Muscle Regeneration in Young and Old Rats: Effects of Motor Nerve Transection With and Without Marcaine Treatment

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We tested the hypothesis that after skeletal muscle regeneration in old compared with young rats damage to the motor nerve rather than damage to muscle fibers determines the magnitude of the deficits in muscle mass and maximum force (P0). The mass and P0 of extensor digitorum longus (EDL) muscles of young (4 months) and old (24 months) male rats were compared two months following (i) Marcaine treatment plus simultaneous motor nerve transection, (ii) motor nerve transection alone, and (iii) Marcaine treatment alone (from data compiled previously). In both the nerve transection-only and Marcaine with nerve transection groups the recovery of mass and P0 was significantly greater in young than in old rats. This is in contrast to our previous data showing that in the absence of nerve damage Marcaine-treated muscle in old rats regenerates as well as that in young rats. Our hypothesis was supported, and we conclude that impaired axonal regeneration, re-establishment of nerve-muscle contact, or both, is the critical component in the impaired regeneration of muscle grafts in old as compared with young rats.

A NUMBER of studies have reported that skeletal muscle regenerates more poorly in old than in young animals (1–5). The reason for the age-related decline in the success of regeneration is not understood, but factors that could be involved are as diverse as a decline in satellite cell populations or proliferative potential (6), altered macrophage function (5), increased connective tissue (3,7), deficient reinnervation (1,8), dietary influences, or decreased activity patterns of the old animals.

A fundamental question is whether an intrinsic difference in the regenerative capacity between old and young muscles is a primary factor or whether the environment in which the muscle regenerates plays an important role. This was investigated by a cross-age muscle transplantation model, in which 24-month-old extensor digitorum longus (EDL) muscles were grafted into legs of young (4-month-old) inbred rats and young muscles were grafted into legs of old host rats (1). After grafting, most of the muscle fibers degenerated and subsequently regenerated. In mature cross-age grafts, muscles grafted from old into young rats (old-into-young muscle grafts) had regenerated as well as young-into-young grafts in the same hosts, whereas young-into-old muscle grafts regenerated no better than old-into-old autografts. From this experiment we concluded that the primary determinant of the age-related success of muscle regeneration is the environment in which the muscle is regenerating and that muscles from old rats can regenerate as well as muscles from young rats if provided with a suitable environment.

A subsequent experiment was performed as a first attempt to evaluate the role of the motor innervation as an environmental factor affecting the success of muscle regeneration in young and old rats. Carlson and Faulkner (8) examined the functional recovery of regenerating EDL muscles in two groups of rats. In the first group, EDL muscles were autografted in young (4-month-old) and old (24-month-old) rats under conditions of local denervation. After 21 days, during which the early stages of muscle regeneration had taken place, there was no difference in the maximum tetanic force developed by non-innervated muscle grafts in young and old rats. This result suggested that at the whole muscle level there were no major intrinsic early differences between muscles regenerating in old and young rats. A second experimental group was designed to examine muscle regeneration in the presence of full innervation. This was accomplished by injecting EDL muscles in young and old rats with the myotoxic anesthetic, Marcaine (9,10). Marcaine causes minimal damage to motor nerves (11,12). Sixty days after Marcaine injection, muscle fibers had regenerated and muscle mass and force development were stable (13). At 60 days, no differences were observed in the return of maximum tetanic force in the regenerated EDL muscles between old and young rats. This experiment showed that in the presence of intact innervation significant differences in muscle regeneration between old and young rats were not demonstrable and suggested indirectly that reinnervation of damaged muscle may be an important factor accounting for age-related differences in muscle regeneration.

The present experiments were designed to follow up the Marcaine experiment (8) as a direct test of the hypothesis that nerve damage and subsequent axonal regeneration are important determinants of the age-related success of muscle fiber regeneration. We hypothesized that following transection and regeneration of the motor nerve into the denervated EDL muscle, the recovery of mass, maximum force, and maximum specific force of EDL muscles of old compared with young rats is impaired equally, whether the
muscle fibers degenerate (as a result of Marcaine injections) or survive and remain intact.

Materials and Methods

Experimental animals and design. — This study was carried out on 31 male rats of the W/LikHicksCar strain (a highly inbred substrain of Wistar rat colony maintained by first author). Young animals were 4 months old and old animals were 24 months old at the time of the original operation. During the aging process, the old rats were maintained under specific pathogen-free conditions at Harlan-Sprague-Dawley, Inc. (Indianapolis). This study consisted of two experiments (i) Marcaine injection of the EDL muscle accompanied by simultaneous motor nerve transection and (ii) transection of the motor nerve in the absence of direct muscle damage. In both cases, reinnervation of the muscles depended upon spontaneous axonal regeneration. In all rats, the contralateral EDL muscles served as controls.

All operations were carried out with the rats under ether anesthesia. The operations and subsequent animal care were carried out in accordance with the guidelines of the University Committee on the Use and Care of Animals at the University of Michigan. After all operations, the rats were placed on oral terramycin for 5 days. At the termination of the experiment, the rats were killed by an overdose of the anesthetic.

Marcaine injection accompanied by motor nerve transection. — For experiments involving Marcaine injections, 8 young and 10 old rats were anesthetized, and the right EDL muscles were surgically exposed. To produce maximum muscle damage, the muscles were injected with as much bupivacaine (Marcaine, Winthrop, New York) as they could hold (usually about 125 μl) through a one-inch, 27-gauge needle. Marcaine injection causes severe muscle damage [90% reduction in maximum tetanic force 2 days after injection (8)] without interrupting the nerve fibers (11,12) or the blood supply (14,15). Although the muscle fibers degenerate, the tendons remain intact. Immediately after Marcaine injection the small motor nerve branches to the EDL muscle were transected in a manner that preserved the accompanying connective tissue and vasculature. This prevented retraction of the proximal nerve stumps and reduced interanimal variation in reinnervation over that seen with complete resection of the neurovascular bundle. Two months later the Marcaine-injected nerve-transected and the contralateral control EDL muscles were removed and contractile properties were determined.

Motor nerve transection only. — In a second series, the motor nerves to the right EDL muscles of five young and eight old rats were transected in the same manner as in the previous experiment. In this experiment, however, the muscles were not injected with Marcaine. Two months postoperatively the experimental and contralateral control EDL muscles were removed and subjected to the same analysis of contractile properties as the muscles of the previous series.

Physiological analysis. — All control and experimental EDL muscles were exposed and dissected free of other tissues. Sutures were placed around the distal and proximal tendons, and the tendons were subsequently severed. The muscle to be tested was then removed from the rat and secured at resting length in a tissue bath with a Krebs-Ringer bicarbonate solution containing 137 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, 24 mM NaHCO₃, and 0.25 mM tubocurarine chloride. The solution was maintained at 25 ± 0.2 °C and gassed with 95% oxygen and 5% carbon dioxide. One tendon of the muscle was tied to a fixed post and the other end to a Cambridge Technology (Cambridge, MA) servomotor/force transducer (model 305). Platinum electrodes were immersed in the bath on either side of the muscle. Muscles were stimulated by the current flow between the two electrodes produced by square wave pulses .2 ms in duration. The voltage was adjusted slightly greater than that necessary to produce a maximum isometric twitch contraction. This has been shown to produce a maximum isometric twitch contraction. This was completed, most muscles were frozen by immersion in a mixture of dry ice and isopentane. These were then cross-sectioned at 8 μm through the plane of the middle of the muscle. The sections were then stained with hematoxylin and eosin. The remainder of the muscles were fixed in 4% paraformaldehyde and embedded in glycol methacylate (Bio-Rad Polaron, Cambridge, MA). Sections cut on a glass knife were then stained in a mixture of Gill's hematoxylin and eosin-phloxine.

Statistical analysis. — For each of the four variables measured on each of the experimental and control groups, means and standard errors were determined. Differences between the body masses of each of the two treatment groups were determined by Student's t tests. Differences between the muscle masses and values for P₀ and specific P₀ of each of the two treatment groups and their respective control EDL muscles and between variables measured on EDL muscles of young and old rats in the two treatment groups were determined separately by a 2 × 2 ANOVA. The method of least squares was used to fit a general linear model (GLM). The GLM method computes the analysis of variance for an unbalanced design of two groups and two conditions, even with unequal numbers

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of observations in groups. An assumption for this test is that the independent group variables are exclusive, which they were in our study. Our dependent variables were continuous scale, which also meets the requirement for this statistical test. A GLM repeated measures option is appropriate when the dependent variable reflects the outcome of the two measurements on the same animals. Consequently, the primary hypothesis that age-related effects exist in the restoration of muscles following recovery after nerve transection was assessed through a $2 \times 2$ ANOVA of the differences between repeated measures. The repeated measure on each variable consisted of data obtained on the treated and control EDL muscles of the same rat in one of the two treatment groups and one of the two young or old age groups. This analysis permitted the assessment of whether these differences were significant between the EDL muscles from the old compared with young rats and between the EDL muscles treated with nerve transection compared with those treated with Marcaine plus nerve transection. Significance for each statistical analysis was set a priori at $p \leq .05$. The result was then compared with our previous observation that when motor innervation remained intact during Marcaine-induced regeneration of EDL muscles, no difference was observed in the muscle mass and $P_o$ of muscles from young and old rats.

Results

For each of the two treatment groups, the body masses of the old rats were greater (117%) than those of the young rats, which simply indicates that the young rats had not yet reached the full maturity of growth. Despite the greater body masses of the old rats in each group, the EDL muscle masses of the old rats were smaller than those of the young rats, and the muscles of the old rats developed lower $P_o$s. These observations are in good agreement with previous comparisons of EDL muscles in young and old rats (1,8,17).

Marcaine treatment accompanied by motor nerve transection. — Based on the $2 \times 2$ ANOVAs for the Marcaine-treated nerve-transacted group, the age effect was significant for the muscle mass and $P_o$ of the old compared with the young rats (Table 1). As with the Marcaine-treated nerve-transacted group, the $P_o$ and the specific $P_o$ of nerve-transacted muscles in old rats were lower than the values for control EDL muscles and for the old rats the mass of nerve-transacted muscles was lower than that of the control muscles.

In young animals, the histological structure of the EDL muscles temporarily denervated by nerve transection returned to an essentially normal condition by 60 days (Figure 2A). On the other hand, EDL muscles in old rats displayed large fields of atrophic muscle fibers (Figure 2B). Muscles subjected to nerve transection alone, whether in old or young rats, only rarely contained central-nucleated muscle fibers. This was a major difference from the Marcaine-treated nerve-transacted groups, in which frequent central nucleiation (Figure 1) reflected cycles of Marcaine-induced muscle fiber degeneration followed by regeneration.

Comparisons among groups with experimental/control data expressed as percentages. — The hypothesis that following transection and regeneration of the motor nerve into the EDL muscle, the recovery of the muscle mass, maximum force, and maximum specific force of EDL muscles of old compared with young rats is impaired equally whether the

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<th>Old</th>
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<tbody>
<tr>
<td></td>
<td>No. of Rats</td>
<td>Muscle Mass (mg)</td>
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<tr>
<td>Control</td>
<td>8</td>
<td>178 ± 4</td>
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<td>Marcaine + nerve transection</td>
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<td>Control</td>
<td>5</td>
<td>180 ± 5</td>
</tr>
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<td>Nerve transection only</td>
<td>5</td>
<td>170 ± 7</td>
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$^a$ The value for old rats significantly is different ($p \leq .05$) from the value for young rats.

$^b$ The value for the variable in the treated muscle is different ($p \leq .05$) from the value for the variable in the control muscle.
Figure 1. (A) Cross-section of the EDL muscle of a young rat two months after Marcaine injection and simultaneous transection of the motor nerve. Most of the muscle fibers contain central nuclei. In the total cross-section of the muscle one fascicle (outlined by dashed line) contains muscle fibers whose diameter is smaller than the others. (B) Cross-section of the EDL muscle of an old rat two months after Marcaine injection and simultaneous transection of the motor nerve. Among the fascicles, there is considerable variability in the diameters of the muscle fibers, including highly atrophic fibers. The maximum size of muscle fibers is considerably less than that in the young muscle depicted in (A). Hematoxylin and eosin stain of frozen sections. Bar = 100 μm.

Figure 2. (A) Cross-section through the EDL muscle of a young rat two months after transection of the motor nerve. The temporarily denervated muscle fibers have become restored to normal size and, in the absence of regeneration, they do not contain central nuclei. (B) Cross-section through the EDL muscle of an old rat two months after transection of the motor nerve. Large regions of atrophic muscle fibers alternate with fascicles containing near-normal sized muscle fibers. Hematoxylin and eosin stain of frozen sections. Bar = 100 μm.

muscle fibers degenerate (as a result of Marcaine injections) or survive and remain intact, was tested by 2 x 2 ANOVAs for repeated measures (see Statistical Analyses). The analysis indicated a significant age effect for muscle mass and for $P_o$, but not for specific $P_e$ (Table 1). Our hypothesis was supported since a significant age effect was coupled with no differences in muscle masses or force development between the two treatments for the same age groups. The lack of any interaction between the age of the rats and the treatment group for muscle mass ($p = .273$) and for $P_o$ ($p = .625$) provides an unambiguous test of the hypothesis.

The age-associated difference in recovery is most evident when the data on the experimental muscles are expressed as a percentage of data from control muscles. In old rats, the mean mass of the Marcaine-treated nerve-transected muscle was restored to only 63% of the control value, whereas in young rats a recovery of 97% of control mass was observed. Similarly, the $P_o$ of Marcaine-treated nerve-transected muscles in old rats returned to 40% of control values, as compared with a 66% return in young rats (Table 2). The nerve-transection experiment was designed as a follow-up experiment to Marcaine treatment associated with nerve transection to identify the effects of nerve transection alone. The results demonstrate that in the absence of direct muscle fiber damage, the mean muscle mass of the temporarily denervated muscles in old rats returned to only 70% of control values after 60 days, whereas in young rats the mean mass returned to 94% of control values (Table 2). The return of $P_o$ as a percentage of the contralateral control was significantly lower in old (48%) than in young (84%) rats. In neither control muscles nor in muscles of either experimental muscle group did specific $P_e$ differ between old and young rats.

In contrast to the dramatic impairments in muscle mass and $P_o$ after nerve transection, either with or without Marcaine treatment, data published previously (8) showed no difference in EDL muscles between young and old rats 60 days following Marcaine treatment alone (Table 2).

**DISCUSSION**

These experiments were part of a series of studies designed to test the role of nerve transection concomitant with muscle damage as a factor accounting for the age-related differences in the success of muscle regeneration between young and old animals. Models of muscle regeneration which reported poorer regeneration in old than in young animals typically involved grafting (1,2) or mechanical trauma in which motor
nerve damage was inevitable (3,5). Cross-age muscle grafting showed that the success of regeneration of muscles from either old or young donors was dependent upon the age of the host into which the muscle was transplanted (1). Thus, muscle from old donors regenerated well in young hosts, but muscle from young donors regenerated poorly when placed in old hosts. Although this experiment demonstrated that muscle from old rats has the intrinsic capacity to regenerate well if placed in a favorable environment, it did not allow identification of the specific environmental factor(s) that accounted for the good regeneration in young rats and the poor regeneration in old rats.

An advance in our understanding of the basis for the poor muscle regeneration that had been reported in old rats occurred when Carlson and Faulkner (8) found that: (i) after Marcaine-induced damage to muscle fibers alone in the presence of an intact vasculature and innervation, muscles in old rats regenerated as well as those in young; and (ii) in the absence of innervation, differences in Po between young and old rats were not seen in early non-innervated muscle grafts. Both the Marcaine injection (with nerves intact) experiment and the result of cross-age grafting of EDL muscles from old into young rats have shown that under favorable environmental conditions muscles in or from old rats can regenerate as well as muscles in young rats (1,8).

The finding that Marcaine-treated muscles with nerves intact regenerated as effectively in old as in young rats allowed a considerable narrowing of options that could account for the poor muscle regeneration that has been frequently reported in old animals. General systemic factors, such as diet and exercise level, which have often been considered to be important variables underlying poor function of a number of systems in old age (18–20), are unlikely to influence muscle fiber regeneration directly because successful Marcaine-induced, nerve-intact muscle fiber regeneration did occur in old rats that had not been exposed to dietary restriction or subjected to exercise. Similarly, the general systemic humoral or cellular (e.g., macrophages) environment in old rats does not seem to be detrimental to good levels of muscle fiber regeneration. Factors that could not be ruled in or out by the simple Marcaine injection model are reinnervation, revascularization or the effects of tenon transsection.

In conjunction with previous experiments involving simultaneous muscle and nerve damage, results of the nerve-transsection only model suggest that the influence of the nerve is most marked during the later stages of muscle fiber regeneration and that local muscle fiber atrophy due to incomplete reinnervation of certain regions of the muscle is a significant factor accounting for the overall reduction in the functional return of regenerating muscles in old animals. Such a conclusion is supported by the histology, which shows fields of highly atrophic muscle fibers in both regenerating and simply denervated muscles (cf. Figures 1B and 2B).

The extent of the deficit in mass and force in Marcaine-treated nerve-transsected EDL muscles in old rats, although significant, was not as great as that reported in muscle grafting experiments in and between young and old animals, where almost three-fold differences between young and old have been reported (1). Because conditions are so different between regeneration in response to muscle grafting and regeneration resulting from Marcaine damage, it is not possible to compare on a strict percentage basis the relative role of the nerve in the poor regeneration of old muscle in these models. Whether or not other factors, such as tendon damage or temporary devascularization, contribute significantly to the large functional deficit seen in muscle grafts in old rats remains to be determined.

The reason for the diminished response of regenerating muscle in the presence of nerve transection in old rats is poorly understood. One option is a slower rate of axonal regrowth to the regenerating muscle fibers. Another is that regenerating axons might be prevented from making functional contacts with muscle fibers. A number of studies have demonstrated a significant reduction in the rate of axonal regeneration in old rodents (21–24). Delayed reinnervation could account for the presence of the intermediate sized muscle fibers seen in the old muscles in both of the experimental series reported here. A reduction in the number of axons reinnervating the muscle (motor unit number) is another option for deficient reinnervation in old rats. Asato et al. (25) have demonstrated a decrease in the number of motor units in EDL muscle grafts in old as compared with young rats. Still another impediment to complete reinnervation of muscles in old animals could be physical access to regions of regenerating muscle fibers in old muscles by deposits of connective tissue or other barriers. This possibility is considered less likely than others, because of the effective reinnervation of cross-transplanted old muscles in young hosts (1). It is also possible that muscle fiber regeneration might have been slower in the old rats of the Marcaine plus nerve transection groups, resulting in incomplete regeneration (and lower Po). However, in the previous Marcaine injection experiment (8) the lack of a difference between old and young regenerates at 60 days militates against a purely myogenic basis for the differences observed in the present experiment. It is possible that in old rats a delay in reinnervation could delay muscle fiber regeneration as well.

### Table 2. Comparison of Degree of Return of Mass and Po in Marcaine-Treated Nerve-Transected, Nerve-Transected Only, and Marcaine-Treated Only EDL Muscles in Young and Old Rats

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<tr>
<td></td>
<td>n</td>
<td>Mass (%)</td>
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<td>Marcaine + nerve transection</td>
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<td>Marcaine only</td>
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*Data from Carlson and Faulkner (8) with no difference between values for young and old rats.
Despite the evidence from the Marcaine injection experiments that in vivo old muscle can regenerate as effectively as young, one should not necessarily conclude that there are no differences in the process of muscle regeneration between old and young animals. McGeachie and Grounds (26) demonstrated no delay in the initiation of replication of precursor cells in damaged muscle in old as opposed to young rats, but they did find a 24-hour delay in the peak level of myogenic precursor cell proliferation. In vitro, several studies have provided evidence of an increased lag phase before the initiation of replication of old vs. young myogenic precursor cells (27,28). For even fundamental properties, such as the proliferative potential of satellite cells, young-old differences might exist and be demonstrable in vitro (6), but they may be masked in the in vivo environment, where the full potential of a cell type or system may not be required for complete regeneration.

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