Myogenic regulatory factors during regeneration of skeletal muscle in young, adult, and old rats

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IN EXPERIMENTAL MODELS of muscle injury, the nature of the muscle damage dictates the extent to which revascularization, phagocytosis, reinnervation, and myogenesis must occur to regenerate the muscle (34). With the exception of phagocytosis of muscle fiber debris, the cellular and molecular events that occur after intramuscular injection of bupivacaine are similar to what must occur during embryonic myogenesis. Bupivacaine injection causes a dissolution of the sarcolemma that leads to rapid muscle fiber necrosis but does not affect basal lamina, satellite cells, intramuscular nerves, and blood vessels (16). The subsequent recapitulation of myogenesis can be used to examine the regenerative capacity of skeletal muscle in young, adult, and old rats.

The extrinsic exposure of skeletal muscle to growth factors is altered during the lifetime of an animal. This change could affect its ability to regenerate during aging (4). For example, in the embryo and in the young rat, hormones and growth factors favor muscle growth (reviewed in Refs. 12 and 14); in the adult, many of these endocrine and growth factors are downregulated (17) because only maintenance of mature muscle cells is required. Skeletal muscle atrophy with senescence is evidence of an alteration in the extracellular and/or intracellular milieu that has diminished the capacity of the muscle to maintain expression of neuromuscular proteins. This may be because of an intrinsic program, but significant modifications of extrinsic factors acting on the muscle, such as voluntary activity patterns, thyroid hormone levels, growth hormone levels, and axoplasmic transport, cannot be discounted.

In young rats injected with bupivacaine, satellite cells proliferate, differentiate, and fuse to form myotubes so that recovery of muscle mass and force is complete within 21 days (29, 30). Differentiation of myogenic cells, during embryogenesis and in muscle culture experiments, has been shown to be regulated by genes, the protein products of which have basic helix-loop-helix sequences that bind to DNA. This family of myogenic factors, which includes MyoD, myogenin, Myf-5, and MRF4 (myf-6, herculin), forms dimers with ubiquitous proteins such as E12 or E47, resulting in heterodimeric complexes that bind to the E-box consensus DNA sequence (5'-CANNTG-3') that is found in the regulatory region of many muscle-specific genes (24).

Increased expression of the above-mentioned myogenic factor genes has been demonstrated in denervated muscles (2, 9), in hindlimb muscle of aged mice (25), and in regenerating skeletal muscle of young rats 2-3 mo of age (13, 15, 28). MRF4 mRNA has recently been shown to decrease in the soleus muscle of immobilized limbs, whereas the level of myogenin mRNA was unaltered (21). Therefore, conditions in which muscle atrophy is prevalent (denervation, aging, limb immobilization) are not always associated with increased expression of myogenic factor mRNAs.

Impaired regeneration of skeletal muscle in old animals has been reported after ischemic necrosis (33), bupivacaine injection (4, 32), and transplantation of muscle grafts (3). A deficiency in reinnervation has been proposed to account for the poor regeneration of autografted muscle observed in aged rats (4). Bupivacaine injection into muscles of young, adult, and aged rats tests the ability of the muscle to reactivate myogenic processes, regenerate muscle fibers, and synthesize muscle proteins. An incomplete recovery of muscle protein in muscle of old rats (22) suggests that, in addition to a possible deficiency in reinnervation, myogenesis is also impaired with aging. A prolonged expression of insulin-like growth factor I (IGF-I) mRNA has been observed in regenerating muscles of adult and old rats compared with that of young rats (22). IGF-I induces myogenin mRNA expression in differentiating muscle cells (11); therefore, elevated expression of myogenic factor mRNAs may also persist in muscles of adult and old rats during regeneration. The myogenin gene has been shown to be involved in the control of muscle cell differentiation (8) and regulation of muscle-specific gene expression (1). Thus it is possible that defective regulation of myogenin mRNA in regenerat-
ing muscles of old rats might be associated with impaired regeneration.

The purpose of the present study was to examine the effect of aging on myogenic factor mRNA expression during muscle regeneration. Muscle regeneration was examined in Fischer 344/Brown Norway F1 hybrid male rats of appropriate ages to obtain muscle in the growth phase (3 mo old), maintenance phase (18 mo old), and atrophic phase (31 mo old). We hypothesized that MyoD, myogenin, and MRF4 mRNA expression would be elevated in regenerating muscles but that the increase would be less in regenerating muscles of old rats compared with that in young rats, relative to contralateral controls. Furthermore, we hypothesized that, like IGF-I mRNA expression, the increased expression of myogenic factor mRNAs would be prolonged in regenerating muscles of adult and old rats compared with young rats. We expected myogenic factor mRNA expression, in regenerating muscles of young rats, to return to control levels within 15 days, which coincides with the period of rapid muscle protein accretion. The present study is a part of our long-term purpose: to use this model of muscle regeneration to gain more insight into the mechanism of muscle atrophy that occurs with aging.

**MATERIALS AND METHODS**

Animals. Specific pathogen-free Fischer 344/Brown Norway F1 hybrid male rats, aged 3 mo (young), 18 mo (adult) and 31 mo (old) of age (n = 20 animals/group), were obtained from the National Institutes of Health Aging Program (Harlan, Indianapolis, IN). Animals were housed two per microisolator cage and maintained in a temperature (21°C)- and light (12:12-h light-dark cycle)-controlled, positive-pressure room. All food, water, and bedding were sterilized by autoclave. All animal protocols were approved by the Institutional Animal Welfare Committee, University of Texas Health Science Center.

Bupivacaine injection. Animals were anesthetized with an injection (0.84 ml/kg ip) containing ketamine (54 mg/ml), xylazine (2.2 mg/ml), and acepromazine (3.5 mg/ml). An incision was made in the skin overlying the distal portion of the tibialis anterior muscle, with care taken not to penetrate the fascia. A 25-gauge, 5/8 (0.5 × 16-mm) needle was inserted along the longitudinal axis of the muscle, and 0.5 ml of 0.75% bupivacaine (Marcaine HCl, Winthrop Pharmaceuticals) was injected slowly while the needle was being withdrawn. The injection was repeated once more with an additional 0.5 ml ~3 mm away at a 15° angle from the original injection, and then the skin was sutured with 4.0 prolene. Varying the injected volume of bupivacaine from 0.5 to 1.5 ml destroyed a similar percentage of muscle fibers (50–70%; data not shown). When the rats had recovered from the anesthetic, they were returned to their cages.

Muscle sampling. After 5, 14, 21, 28, or 56 days of bupivacaine injection (n = 5/day/age group), rats were anesthetized with an intramuscular injection (1.4 ml/kg) of a mixture containing ketamine, xylazine, and acepromazine as described earlier. The entire tibialis anterior muscle was removed from each limb and immediately frozen with liquid nitrogen-cooled Wollenberger tongs. Muscle samples were kept at −80°C until further analysis. Rats were euthanized by cervical dislocation while under anesthesia.

Total RNA isolation. Entire tibialis anterior muscles were powdered under liquid nitrogen with a mortar and pestle. Total RNA was extracted from an aliquot of 150–300 mg of powdered muscle by using the guanidinium thiocyanate method of Chomczynski and Sacchi (6) with Trisolve (Biotech Laboratories). The extracted RNA was dissolved in diethylpyrocarbonate-treated water and quantified spectrophotometrically at 260-nm wavelength. The integrity and concentration of the RNA were confirmed by visual inspection of ethidium bromide-stained 18S and 28S ribosomal (r)RNAs before use of the total RNA for Northern blots.

mRNA determination. Northern blot analysis was used to assess the relative abundance of myogenin, MyoD, MRF4, and 18S mRNAs in contralateral control and bupivacaine-injected muscles and in control tibialis anterior muscles. Extracted RNA (15 µg) for each muscle was loaded onto a denaturing 1% agarose gel [1 × 3-(N-morpholino)propanesulfonic acid and 6.7% formaldehyde] and electrophoresed at 4 V/cm for 3 h. The RNA was then transferred to a nylon membrane (Hybond-N+, Amersham) by capillary action and ultraviolet cross-linked to the membrane when transfer was complete. A rat myogenin cDNA probe was prepared by random priming the full-length cDNA cut from Bluescript plasmid (37). Similarly, probes for the full-length cDNAs for MyoD and MRF4 were prepared from EMSV plasmids generously provided by Eric Olson. The RNA-containing membrane was prehybridized with 12 ml hybridization buffer (QuickHyb, Stratagene) for 40 min at 68°C. One of the myogenin, MyoD, or MRF4 probes (≥3 × 107 counts/min (cpm); sp. act. >1 × 108 cpm/µg DNA) was mixed with the hybridization buffer and incubated for 2 h at 68°C. The membrane was washed two times with 2× sodium chloride-sodium citrate (SSC) with 0.1% sodium dodecyl sulfate (SDS; 22°C, 15 min) and once with 0.1× SSC with 0.1% SDS (55°C, 30 min) (5). The membrane was then visualized autoradiographically after an exposure time of 1 h (18S), 48 h (MRF4), or 65 h (MyoD, myogenin) with one intensifying screen at −80°C, and the bands corresponding to 18S, myogenin, MyoD, and MRF4 mRNAs were quantified by densitometry scanning (BioImage, Millipore) as integrated optical density (IOD). The IOD values of 18S mRNA were used to correct loading efficiencies for myogenin, MyoD, and MRF4 mRNAs. Data are expressed per milligram muscle RNA loaded into gel.

Statistics. An analysis of variance was used to determine whether significant differences occurred at the P < 0.05 level for body weights, muscle mass, and myogenin, MyoD, and MRF4 mRNAs among control young, adult, and old rat muscles that received no bupivacaine injections. A multifactorial analysis of variance (age, bupivacaine treatment, and recovery day) was used to determine whether significant differences occurred at the P ≤ 0.05 level for muscle mass and mRNAs for myogenin, MyoD, and MRF4 among young, adult, and old tibialis anterior muscles after bupivacaine injection. When significant interaction of main effects occurred, Newman-Keuls post hoc tests were used to assess significance at P < 0.05.

**RESULTS**

Muscle mass. The tibialis anterior muscle of adult control rats (0.81 ± 0.02 g) was significantly greater in mass than that of young (0.58 ± 0.02 g) or old rats (0.48 ± 0.03 g), demonstrating muscle growth during maturation (3–18 mo) and aging-associated muscle atrophy (18–31 mo). Atrophy in the gastrocnemius
muscle (adult: 1.869 ± 0.030 g; old: 1.062 ± 0.043 g) was similar to that in the tibialis anterior despite their different physiological functions. Muscle mass was decreased significantly in young, adult, and old rats by the day 14 postbupivacaine injection (Fig. 1). At this time, the bupivacaine-injected tibialis anterior muscle of young rats was 77% of its contralateral control and, by 21 days postinjection, mass was not different from control mass (Fig. 1). Adult and old muscle mass were only 60 and 68% of their respective contralateral controls by 28 days postbupivacaine (Fig. 1). Furthermore, adult rat tibialis anterior muscle mass had only recovered to 78% of contralateral control mass when recovery was extended to 56 days. Recovery of muscle mass did not differ between adult and old rats at 14, 21, or 28 days after bupivacaine injection. Regenerating muscles of young rats were significantly different, as a percentage of contralateral controls, compared with adult and old muscles at each time point of recovery (Fig. 1). These observations confirm findings in the Fisher 344 strain (22).

Myogenin, MyoD, and MRF4 mRNAs. Myogenin, MyoD, and MRF4 mRNA expression was similar between the uninjected tibialis anterior muscles (control) of young and adult rats. In contrast, expression of myogenin, MyoD, and MRF4 mRNAs was greater in the control muscle of old rats compared with that of adult and young rats (Figs. 2–4). The greatest relative increase occurred for myogenin mRNA, which was 1,090% greater in old control muscle, whereas MyoD and MRF4 mRNAs were increased by 187 and 66%, respectively, relative to young control muscle.

During muscle regeneration, expression of myogenin, MyoD, and MRF4 mRNAs was altered in young, adult, and old rat muscles recovering from bupivacaine injection. In young rats, myogenin mRNA expression was dramatically increased (18-fold relative to control) at 5 days of recovery, attenuated by 14 days (2.7 fold), and then returned to its respective control levels by 21 days (Fig. 2). The temporal expression of myogenin mRNA in regenerating muscles of adult and old rats differed from that of young rats. In adult rat muscle, myogenin mRNA expression was dramatically increased (13-fold relative to control) at 5 and 14 days of regeneration. At 28 days postbupivacaine injection, myogenin mRNA levels in adult rat muscle remained fourfold greater than its age-group control (Fig. 2). In contrast to young (18-fold) and adult (13-fold) rat muscle, myogenin mRNA expression in old rat muscle

![Fig. 1. Time course of recovery of muscle mass (wet wt) of tibialis anterior muscle of young (3 mo of age; ○), adult (18 mo of age; ■), and old (31 mo of age; ▲) Fischer 344/Brown Norway F1 rats after damage produced by an intramuscular injection of bupivacaine. Values are means ± SE; n = 15 rats/age group, 5 rats/day. Data are presented as a percentage of contralateral control muscle. Absolute values for each control are presented in RESULTS. Contralateral control values for each point of recovery in an age group are averaged, and mean ± SE is shown as day 0 of recovery. *Significantly different from adult and old rats, P < 0.05.

![Fig. 2. Myogenin mRNA in tibialis anterior muscles of rats as described in Fig. 1. A: representative Northern blots of myogenin and 18S rRNA from young, adult, and old rat tibialis anterior muscle. Each lane was loaded with 15 µg of total RNA extracted from muscle 5 days postbupivacaine injection (regenerating) and paired with its contralateral control. Migration position of each band of hybridized probe is marked relative to migration of 18S ribosomal (r)RNA. E18, embryonic day 18; YC, young control; YR, young regenerating; AC, adult control; AR, adult regenerating; OC, old control; and OR, old regenerating. B: integrated optical density (IOD) of probe hybridized to 15 µg of total RNA and expressed per milligram muscle mass. Values are means ± SE; n = 20 rats/age group, 5 rats/day. Contralateral control values for each point of recovery in an age group are averaged, and mean ± SE is shown as day 0 of recovery. E18, single observation for rat hindlimb muscles pooled from embryonic day 18. *Significantly different from respective age-group control values, P < 0.05. **Significantly different from young control values, P < 0.05.](http://jap.physiology.org/Downloadedfrom10.22033.3onOctober15,2017)
was only increased 2.5-fold at 5 days and remained elevated 1.6- to 2-fold above its age-group control at 14 and 21 days postbupivacaine injection. Myogenin mRNA levels in old regenerating muscle was not different from its respective control by 28 days of recovery (Fig. 2).

MyoD mRNA expression in regenerating muscles of young rats was significantly elevated (3-fold) relative to control muscle at 5 days postbupivacaine injection (Fig. 3). At all subsequent time points in young rats, MyoD mRNA levels were similar to their age-matched control muscle. The temporal expression of MyoD mRNA in regenerating muscles of adult rats was similar to that of young rats. In contrast, MyoD mRNA expression in regenerating muscles of old rats was elevated two- to threefold above its age-group control at 5, 14, 21, and 28 days postbupivacaine injection (Fig. 3).

The temporal expression of MRF4 mRNA during regeneration differed from that of myogenin and MyoD. In regenerating muscles of young rats, MRF4 mRNA levels were significantly decreased by 46% at day 5 when the expression of myogenin and MyoD mRNAs was maximal, significantly increased by 239% at day 14, coincident with decreased expression of myogenin and MyoD mRNAs, presumably once myotubes have been formed, and similar to control values by day 21 of recovery (Fig. 4). In contrast to expression of myogenin and MyoD mRNAs, there was no difference in the temporal pattern of MRF4 mRNA expression among regenerating muscles of young, adult, or old rats. It has been reported in a recent review that the physiological role of MRF4 remains unclear (23).

**DISCUSSION**

The novel finding in this study was that myogenin and MyoD mRNAs remained upregulated in bupivacaine-injected muscles of old rats compared with those of young and adult rats. Although not mutually exclusive, the expression of MyoD mRNA has been related to proliferation of satellite cells, whereas myogenin mRNA expression has been related to muscle differentiation and expression of muscle-specific proteins (26). Thus it is paradoxical that the mRNA expression of MyoD and myogenin remains elevated during impaired regenera-
tion. The persistent elevation of myogenic factor mRNA levels may reflect a continued attempt to form new muscle cells. Negative feedback, which in regenerating muscles of the young rat downregulates IGF-I (22), MyoD, and myogenin mRNA (Figs. 2 and 3) levels to control values by day 14 to day 15 of recovery, does not seem to be present or is ineffective in regulating these mRNA levels in regenerating muscles of old rats. The IGF-I, MyoD, and myogenin gene products have been demonstrated to be critical for muscle cell determination, differentiation, and activation of muscle-specific gene expression (5, 20, 23, 26). However, there are many sites of protein regulation beyond mRNA levels, e.g., mRNA stability, translation efficiency, phosphorylation, and signal transduction targets, all of which may be modified within the aged muscle.

An alternative explanation is that a denervation-like phenomenon is responsible for the elevated MyoD and myogenin mRNAs. MyoD and myogenin mRNA levels are higher in embryonic muscle before its innervation and then are reexpressed at high levels in denervated adult muscles (9, 35). Therefore, it is possible that their prolonged expression during regeneration of old muscle could be related to incomplete reinnervation. Terminal nerve branches retract from the neuromuscular junction immediately after bupivacaine injection (32a), so a failure to reinnervate is possible. We have previously demonstrated that IGF-II mRNA levels are elevated at day 10 and are diminish in denervated muscles of young, adult, and old rats (22). Elevated levels of IGF-II mRNA after peripheral nerve damage return to control values once the nerve regrows and connects back to the muscle (27), which indirectly supports a successful reinnervation in regenerating muscles of old rats.

Limb muscles have been reported to regenerate less well in old compared with young mammals (3, 4, 32, 38). However, recovery from bupivacaine varies between dorsiflexor muscles of older animals. The extensor digitorum longus (EDL) muscle recovers better from bupivacaine-induced damage than the tibialis anterior muscle, even though they have similar functions and are composed of predominantly fast-twitch fiber types. The bupivacaine-injected tibialis anterior of 18 mo-old rats had not recovered muscle wet weight by 56 days (Fig. 1), whereas the EDL had recovered its muscle wet weight by 28 days (data not shown). This concurs with previous reports of complete recovery of EDL muscle wet weight and force at 60 days in young and old rats (4) and with unsuccessful regeneration of muscle fibers in the tibialis anterior of old rats after bupivacaine injection (Ref. 32; see also RESULTS). Inherent differences likely exist between the tibialis anterior and the EDL muscles to account for this discrepancy in regenerative capacity. One potential explanation for this difference could be the success of reinnervation (4). Although axonal regeneration does not need to occur in bupivacaine-injected muscles, neuromuscular synapses must be reformed between the retracted terminal nerves and the myotubes regenerating within the basal laminae (32a). The reformation of synapses in bupivacaine-injected EDL muscles may be superior to that occurring in the tibialis anterior.

During embryonic development of muscle, expression of myogenin and MyoD mRNA is elevated. Myogenin and MyoD mRNA levels then decrease from embryonic day 19 to an almost undetectable signal in muscles of young rats (3) and mice (2, 7, 9). In contrast, MRF4 mRNA expression increases postnatally until it is the dominantly expressed myogenic factor in adult muscle (2, 7, 9). Myogenin and MyoD mRNA levels were thought to remain low throughout adulthood; however, myogenin and MyoD mRNA levels in old mouse muscle are increased to levels similar to those in the newborn (25). Our present results confirm elevated myogenin and MyoD mRNA levels with aging, but we also observed an increased MRF4 mRNA expression in old rat muscle. Thus both embryonic myogenesis and senescence of skeletal muscle are associated with an increased expression of mRNAs for myogenin and MyoD. A loss of motoneurons occurs with aging (18) and results in denervated muscle fibers in old muscle. Denervation and/or electrical inactivity increases myogenin, MyoD, and MRF4 mRNA expression in skeletal muscle (9, 35), whereas electrical stimulation of denervated muscle represses myogenin and MyoD mRNA expression (9). Therefore, it is possible that alterations at the neuromuscular junction and/or the physical inactivity of old rats could be causative of the increased myogenin, MyoD, and MRF4 mRNA levels in muscles of old rats.

In summary, myogenin, MyoD, and MRF4 mRNAs demonstrated distinct patterns of expression during regeneration of skeletal muscle after bupivacaine injection. Aging had a pronounced effect on the temporal pattern of myogenin and MyoD mRNA expression but had no effect on MRF4 mRNA expression during regeneration. The prolonged elevation of myogenin and MyoD mRNA expression in regenerating muscles of old rats is a novel finding. Regeneration of growing muscle in young rats was accompanied by transient elevations of myogenin and MyoD mRNA expression and complete recovery of muscle mass. Regeneration of nongrowing and atrophic muscle in adult and old rats, respectively, was accompanied by a prolonged elevation of myogenin and MyoD (old rats only) mRNA expression and incomplete recovery of muscle mass. Thus the regenerative capacity of skeletal muscle is reduced with maturation concomitant with alterations in the regulation of myogenin mRNA, whereas alterations of MyoD mRNA regulation occur with senescence. The results of the present study demonstrate that a deficiency in myogenesis is at least partly accountable for the poor regeneration of the tibialis anterior muscle observed in old rats. Furthermore, elevated MyoD and myogenin mRNA levels in old control muscle may reflect a continued attempt to generate new muscle fibers and ameliorate muscle atrophy.

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