Ref. 3). There are, however, reports that DNA content (52) and myonuclear number (27) do not change during HS-induced atrophy of the soleus. In these studies, DNA content or myonuclear number was normalized to protein content or myofiber number.

**DISCUSSION**

In this study, multiple assays were used to assess muscle growth after HS-induced atrophy and the cellular mechanisms responsible for this growth. We find that the soleus muscle doubles in size, returning to control values after 2 wk of reloading without pharmacological or physiological intervention. Myonuclear number is decreased during HS and similarly returns to control values after 2 wk of recovery, indicating that MPC proliferation and fusion occurs during this period. When γ-irradiation is used to block proliferation of MPCs, recovery of the soleus is attenuated by ~50%.

Fig. 5. γ-Irradiation attenuates recovery of the soleus. Hindlimbs were γ-irradiated as in Fig. 4. γ-Irradiation (+) attenuates restoration of muscle weight (A) and protein content (B) at 2 wk recovery but not at 1 wk (n = 6). *Statistically significant (P < 0.05) from Rec 1 wk (±) and Rec 2 wk (+). C: restoration of myofiber CSA after 2 wk of recovery is attenuated by γ-irradiation. D: γ-irradiation inhibits the increase in myonuclear number normally observed at 2 wk recovery. Data are means ± SE; *Significantly different (P < 0.05) from control (±); **significantly different (P < 0.05) from *.

Fig. 6. γ-Irradiation has no effect on recovery of the plantaris muscle after HS. A: muscle weight is decreased after 2 wk HS. B: myofiber CSA is decreased after 2 wk HS and recovers normally in the presence of γ-irradiation. C: myonuclear number is not decreased significantly after 2 wk HS and myonuclear number does not increase during recovery. Data are means ± SE; n = 3–4.
CSA, respectively. Because muscle protein content and myofiber CSA decrease significantly during HS-induced atrophy, the fact that these normalized values do not change or even increase is not surprising. Our methods, and those of other groups (4, 16, 24), provide a more accurate indication of whether the muscle as a whole loses myonuclei.

During recovery of the soleus on reloading, the 36% loss of myonuclear number observed after HS is restored to control values, suggesting that MPCs fuse with myofibers during the recovery process. Consistent with this finding, RT-PCR analyses show that markers of MPC activation/proliferation and differentiation increase during the recovery period. Moreover, γ-irradiation inhibits the increases in MyoD and myogenin mRNA levels normally observed during the recovery period, suggesting that MPCs and not the myofiber are the source of these increased mRNA levels. γ-Irradiation of the hindlimb attenuates the restoration of muscle mass by 50% and completely inhibits the increase in myonuclear number normally seen after 2 wk of recovery. These results suggest that growth of atrophied muscle is quite dependent on MPCs, similar to other forms of muscle growth (7, 23, 46, 47).

To determine whether MPCs are required for recovery of all muscles after atrophy, we performed similar experiments on the plantaris muscle. The plantaris muscle is composed of predominantly type II myofibers and atrophies by only ~25% after 2 wk HS. In contrast to the soleus, myonuclear number of plantaris myofibers does not decrease after 2 wk HS and γ-irradiation has no effect on recovery of the plantaris. Our findings are in agreement with a recent study (16), showing that myonuclei are not lost from plantaris myofibers during spinal cord transection-induced atrophy. This is most likely due to the fact that this muscle does not atrophy to the same extent as the soleus, thereby eliminating the requirement for MPCs during the recovery process. Furthermore, the myonuclear domain of type II myofibers is larger than that of type I myofibers (54) and may not be as tightly regulated as in type I myofibers.

Our conclusions on the involvement of MPCs in the growth of atrophied muscle are based on the use of localized γ-irradiation. γ-Irradiation has previously been used to study the requirement for proliferating MPCs in different forms of muscle growth (37, 39, 41, 45–47). Attenuation of muscle growth is not due to general toxic effects on skeletal muscle. Heslop et al. (23) demonstrated that 1,800 rads of γ-irradiation did not adversely affect myofiber permeability. Their conclusion was based on the lack of muscle creatine kinase leakage and the ability of irradiated myofibers to exclude Fcrocion Orange dye. An earlier study demonstrated that 10,000 rads of γ-irradiation did not cause necrosis of myofibers (20). Furthermore, in the current study we show that 2,500 rads of γ-irradiation does not adversely affect soleus myofiber growth during the first week of reloading.

γ-Irradiation may attenuate muscle growth by either direct or indirect effects on MPC activity. DNA lesions in MPCs themselves could directly affect either activation or proliferation. Alternatively, DNA lesions in nonmuscle cells may prevent these cells from secreting factors that regulate activation, proliferation, or differentiation of MPCs. Such nonmuscle cell types could include macrophages (10, 11) or fibroblasts, both of which are known to secrete products that modulate MPC activity.

Earlier attempts to inhibit proliferation of MPCs in muscle growth have used systemic administration of hydroxyurea to inhibit DNA synthesis (17, 18). Systemic administration of hydroxyurea is likely to have deleterious effects in tissues other than the intended muscle, whereas localized γ-irradiation has minimal effects on tissues outside the irradiated region. In both these studies, inhibition of DNA synthesis with hydroxyurea had no effect on muscle hypertrophy. In the study by Fleckman et al. (17), inhibition of DNA synthesis by hydroxyurea had no effect on the growth of the soleus after 2 days of a hypertrophic stimulus. Inhibition of DNA synthesis would be unlikely to affect muscle growth at this early time point, since our results indicate that early muscle growth can occur independently of myonuclear accretion. The study by Fortado et al. (18) examined hypertrophy of the chicken patagialis muscle after 7 days of passive stretch with daily drug treatment. However, recent reports indicate that avian muscle hypertrophy occurs in the absence of proliferating MPCs (32, 37), thus explaining the lack of effect of hydroxyurea.

An important finding from the current study is the stage of recovery at which MPCs play a role in soleus muscle growth after atrophy. Muscle growth after HS-induced atrophy can occur independently of an increase in myonuclear number during the first week, whereas the second week of growth is dependent on irradiation-sensitive MPCs. These results highlight the importance of selecting the appropriate time point(s) when analyzing the role of MPCs during muscle growth. Taken together, our data suggest that myofibers, in which the myonuclear domain is tightly regulated, increase in size until a “threshold” value is attained; further growth is then dependent on the fusion of MPCs with myofibers. Thus therapeutic strategies for the restoration of muscle mass after atrophy may be optimized by the appropriate timing of such treatment. Increasing the proliferative capacity of MPCs coupled with modulating the anabolic capacity of myofibers during specific stages of the recovery process may enhance restoration of muscle mass after atrophy.

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