Apoptosis: a mechanism contributing to remodeling of skeletal muscle in response to hindlimb unweighting

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[1]*Department of Physiological Science and* [2]*Brain Research Institute, University of California, Los Angeles 90095-1527;* [3]*National Aeronautics and Space Administration-Ames Research Center, Moffett Field 94035-1000; and* [4]*Genentech, South San Francisco, California 94080*

Allen, David L., Jon K. Linderman, Roland R. Roy, Allison J. Bigbee, Richard E. Grindeland, Venkat Mukku, and V. Reggie Edgerton. Apoptosis: a mechanism contributing to remodeling of skeletal muscle in response to hindlimb unweighting. *Am. J. Physiol.* 273 (*Cell Physiol.* 42): C579–C587, 1997.—The role of apoptosis in the elimination of myonuclei during hindlimb unloading-induced atrophy and the inhibition of apoptosis in the prevention of muscle atrophy were examined. The number of nuclei demonstrating double-stranded DNA fragmentation seen by terminal deoxynucleotidyl transferase (TDT) histochemical staining, an indicator of apoptosis, was significantly increased after 14 days of suspension. Double staining with TDT and antilaminin immunohistochemistry revealed that some TDT-positive nuclei were within the fiber lamina and were most likely myonuclei. The number of fibers containing morphologically abnormal nuclei was also significantly greater in suspended compared with control rats. Combined treatment with growth hormone and insulin-like growth factor I (GH/IGF-I) and resistance exercise attenuated the increase in TDT-positive nuclei (~26%, $P > 0.05$) and significantly decreased the number of fibers with morphologically abnormal nuclei. The data suggest that *1*) "programmed nuclear death" contributes to the elimination of myonuclei and/or satellite cells from atrophying fibers, and *2*) GH/IGF-I administration plus muscle loading ameliorates the apoptosis associated with hindlimb unloading.

skeletal muscle plasticity; programmed cell death; hindlimb suspension; soleus; myonuclear number

REDUCTION OF NEUROMUSCULAR activity and/or loading results in rapid and substantial atrophy of skeletal muscle fibers (28), and the mechanisms by which muscle fiber size are reduced include transcriptional, translational, and posttranslational pathways (16, 33). It now appears that muscle fiber atrophy is accompanied by a reduction in the mean number of myonuclei per fiber (1–3). Elimination of myonuclei may be another mechanism for modulating the transcriptional potential in muscle fibers. However, the mechanisms by which myonuclei are eliminated from adult muscle fibers are unknown.

One possibility is that myonuclei are eliminated from atrophying skeletal muscle by a form of apoptosis. Apoptosis is one process by which cells are eliminated in a regulated manner (37). Apoptosis plays a role in shaping a number of tissues and structures during development (27) and is characterized by targeted destruction of the chromatin by an endonuclease followed by nuclear fragmentation and destruction, eventually resulting in cellular fragmentation (37). A number of studies have demonstrated a role for apoptosis in the involution of adult tissues after removal of a trophic signal and in the regression of hypertrophied tissue on elimination of the hypertrophic stimulus. For example, apoptosis is responsible for the involution of breast tissue during weaning in mice and rats (36) and for the atrophy of the prostate gland in response to castration (17). Finally, apoptosis is involved in the regression of several tissues after hypertrophy/hyperplasia, including the pancreas in response to soya flour feeding (24) and the liver after lead nitrate-induced hyperplasia (8). Thus apoptosis is a normal feature of tissue atrophy or regression and may assist in tissue remodeling in response to reductions in growth stimuli. Moreover, apoptosis is characterized by the coordinated deletion of cells without interference with tissue function or activation of an inflammation response, allowing tissue function to be uncompromised during remodeling. Muscle atrophy during hindlimb unloading is also characterized by an orderly reduction in muscle mass and remodeling of the muscle tissue without a significant immune response (32).

Apoptosis can occur in fully differentiated skeletal muscle fibers in vivo. Recent studies have demonstrated apoptosis of muscle cells in the degeneration associated with dystrophin-deficient muscular dystrophy (34), and electron microscopy studies have suggested that apoptosis may occur in chronically denervated adult muscle (20). For example, Lee and Altschel (20) observed a number of myonuclear ultrastructural abnormalities consistent with apoptosis after long-term denervation, including chromatin condensation, nuclear shrinkage, and nuclear fragmentation. In a more recent report (6), long-term denervation resulted in the elimination of myonuclei via both necrotic and apoptotic processes, as shown with electron microscopy, nuclear morphological analysis, and in situ labeling of double-stranded DNA breaks. These studies suggest that apoptosis occurs in response to chronic degenerative conditions such as muscular dystrophy or long-term denervation. Although it is clear that myonuclei are lost in normal muscle in response to unloading (2, 3), it is currently unclear whether apoptosis plays a role in the remodeling occurring in normal adult skeletal muscle in response to reduced neuromuscular activation and/or loading.

The purpose of the present study was to evaluate whether apoptosis can play a role in the remodeling of normally innervated, nonpathological skeletal muscle during hindlimb suspension-induced atrophy. We previously demonstrated (1) that mean myonuclear number

is significantly decreased in rat soleus muscle fibers after 14 days of hindlimb suspension and that a combination of brief bouts of daily climbing exercise and injection of growth hormone (GH) and insulin-like growth factor I (IGF-I) attenuated the loss of myonuclei in response to hindlimb suspension. Using terminal deoxynucleotidyl transferase (TDT) in situ end labeling (TDT-ISEL) on these same tissues, we now show that 14 days of hindlimb suspension results in an increase in the number of apoptotic nuclei compared with control and that a combination of GH/IGF-I and exercise treatments partially attenuates the increase in TDT-positive nuclei after suspension. Together, these data support the hypothesis that apoptotic death of myonuclei contributes to the loss of myonuclei associated with a decrease in neuromuscular activity and/or loading.

METHODS

Experimental animals and design. Adult female Sprague-Dawley albino rats (~250 g body wt; Simonsen, Gilroy, CA) were used in all experiments. Animal care and use were in accord with the Ames Research Center Animal Users Guide (AHB-7180) and the guidelines of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee at the Ames Research Center and the Animal Research Committee at the University of California, Los Angeles. The rats were housed in pairs and maintained on a standard 12-h dark-light cycle in a room maintained at 24 ± 1°C. Standard rat chow and water were provided to rats ad libitum. Hindlimb suspension was performed using a tail cast procedure described in detail previously (15).

Three separate experiments were performed. In *experiment 1*, rats were assigned randomly to one of three groups (n = 5/group): *1*) control, *2*) hindlimb suspension, or *3*) hindlimb suspension + GH/IGF-I + exercise. The soleus muscles from these rats had been analyzed previously for changes in mean myonuclear number and fiber cross-sectional area (1). Mean muscle mass and myonuclear number were significantly reduced in the hindlimb-suspended group compared with control, whereas combined growth factor and exercise treatment attenuated these decreases. Therefore, this combination of experimental groups allowed us to examine the effects of 2 wk of unloading, with and without a countermeasure, on the presence of apoptosis in atrophying soleus muscles. The suspended rats in *experiment 1* also were subjected to functional overload of the soleus by ablation of the gastrocnemius and plantaris 7 days before a 14-day suspension period. Functional overload was employed to enhance the loading of the soleus during the brief daily bouts of resistance exercise. Combined GH/IGF-I injections were used because previous studies demonstrated that optimal elevation of serum IGF-I levels and minimal effects on insulin secretion are achieved in normal, nonhypophysectomized rats with coinjection compared with either growth factor alone (19). Combined GH/IGF-I injection also results in the optimal secretion of IGF-I binding proteins from the liver (19), thus increasing the half-life of injected IGF-I. Two additional hindlimb suspension experiments (*experiments 2* and *3*, see below) were conducted without functional overload to evaluate the role of apoptosis during atrophy in muscles that were not functionally overloaded.

In *experiment 2*, rats were suspended for 3, 7, or 14 days (n = 5/time point) to examine the time course of the presence of apoptosis during hindlimb suspension. Five age-matched control rats were studied at each time point. To eliminate any possible effects of functional overload (see *experiment 1* above), the soleus muscles of these animals were not functionally overloaded before suspension.

In *experiment 3*, rats were assigned randomly to one of five groups (n = 4/group), *1*) control, *2*) hindlimb suspension, *3*) hindlimb suspension + IGF-I, *4*) hindlimb suspension + exercise, and *5*) hindlimb suspension + IGF-I + exercise, to determine the role of IGF-I and/or exercise in preventing atrophy-induced apoptosis. As in *experiment 2*, the soleus muscle was not functionally overloaded in these rats. In addition, treatment with IGF-I alone was used to attempt to isolate the effects of IGF-I from those of GH.

Exercise protocol. Rats from each of the exercised groups were subjected to brief bouts of climbing exercise three times daily (0800, 1200 and 1600) as described in detail in Grindeland et al. (15). Briefly, during each bout of exercise, the rats climbed a 1-m wire mesh grid (85° incline) three times in succession with a weight of ~40% of body weight attached to a tail cast to increase the load on the muscle. Each rat spent a total of ~5–10 min per day preparing to climb or climbing.

Growth factor protocol. Immediately before each exercise bout, the growth factor-treated rats were given an injection (sc) of combined recombinant human GH and IGF-I (*experiment 1*) (Genentech, San Francisco, CA) of 1 mg/kg body wt each dissolved in 0.85% NaCl at a concentration of 250 μg/ml or IGF-I alone (*experiment 3*). The rats in both the control and hindlimb-suspended groups received the same volume of saline injected at the same times.

Muscle preparation. At the end of the hindlimb suspension period, the rats were killed and the soleus muscles were removed bilaterally, cleaned of excess fat and connective tissue, wet weighed, frozen in freon cooled in liquid nitrogen, and stored at −70°C.

TDT-ISEL staining of skeletal muscle cross sections. The number of apoptotic nuclei was quantified by the histochemical method for staining double-stranded DNA breaks described by Gavrieli et al. (14), as modified by Tidball et al. (34). This technique uses TDT enzyme to incorporate biotinylated dUTP nucleotides to the end of the double-stranded DNA breaks with an avidin-peroxidase-precipitate visualization process. Double-stranded DNA fragmentation is a hallmark of apoptosis (37) and has been used on dystrophic skeletal muscle to label apoptotic myonuclei (34). Frozen sections (20-μm thick) were cut from the midbelly of the soleus muscle of each rat, fixed in 2% paraformaldehyde for 15 min, and rinsed twice in phosphate-buffered saline (PBS). Sections were digested with 0.1 μg/ml proteinase K for 10 min, and endogenous peroxidase activity was quenched in 0.3% hydrogen peroxide for 5 min. After a rinse in TDT buffer (30 mM tris(hydroxymethyl)aminomethane, pH 7.4, 140 mM Na cacodylate, 1 mM cobalt chloride), sections were incubated for 1 h at 37°C in the TDT buffer with 300 U/ml TDT and 4 μM biotinylated UTP. After several rinses in PBS and a blocking for 1 h in 1% gelatin, sections were incubated in avidin-peroxidase (Vector Laboratories, Burlingame, CA) diluted 1:1,000 (vol/vol) in PBS for 30 min. Staining was visualized with a 3-amino 9-ethyl carbazole kit (Vector Laboratories) per manufacturer's instructions; the reaction creates a distinct red precipitate. Sections were rinsed with double-distilled H_2O for 5 min and mounted in 100% glycerol. Negative control slides that received all steps except TDT enzyme were run with every staining group and showed no specific staining and low background (data not shown). Sections were examined with a bright-field microscope using Nomarski optics, and the total number of apoptotic myonuclei was determined by counting the number of TDT-positive nuclei per section. In

addition, the number of muscle and nonmuscle TDT-positive nuclei was determined. Nuclei that were clearly within the muscle fiber boundary and corresponded in shape and position (i.e., round or oval and peripheral to the fiber) to myonuclei were counted as muscle nuclei. All other nuclei were counted as interstitial, which included fibroblasts, immune cells, and vascular endothelial cells. A mean number of TDT-positive nuclei per cross section was determined from nine sections from each rat in *experiment 1* and from six sections from each rat in *experiments 2* and *3*. Studies on apoptosis in vivo often report the number of apoptotic cells per unit volume (34). The dramatic differences in tissue volume between the various groups, however, made this value of limited use in comparing across conditions. Therefore, the mean number of TDT-positive nuclei per section was chosen as the value for comparison.

Several sections from three animals in *experiment 1* were fluorescently double-labeled for apoptotic nuclei and laminin, a component of the muscle fiber basal lamina, to further demonstrate the presence of apoptotic nuclei within the fiber boundary. Frozen sections were fixed for 10 min in 2% paraformaldehyde and then rinsed for 5 min in PBS. Sections were quenched for 15 min in sodium borohydride, permeabilized with 0.1% Triton X-100, and blocked for 15 min in 0.5% bovine serum albumin and 0.5% nonfat dry milk. After a 5-min wash in TDT buffer, sections were incubated in the TDT reaction medium described above, to which polyclonal rabbit anti-laminin antibodies (Sigma) diluted 1:40 had been added. Incubation was for 2 h at 37°C, and the reaction was stopped by the addition of buffer (30 mM sodium citrate, 300 mM NaCl) for 15 min. Sections were rinsed twice with PBS and incubated with fluorescein isothiocyanate-conjugated streptavidin (Sigma) diluted 1:100 and goat anti-rabbit-tetramethylrhodamine isothiocyanate-conjugated antibodies (Sigma) diluted 1:40 for 1 h at room temperature. Sections were rinsed with PBS several times, stained for nuclei using Hoechst 33258, and mounted in 100% glycerol. A fluorescent microscope was used to evaluate the location of the TDT-positive nuclei relative to the fiber basal lamina. Nuclei within the muscle fiber basal lamina were taken to be myonuclei. Because the basal lamina was used as the boundary, it is possible that some of the nuclei may reside in satellite cells, which lie adjacent to the muscle fiber and within the basal lamina (21). These nuclei, therefore, are referred to as "muscle nuclei" and include both satellite cell nuclei and myonuclei.

Analysis of myonuclear shape abnormalities using confocal microscopy. Apoptosis in the absence of internucleosomal DNA fragmentation has been reported previously (5, 7), and it is possible that myonuclear destruction is not characterized by TDT labeling. Another characteristic of apoptosis is the presence of nuclear morphological abnormalities, including alterations in nuclear shape and size (37). Single muscle fibers from control, hindlimb-suspended, and hindlimb-suspended + GH/IGF-I + exercise soleus muscles from *experiment 1* were evaluated for myonuclear abnormalities. Single muscle fibers were isolated and stained for myonuclei as described previously (2, 3). Briefly, frozen muscle samples were progressively thawed to room temperature, and single muscle fiber segments were mechanically dissected in low-calcium relaxing solution and placed on gelatin-coated coverslips. Fiber nuclei were stained with propidium iodide and acridine orange and examined using confocal microscopy.

The number of abnormal myonuclei in three different randomly chosen fields (175 μm^2) per fiber was quantified. Abnormal myonuclei were defined as having one or more of the following characteristics: *1*) size abnormalities, i.e., nuclei that were less than one-half the length or twice as long as normal nuclei (5, 37); *2*) shape abnormalities, i.e., nuclei with scalloped or wavy edges and/or sharp, angulated edges (37); and/or *3*) location abnormalities, i.e., nuclei located in either the direct fiber center or slightly deep to the fiber periphery. Approximately 100–200 fibers (n = 139, 107, and 150 fibers for control, hindlimb-suspended, and hindlimb-suspended + GH/IGF-I + exercise, respectively) were analyzed from four rats from each condition in *experiment 1*, for a total of ~30,000 nuclei analyzed. The number of muscle fibers containing abnormal myonuclei was expressed as a percentage of the total number of fibers analyzed per animal. Mean values per animal and per group were used for statistical evaluation.

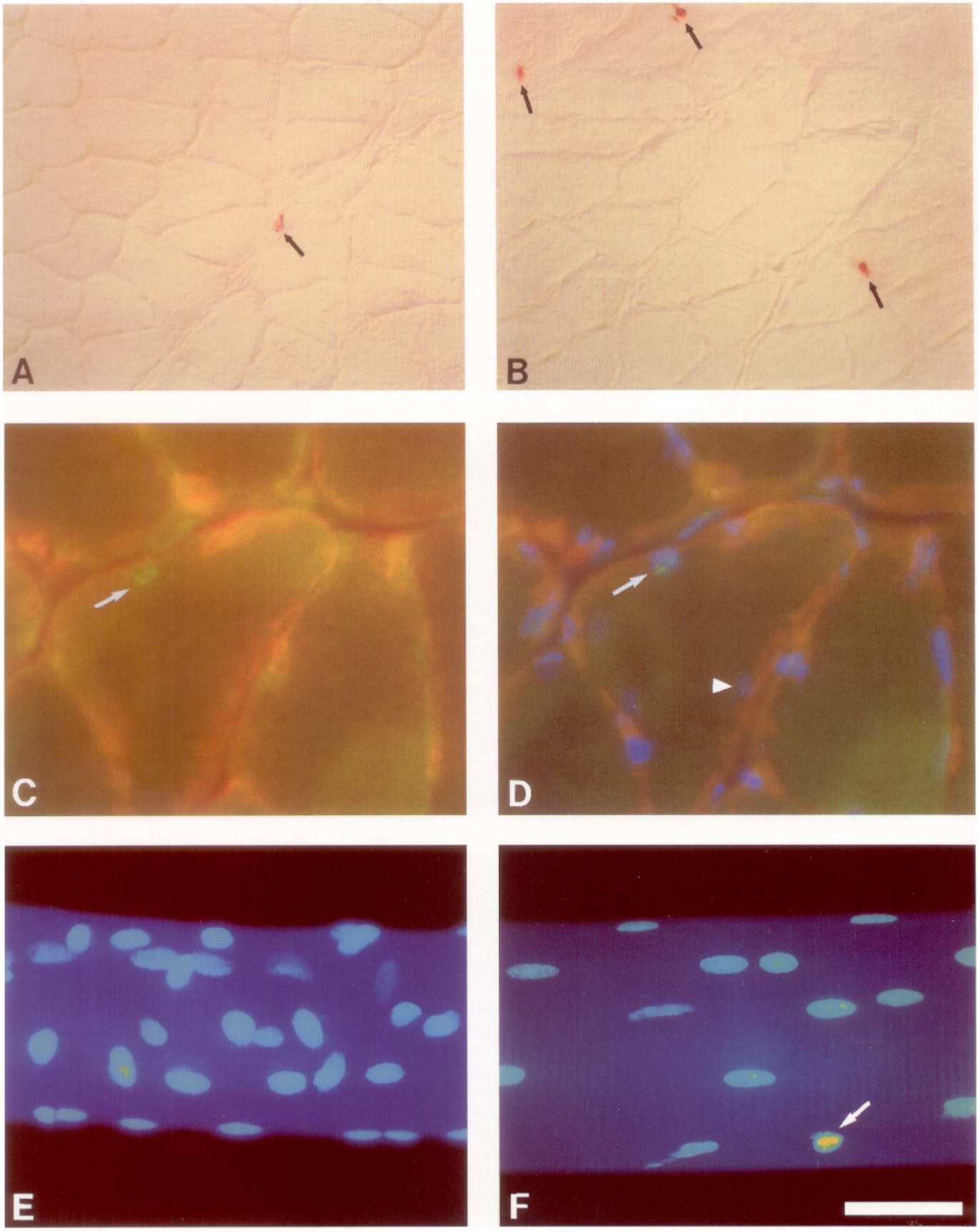
Statistical analyses. All values are expressed as means ± SE. Differences between groups were determined using analysis of variance, with Fisher's post hoc test used for individual comparisons. For all tests, an α-level of 0.05 was chosen for significance. For the time-course study, Student's unpaired t-test was used to compare suspended versus control values at each time point.

RESULTS

Experiment 1: Hindlimb suspension and apoptosis. A distinct red precipitate labeled TDT-positive nuclei (Fig. 1, *A* and *B*). Most of the TDT-positive nuclei in all conditions were located in the interstitial spaces between muscle fibers or within connective tissue bands, although some TDT-positive nuclei were located within the muscle fiber and were considered to be muscle nuclei (Fig. 1, *A–D*). The cross sections of fibers containing TDT-positive nuclei were not noticeably different in shape or size from fibers that did not contain TDT-positive nuclei, suggesting that these fibers were still viable and not undergoing large-scale degeneration or death (Fig. 1, *A–D*).

Some TDT-positive nuclei were clearly within the muscle fiber basal lamina and were therefore considered either true fiber myonuclei or satellite cell nuclei (Fig. 1, *C* and *D*). Hoechst staining of the nuclei revealed three additional points: *1*) TDT-positive staining corresponded spatially to the presence of Hoechst-positive nuclei, confirming that actual nuclear staining was being observed; *2*) fibers with TDT-positive nuclei were not in the process of undergoing widespread infiltration by immune cells, which are known to undergo apoptosis after phagocytosis of dead muscle fibers (32); and *3*) TDT-positive muscle nuclei were observed within the cross section of fibers that also contained normal myonuclei (Fig. 1, *C* and *D*). In addition, examination of the same fibers in serial sections revealed that fibers containing TDT-positive muscle nuclei at one level were morphologically normal and contained myonuclei that were not TDT-positive at other levels along the fiber length. It is apparent that individual myonuclei can undergo apoptosis within a single fiber while other myonuclei remain unaffected.

The total number of TDT-positive nuclei per muscle cross section was ~15-fold higher in hindlimb-suspended and ~10-fold higher in hindlimb-suspended + GH/IGF-I + exercise compared with control rats (Fig. 2*A*). The mean number of TDT-positive total nuclei in hindlimb-suspended + GH/IGF-I + exercise rats was not significantly different from control or hindlimb-


A
B
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suspended rats. The number of TDT-positive muscle nuclei per cross section was significantly higher in hindlimb-suspended and hindlimb-suspended + GH/IGF-I + exercise rats than in control rats (Fig. 2*B*).

Single muscle fibers were isolated from the same muscles used for TDT histochemistry and were evaluated for myonuclear morphological abnormalities. Morphological abnormalities characteristic of apoptosis were observed in myonuclei within isolated muscle fibers. For example, abnormal myonuclei were often shrunken and intensely stained with propidium iodide (Fig. 1, *E* and *F*), a hallmark of apoptosis (5). In addition, unusually elongated and angulated myonuclei were seen, as were myonuclei with convoluted nuclear membranes (not shown). Hindlimb-suspended rats had a significantly higher percentage of fibers containing myonuclear abnormalities compared with control and hindlimb-suspended + GH/IGF-I + exercise rats (Fig. 3). It should be noted that the total number of abnormal myonuclei in any group was very small, i.e., ~1 abnormal myonucleus for every 400 normal myonuclei, consistent with the rarity of TDT-positive muscle nuclei observed in the muscle cross sections from hindlimb-suspended rats.

Experiment 2: Time course of apoptosis during hindlimb suspension. The mean numbers of total and muscle TDT-positive nuclei were significantly increased after 3 days of suspension (Fig. 4). The mean number of TDT-positive muscle nuclei then remained consistent throughout the 14-day period (Fig. 4*B*), whereas the number of total TDT-positive nuclei was significantly increased further at 7 days and remained elevated at 14 days (Fig. 4*A*). The increase in TDT-positive nuclei (appearing at 3 days) preceded significant muscle atrophy (appearing at 7 days) (Fig. 5).

Experiment 3: Role of IGF-I and/or exercise on hindlimb suspension-induced apoptosis. Because 3 days of suspension showed a significant incidence of apoptosis, this time point was studied to determine whether IGF-I and/or exercise could ameliorate this effect. Three days of hindlimb suspension resulted in a significant increase in the mean number of total and muscle TDT-positive nuclei per cross section compared with control (Fig. 6). After IGF-I or exercise treatment alone, the number of muscle TDT-positive myonuclei per cross section was similar to control (Fig. 6*B*), although the number of total TDT-positive nuclei per section remained elevated (Fig. 6*A*). Combined treatment with IGF-I + exercise resulted in both the total and muscle TDT-positive nuclei per section being significantly lower than in hindlimb-suspended rats and similar to control values.

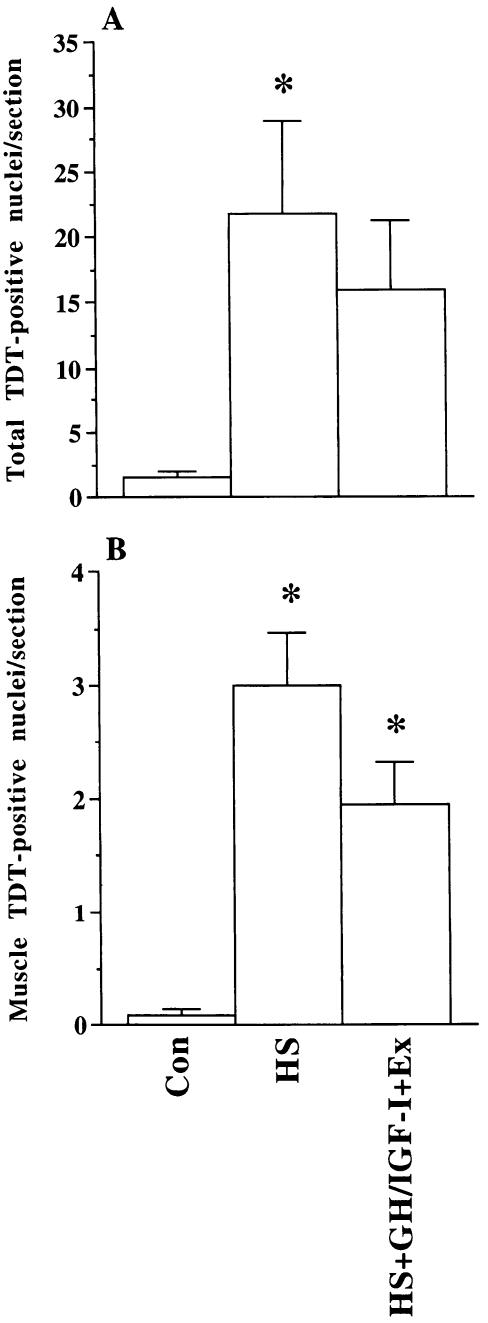

Fig. 2. Mean number of TDT-positive nuclei per section is shown for the 3 groups of rats from *experiment 1*. Total number of TDT-positive nuclei (*A*) and TDT-positive muscle nuclei (*B*) are shown for each group. Values are means ± SE. Con, control; HS, hindlimb suspended; HS + GH/IGF-I + Ex, hindlimb suspended + growth hormone/insulin-like growth factor I + exercised. *Significantly different from control ($P < 0.05$).

Fig. 1. Histochemical and morphological evidence for myonuclear apoptosis. Cross sections of a soleus muscle from a control (*A* and *C*) and a 2-wk hindlimb-suspended (*B* and *D*) rat were histochemically labeled with terminal deoxynucleotidyl transferase (TDT) in situ end-labeling with either a precipitate-based labeling kit (*A* and *B*) or with fluorescein-conjugated avidin (*C* and *D*). Arrows, apoptotic nuclei. Number of TDT-positive nuclei was significantly higher in hindlimb-suspended compared with control soleus muscles. Triple labeling with TDT-fluorescein isothiocyanate (green), laminin-tetramethylrhodamine isothiocyanate (reddish orange), and Hoechst 33258 (blue) is shown in *C* and *D*. A TDT-positive nucleus (arrow) can be clearly seen by TDT staining (*A*) that corresponds to a Hoechst-positive nucleus (*B*) within muscle fiber basal lamina. Arrowhead indicates a muscle nucleus within same muscle fiber that is TDT-negative. False-color confocal images of mechanically isolated single muscle fibers from control and 2-wk hindlimb-suspended rat soleus muscles stained with propidium iodide and acridine orange are shown in *E* and *F*, respectively. Control fiber (*E*) contains a multitude of normal, oval-shaped nuclei. Fiber from hindlimb-suspended rat (*F*) contains a rounded, intensely stained nucleus (arrow in *B*). Bar: 100 μm in *A–D*, 30 μm in *E* and *F*.

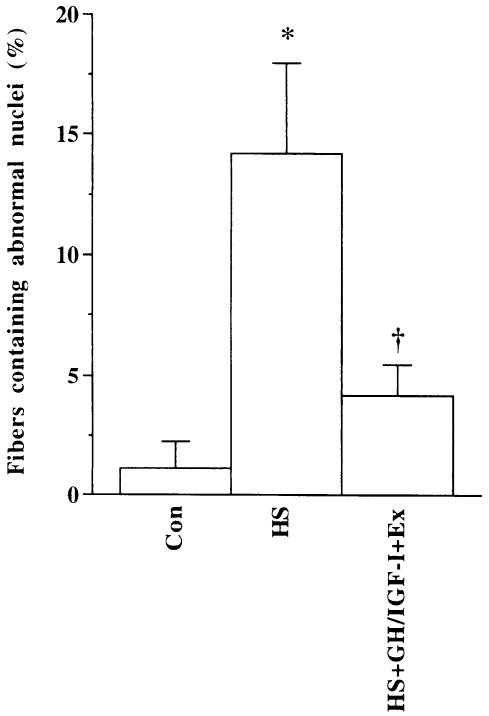

Fig. 3. Percentage of fibers containing morphologically abnormal myonuclei in Con, HS, and HS + GH/IGF-I + Ex rats. Values are means ± SE. *, † Significantly different from control or hindlimb suspended, respectively ($P < 0.05$).

DISCUSSION

Mean myonuclear number is significantly lower than controls after skeletal muscle atrophy associated with unloading in adult rats (1–3). Schultz and co-workers (9, 30) also reported a significant decrease in mean myonuclear number after hindlimb suspension in very young postnatal rats (20 days old on day of suspension). The mechanism responsible for the lower myonuclear number in suspended versus control groups in these young rats was a reduction in satellite cell proliferation, which in turn reduced the rate of formation of new myonuclei via fusion of satellite cells to existing myofibers during the postnatal phase of muscle growth. In the present study adult rats were used, and the available evidence indicates that satellite cell proliferation is extremely low at this stage of development. Therefore, in adults, it would appear that something other than reduced formation of new myonuclei was responsible for the loss of myonuclei that we observed. In the present study, 14 days of hindlimb suspension resulted in a significant increase in the number of TDT-positive muscle nuclei and in the percentage of fibers containing myonuclear abnormalities in the rat soleus compared with control. Moreover, in the three separate experiments (a total of 24 rats), the number of TDT-positive total or muscle nuclei relative to the number of normal nuclei was extremely small in the soleus of control rats, suggesting that normal "turnover" of myonuclei is low. Together, these data suggest that the elimination of existing myonuclei by apoptosis is at least partially responsible for the reduction in myonuclear number accompanying hindlimb suspension-induced atrophy.

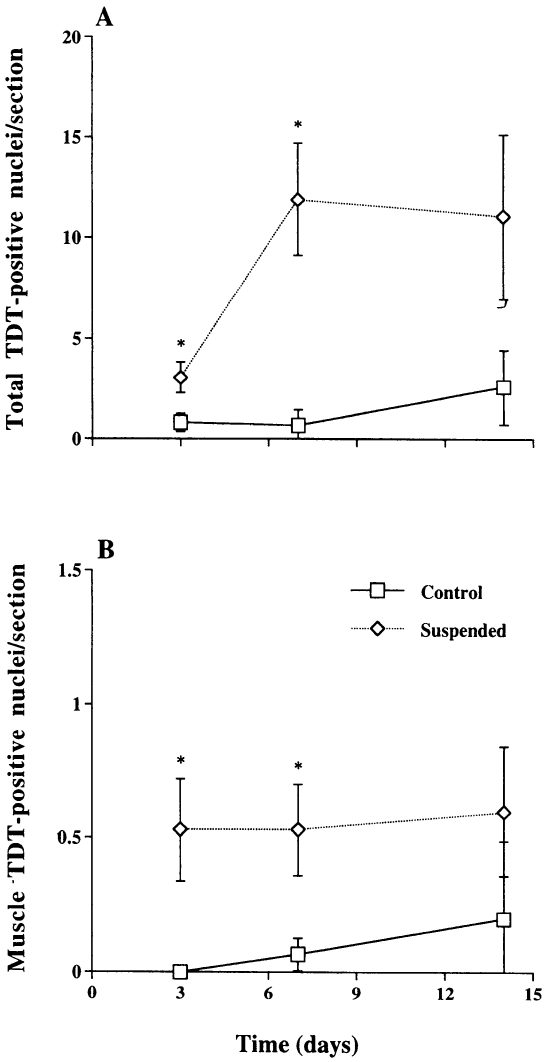

Fig. 4. Relationship between number of TDT-positive total (*A*) and muscle (*B*) nuclei and duration of hindlimb suspension. Control and suspended rats were studied at 3, 7, or 14 days. Soleus muscle cross sections were stained with TDT as described in METHODS. Each point represents mean ± SE for 5 rats. Note different scales for *A* and *B*. *Significantly different from control ($P < 0.05$).

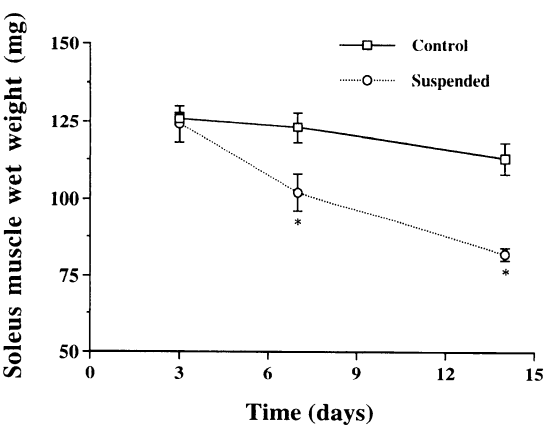

Fig. 5. Relationship between mean soleus mass and duration of hindlimb suspension. Each point represents mean ± SE for 5 rats. *Significantly different from control ($P < 0.05$).

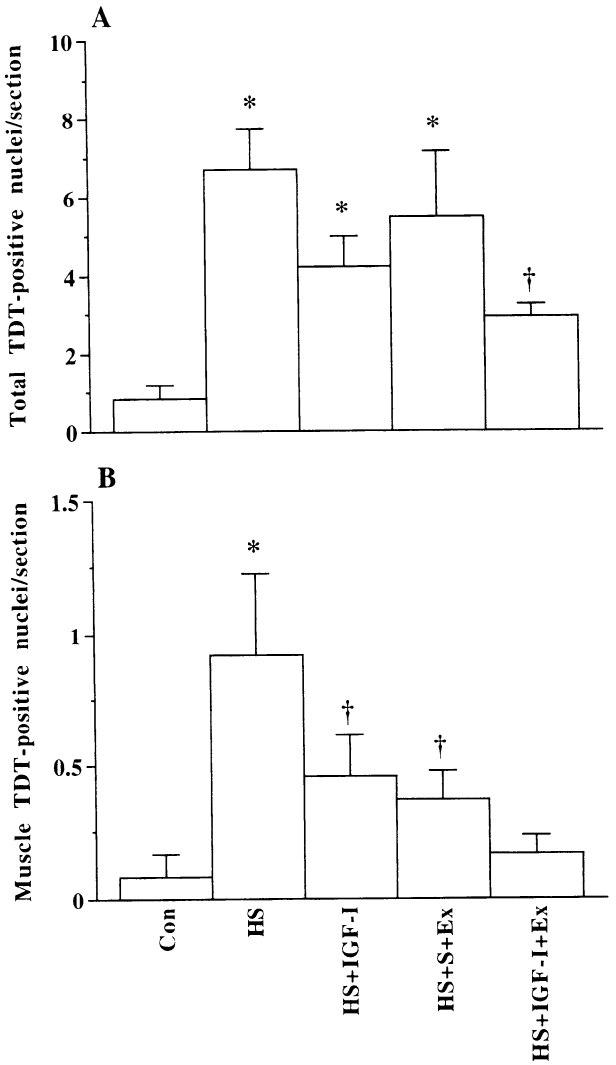

Fig. 6. Mean number of TDT-positive total (*A*) and muscle (*B*) nuclei after 3-day hindlimb-suspension period in *experiment 3*. Values are means ± SE for 4 rats/group. HS + IGF-I, hindlimb suspended + insulin-like growth factor; HS + S + Ex, hindlimb suspended + exercised; HS + IGF-I + Ex, hindlimb suspended + insulin-like growth factor-I + exercised. *, †Significantly different from control or hindlimb suspended, respectively ($P < 0.05$).

The time-course data from *experiment 2* indicate that, at least during this 2-wk period, the total number of TDT-positive nuclei progressively increased up to 7 days and then reached a plateau. It is clear that within 2 wk of suspension the majority of the atrophy associated with hindlimb suspension is complete (28). Also within 2 wk of either hindlimb suspension or spaceflight, there is a loss of myonuclei in the soleus muscle (1–3). However, whether this is a true plateau in the total number of TDT-positive nuclei at this time is unknown.

In hindlimb-suspended rats, little evidence for gross pathology of muscle fibers was evident, suggesting that individual myonuclei underwent apoptosis in the absence of widespread or regional degeneration of the muscle fiber. In fact, apoptotic myonuclei appeared to be distributed randomly within muscle fibers, i.e., normal myonuclei (TDT negative) were observed within the same section and in adjacent sections of the same fiber containing TDT-positive myonuclei. Davis et al. (10) reported apoptosis in some nuclei in the multinucleated protozoan *Tetrahymena*, and others have shown apoptosis in individual nuclei within multinucleated heterokaryons in vitro (11, 26). These findings and the present data argue that individual nuclei can undergo apoptotic degradation within multinucleated cells, suggesting that at least some of the critical steps for apoptotic nuclear destruction must occur within the nucleus itself. Conversely, it is possible that individual myonuclei within a common cytoplasm may sense and respond to extracellular signals individually and thus may possess "signaling domains" within the cytoplasmic syncytium that allow inputs to affect only one or a few myonuclei (23).

The number of fibers containing morphologically abnormal myonuclei was significantly increased in hindlimb-suspended animals compared with control. These morphological abnormalities have been used previously as an indication of apoptotic death (37). We have done extensive studies using mechanically isolated fibers and, using anti-vimentin immunohistochemistry to identify mesenchymal cells, we demonstrated that the mechanical fiber dissection procedure stripped off virtually all adhering mononucleated cells, including satellite cells and fibroblasts (Allen, unpublished observations). These findings are similar to those of Konigsberg et al. (18). Thus it is extremely likely that the abnormal nuclei were true myonuclei and not satellite cell nuclei and that these abnormal myonuclei were undergoing apoptosis.

The contribution of apoptosis to the elimination of myonuclei during hindlimb suspension can be estimated using a few assumptions. Given that the mean myonuclear number decreased from 162 myonuclei/mm in control to 134 myonuclei/mm in 14-day hindlimb-suspended rat soleus muscle fibers (2) and assuming that the myonuclei were lost homogeneously along the length of the fiber, there was an average loss of 28 myonuclei·mm^{-1}·fiber^{-1} or 0.028 myonuclei·μm^{-1}·fiber^{-1}. This assumption is probably valid, because there was no apparent indication of elimination of myonuclei from a single area. Assuming that the soleus has ~3,000 fibers (25) and that the percentage of slow fibers in the rat soleus is ~80%, then ~1,344 myonuclei should be lost per 20-μm-thick soleus cross section (3,000 fibers/section × 0.8% slow fibers × 0.028 myonuclei lost·fiber^{-1}·μm section^{-1} × 20 μm/section). Finally, the time-course data suggest that the number of TDT-positive muscle nuclei was relatively constant over a 14-day period. If a constant rate of loss is assumed and the mean length of time for apoptosis to occur is ~2 h (14), then eight TDT-positive muscle nuclei should be evident per 2-h time point per 20-μm-thick section. Approximately three TDT-positive muscle nuclei per 20-μm section were found at the 14-day time point in the first study, or ~40% of the number of muscle nuclei expected if the assumptions listed above are correct. This discrepancy may indicate that apoptosis is not solely responsible for the elimination of myonuclei. On

the other hand, the 2-h duration for apoptosis was determined from studies on apoptosis of epithelial cells in vitro (14), and it is possible that this assumption is not valid for muscle nuclei in vivo. The time course for myonuclear apoptosis can be estimated using these same data if it is assumed that the number of TDT-positive muscle nuclei per section represents the total number of lost myonuclei. This calculation yields a value of ~45 min for the average duration of myonuclear apoptosis, slightly shorter than the 1–3 h previously reported (14). However, because only the myonucleus is destroyed in atrophying muscle fibers rather than the entire cell, then the time required for apoptosis may be somewhat shorter.

A large number of TDT-positive nuclei was observed in the fiber interstitial areas and connective tissue bands in hindlimb-suspended rats in *experiment 1*. Presumably, nonmuscle cells, such as fibroblasts and immune cells, underwent apoptosis as well. These rats were first exposed to 1 wk of functional overload of the soleus by ablation of its synergists before experiencing 2 wk of hindlimb suspension. Previous studies demonstrated an increase in immune cell infiltration and inflammation during the first week of functional overload (4), whereas hindlimb suspension alone does not result in an increase in immune cell infiltration unless reloading occurs (32). Infiltrating immune cells can undergo apoptosis in skeletal muscle (35), and thus at least part of the substantial increase in apoptotic nuclei probably represents apoptosis of macrophages and other leukocytes recruited to assist in muscle remodeling and fiber degeneration accompanying functional overload. Conversely, the presence of significant numbers of TDT-positive nonmuscle nuclei in the studies employing hindlimb suspension alone (*experiment 2*) argues that other nonmuscle cells, such as fibroblasts and vascular cells, also undergo apoptosis during hindlimb suspension-induced atrophy. Presumably, elimination of nonmuscle as well as muscle nuclei occurs as the tissue undergoes remodeling during atrophy. These observations suggest that nonmuscle cells within skeletal muscle are sensitive to alterations in neuromuscular activation and loading and can undergo apoptosis in response to these alterations. Furthermore, these data suggest that the survival of nonmuscle cells is regulated coordinately with that of muscle nuclei. Coordinate modulation of both muscle and nonmuscle cells within a muscle may reflect an in-parallel remodeling of the force-generating components (myofibers) and the components that transmit force (fibroblasts, connective tissue). On the other hand, in *experiment 3*, the exercise and growth factor interventions alone had differential effects on total and muscle nuclei. Both exercise and IGF-I injection alone were able to partially ameliorate the increase in TDT-positive muscle nuclei but not total nuclei, suggesting that nonmuscle nuclei do not always respond similarly to muscle nuclei to a given stimulus.

The number of muscle and nonmuscle TDT-positive nuclei was substantially lower in the studies using hindlimb suspension alone compared with hindlimb suspension after functional overload. Functional overload is accompanied by satellite cell proliferation (31) and incorporation of satellite cell nuclei into the muscle fibers (29). Thus mean myonuclear number may have been elevated in the soleus of functionally overloaded compared with control rats before suspension, and the greater number of TDT-positive nuclei in these muscles may reflect the loss of a greater number of nuclei during the atrophic phase in functionally overloaded-suspended compared with control-suspended rats. In addition, unfused satellite cells, newly fused myonuclei associated with functional overload, or immune cells may be more susceptible to apoptotic death after initiation of the hindlimb suspension (13). A mechanistic role for immune cells in the apoptotic death of myonuclei accompanying muscular dystrophy has been suggested (J. Tidball and M. Spencer, personal communication).

In a previous study, combined GH/IGF-I treatment plus muscle loading by ladder climbing was successful in preventing much of the decrease in mean myonuclear number, muscle mass, and fiber size associated with hindlimb suspension (1). The present data are consistent with the hypothesis that GH/IGF-I and exercise treatment ameliorate the decrease in myonuclear number, at least in part, by inhibiting the apoptotic death of individual myonuclei. The data from *experiment 3* suggest that the survival effect resulting from combined GH/IGF-I treatment was at least partially mediated by IGF-I, a conclusion supported by in vitro studies (5, 12). In addition, injection of IGF-I has been successful in preventing apoptosis of developing motoneurons in vivo (22). The present data are consistent with the hypothesis that depletion of survival factors could be responsible for apoptotic cell death in vivo as well as in vitro.

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REFERENCES

1. **Allen, D. L., J. K. Linderman, R. E. Grindeland, V. R. Mukku, and V. R. Edgerton.** Growth hormone/IGF-I and muscle loading prevent the loss of myonuclear number and fiber size during hindlimb suspension (Abstract). *Med. Sci. Sports Exerc.* 59, *Suppl.*: S166, 1996.
2. **Allen, D. L., S. R. Monke, R. J. Talmadge, R. R. Roy, and V. R. Edgerton.** Plasticity of myonuclear number in hypertrophied and atrophied mammalian skeletal muscle fibers. *J. Appl. Physiol.* 78: 1969–1976, 1995.
3. **Allen, D. L., W. Yasui, T. Tanaka, Y. Ohira, S. Nagoaka, C. Sekiguchi, W. E. Hinds, R. R. Roy, and V. R. Edgerton.** Myonuclear number and myosin heavy chain expression in rat soleus muscle fibers following spaceflight. *J. Appl. Physiol.* 81: 145–151, 1996.
4. **Armstrong, R. B., P. Marum, P. Tullson, and C. Saubert.** Acute hypertrophic response of skeletal muscle to removal of synergists. *J. Appl. Physiol.* 46: 835–842, 1979.
5. **Barres, B. A., I. K. Hart, H. S. Coles, J. F. Burne, J. T. Voyvodic, W. D. Richardson, and M. C. Raff.** Cell death and

control of cell survival in the oligodendrocyte lineage. *Cell* 70: 31–46, 1992.

6. **Borisov, A. G., and B. M. Carlson.** Loss of nuclei in denervated skeletal muscle: possible cellular mechanisms (Abstract). *FASEB J.* 9: 825A, 1995.
7. **Cohen, G. M., X. M. Sun, R. T. Snowden, D. Dinsdale, and D. N. Skilleter.** Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. *Biochem. J.* 286: 331–334, 1992.
8. **Columbano, A., G. M. Ledda-Columbano, P. P. Coni, G. Faa, C. Liguori, G. Santa Cruz, and P. Pani.** Occurrence of cell death (apoptosis) during the involution of liver hyperplasia. *Lab. Invest.* 52: 670–675, 1985.
9. **Darr, K. C., and E. Schultz.** Hindlimb suspension suppresses muscle growth and satellite cell proliferation. *J. Appl. Physiol.* 67: 1827–1834, 1989.
10. **Davis, M. C., J. G. Ward, G. Herrick, and C. D. Allis.** Programmed nuclear death: apoptotic-like degradation of specific nuclei in conjugating *Tetrahymena*. *Dev. Biol.* 154: 419–432, 1992.
11. **Dipasquale, B., and R. J. Youle.** Programmed cell death in heterokaryons. A study of the transfer of apoptosis between nuclei. *Am. J. Pathol.* 141: 1471–1479, 1992.
12. **D'Mello, S. R., C. Galli, T. Ciotti, and P. Calissano.** Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. *Proc. Natl. Acad. Sci. USA* 90: 10989–10993, 1993.
13. **Duke, R. C.** Apoptosis in cytotoxic T lymphocytes and their targets. *Semin. Immunol.* 4: 407–412, 1992.
14. **Gavrieli, Y., Y. Sherman, and S. A. Ben-Sasson.** Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119: 493–501, 1992.
15. **Grindeland, R. E., R. R. Roy, V. R. Edgerton, E. J. Grossman, V. R. Mukku, B. Jiang, D. J. Pierotti, and I. Rudolph.** Interactive effects of growth hormone and exercise on muscle mass in suspended rats. *Am. J. Physiol.* 267 (*Regulatory Integrative Comp. Physiol.* 36): R316–R322, 1994.
16. **Howard, G., J. M. Steffen, and T. E. Geoghegan.** Transcriptional regulation of decreased protein synthesis during skeletal muscle unloading. *J. Appl. Physiol.* 66: 1093–1098, 1989.
17. **Kerr, J. F., and J. Searle.** Deletion of cells by apoptosis during castration-induced involution of the rat prostate. *Virchows Arch.* 13: 87–102, 1973.
18. **Konigsberg, U. R., B. H. Lipton, and I. R. Konigsberg.** The regenerative response of single mature muscle fibers isolated in vitro. *Dev. Biol.* 45: 260–275, 1975.
19. **Kupfer, S. R., L. E. Underwood, R. C. Baxter, and D. R. Clemmons.** Enhancement of the anabolic effects of growth hormone and insulin-like growth factor I by use of both agents simultaneously. *J. Clin. Invest.* 91: 391–396, 1993.
20. **Lee, J. C., and R. Altschel.** Electron microscopy of the nuclei of denervated skeletal muscle. *Z. Zellforsch. Mikrosk. Anat.* 61: 168–182, 1963.
21. **Mauro, A.** Satellite cells of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* 9: 493–494, 1961.
22. **Neff, N. T., D. Prevette, L. J. Houenou, M. E. Lewis, M. A. Glicksman, Q. W. Yin, Q., and R. W. Oppenheim.** Insulin-like growth factors: putative muscle-derived trophic agents that promote motoneuron survival. *J. Neurobiol.* 24: 1578–1588, 1993.
23. **Nicotera, P., B. Zhivotovsky, and S. Orrenius.** Nuclear calcium transport and the role of calcium in apoptosis. *Cell Calcium* 16: 279–288, 1994.
24. **Oates, P. S., R. G. Morgan, and A. M. Light.** Cell death (apoptosis) during pancreatic involution after raw soya flour feeding in the rat. *Am. J. Physiol.* 250 (*Gastrointest. Liver Physiol.* 13): G9–G14, 1986.
25. **Parsons, D., M. Riedy, R. L. Moore, and P. D. Gollnick.** Acute fasting and fiber number in rat soleus muscle. *J. Appl. Physiol.* 53: 1234–1238, 1982.
26. **Polunovsky, V. A., D. H. Ingbar, M. Peterson, and P. B. Bitterman.** Cell fusion to study nuclear-cytoplasmic interactions in endothelial cell apoptosis. *Am. J. Pathol.* 149: 115–128, 1996.
27. **Raff, M. C., B. A. Barres, J. F. Burne, H. S. Coles, Y. Ishizaki, and M. D. Jacobson.** Programmed cell death and the control of cell survival. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 345: 265–268, 1994.
28. **Roy, R. R., K. M. Baldwin, and V. R. Edgerton.** The plasticity of skeletal muscle: effects of neuromuscular activity. *Exerc. Sport Sci. Rev.* 19: 269–312, 1991.
29. **Schiaffino, S., S. P. Bormioli, and M. Aloisi.** The fate of newly formed satellite cells during compensatory hypertrophy. *Virchows Arch.* 21: 113–118, 1976.
30. **Schultz, E., K. C. Darr, and A. Macius.** Acute effects of hindlimb unweighting on satellite cells of growing skeletal muscle. *J. Appl. Physiol.* 76: 266–270, 1994.
31. **Snow, M.** Satellite cell response in rat soleus muscle undergoing hypertrophy due to surgical ablation of synergists. *Anat. Rec.* 227: 437–446, 1990.
32. **St. Pierre, B. A., and J. G. Tidball.** Macrophage activation and muscle remodeling at myotendinous junctions after modifications in muscle loading. *Am. J. Pathol.* 145: 1463–1471, 1994.
33. **Thomason, D. B., P. R. Morrison, V. Oganov, E. Ilyina-Kakueva, F. W. Booth, and K. M. Baldwin.** Altered actin and myosin expression in muscle during exposure to microgravity. *J. Appl. Physiol.* 73, *Suppl.*: S90–S93, 1992.
34. **Tidball, J. G., D. E. Albrecht, B. E. Lokensgard, and M. J. Spencer.** Apoptosis precedes necrosis of dystrophin-deficient muscle. *J. Cell Sci.* 108: 2197–2204, 1995.
35. **Tidball, J. G., and B. St. Pierre.** Apoptosis of macrophages during the resolution of muscle inflammation. *J. Leukoc. Biol.* 59: 380–388, 1996.
36. **Walker, N. I., R. E. Bennett, and J. F. Kerr.** Cell death by apoptosis during involution of the lactating breast in mice and rats. *Am. J. Anat.* 185: 19–32, 1989.
37. **Wyllie, A. H., J. F. Kerr, and A. R. Currie.** Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68: 251–306, 1980.