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Skeletal Muscle Regeneration in Very Old Rats

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This study was undertaken to assess the regenerative capacity of skeletal muscle in rats near the end of their normal life span. Two experiments were performed. In the first, extensor digitorum longus (EDL) muscles were cross-age transplanted from 32-month-old male inbred Wistar (WI/HicksCar) rats in place of an EDL muscle in 4-month-old hosts. The other EDL muscle in the hosts was autotransplanted. After 60 days, the old-into-young muscle transplants regenerated as well as the young-into-young autotransplants. In the second experiment, EDL muscles in young adult (4 months) and old rats (32 and 34 months) of WI/HicksCar and Brown Norway (BN) were injected with a local anesthetic, bupivacaine, and allowed to regenerate for 41 days. In all cases, the masses and absolute maximum tetanic force of the regenerates equaled or exceeded those of untouched contralateral control muscles. These experiments showed that under appropriate conditions, very old muscles can regenerate to equal or exceed the contralateral control values, which in old rats are much less than those in muscles of young rats.

OVER the past decade, the substantial deterioration that occurs in the structure and function of skeletal muscles between maturity and old age has been well documented in mice (1), rats (2,3), and human beings (for a review, see Ref. 4). More recently, even more dramatic impairments have been observed near the end of the life span (5,6). At 24 months, both Wistar (WI/HicksCar) and Brown Norway (BN) male rats enter the steep slope of their survival curve (7). Similarly, 20–25% survival is at 32 months for the WI/HicksCar rats and at 34 months for BN rats. The deterioration of skeletal muscle structure and function can be measured more precisely in rodents than in human beings (1). The skeletal muscle characteristics of the 20% of oldest surviving male rats expressed as a percent of the value for adult male rats are as follows: muscle mass is ~65%, absolute maximum force is ~40%, and specific force (kilonewtons per square meter) is ~50% (2,5,6). A substantial reduction in the number of motor units, primarily of the fast-fatigable type (5,8,9), is also observed. An electron microscopic analysis has shown substantial atrophy and degeneration of muscle fibers in rats over 3 years of age (10).

Muscle regeneration is also affected by the aging process (11–13). After severe damage, such as that associated with free, whole skeletal muscle transplantation (2,3,14,15) or mechanical trauma (16–19), muscles regenerate much more poorly in old than in young rodents. In contrast, when muscle fibers alone are damaged as a result of bupivacaine injection, regeneration of the extensor digitorum longus (EDL) muscle in 2-year-old rats returns to near control levels of maximum tetanic force (20). However, when the motor nerve to the EDL muscle is damaged concomitantly with bupivacaine injection, a significant deficit in regeneration is seen in old as compared with young rats (21).

With cross-age transplantation of EDL muscles between 4-month-old and 24-month-old inbred rats, the success of muscle regeneration is related to the age of the host rather than to the age of the grafted muscle (3). EDL muscles from old rats cross-age transplanted into young hosts regenerate as well as the EDL muscles autotransplanted within young hosts, whereas EDL muscles cross-age transplanted from young into old hosts regenerate no better than muscles autotransplanted within old rats. These results demonstrate conclusively that when placed into a suitable environment, muscles from 24-month-old rats, even though the muscles are atrophied and weak (1,5,6), maintain a substantial capacity for regeneration.

The present study is focused on the regenerative capacity of EDL muscles from rats near the end of their normal life span (32–34 months of age) when 20% of cohorts are surviving. The specific question asked was whether, in the face of the massive deterioration of control muscles in these old rats, the muscles still retain the capacity for significant regeneration. Two experimental models were used. In the first, EDL muscles from premorbid inbred WI/HicksCar rats were cross-age transplanted into young adult hosts, and the success of regeneration was compared with that of EDL autografts in the same young hosts. In the second model, EDL muscles of 32-month-old WI/HicksCar rats and 34-month-old BN rats were injected with the myotoxic agent, bupivacaine (Marcaine, Winthrop, NY), to determine the success of regeneration (1) in comparison with regeneration in 4-month-old rats of the same strains and (2) in comparison with the maximum tetanic force of contralateral control muscles. The working hypothesis was that the capacity for regeneration in the muscles from rats over 32 months of age would be reduced in relation to that previously seen in young adult or 24-month-old rats.

Materials and Methods

Experimental Animals and Operative Techniques

This study consisted of two experiments, each designed to test the regenerative capacity of the old muscles. The ex-

periments were carried out on rats of two different strains. The first experiment was a cross-age transplantation study involving young (4-month-old) and old (32-month-old) male rats of the highly inbred WI/HicksCar strain of Wistar rat. In each of eight young host rats, the left EDL muscle was removed and discarded. Eight EDL muscles from four 32-month-old donor animals were then cross-age transplanted into the bed of a removed EDL muscle (for details of the transplant operation, see Ref. 3). In the contralateral leg of each young host rat, the existing EDL muscle was autotransplanted orthotopically. In both the old-into-young and young-into-young grafts, the motor nerve of the host was implanted by pulling the stump of the motor nerve completely through the belly of the transplanted muscle. Because of the advanced age and anticipated mortality of the old rats over a 2-month postoperative period, muscle grafts into old hosts were not done. As in previous cross-age transplantation experiments (3), all muscle grafts were left in place for 2 months before they were removed for physiological analysis.

Survival and pathology data on the WI/HicksCar rats have not been previously published. Figure 1 illustrates both the survival and weight characteristics of a cohort of 50 male rats of this strain, which were allowed to live a normal life span under specific pathogen-free (SPF) conditions, housed two per shoebox cage. In a pathology study on the same animals, conducted by Dr. Clarence Chrisp of the Unit for Laboratory Animal Medicine at the University of Michigan, probable causes of death were analyzed (Table 1). Additional non-lethal pathology on these animals is listed in Table 2. Survival curves for male BN rats have already been published (7).

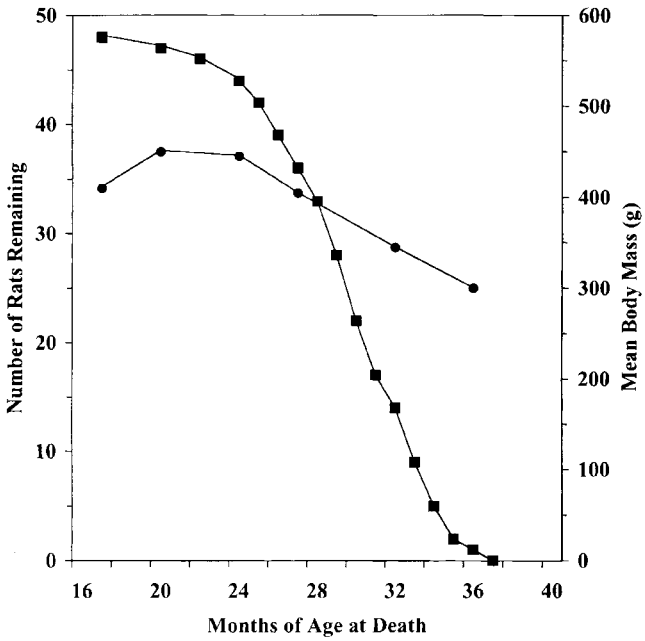

Figure 1. Survival curve (squares) of 50 male WI/HicksCar rats that were allowed to live a normal life span under specific pathogen-free (SPF) conditions. The curve connected by circles represents the mean body mass of all surviving animals at the age indicated.

Table 1. Probable Causes of Death in the Aging Study of WI/HicksCar Rats

Pathology	No. of Rats
Pituitary adenoma	11
Mononuclear cell leukemia	11
Undetermined	8
Polyradiculoneuropathy	6
Aspiration pneumonia	3
Euthanized	3
Congestive heart failure	2
Urinary bladder calculi	1
Other leukemia	1
Metastatic liposarcoma	1
Prostatitis	1
Fibrosarcoma	1
Thymoma	1

A second series of experiments consisted of direct injections of one EDL muscle in both old and young rats with equivalent volumes of a 0.75% solution of the myotoxic local anesthetic, Marcaine, according to the same method used by Carlson and Faulkner (20). The contralateral muscle was left untouched as a control. For reasons of survival in the old animals, 41 days rather than the normal period of 60 days elapsed between the time of Marcaine injection and removal of both experimental and control muscles for physiological analysis. Because of a short supply of 32-month-

Table 2. Other Nonlethal Pathology in Aging WI/HicksCar Rats

Pathology	No. of Rats
Interstitial tumor of testis	32
Pituitary adenoma	13
Mononuclear cell leukemia	9
Subcutaneous fibroma (sarcoma)	8
Polyradiculoneuropathy	7
Testicular atrophy	5
Nephrosis	3
Hemangiosarcoma	3
Renal adenocarcinoma	2
Pheochromocytoma	2
Malignant pheochromocytoma	2
Thymic lymphoma	2
Nephropathy	1
Gastric ulcer	1
Foreign body pneumonia	1
Histiocytosis of lung	1
Keratoancanthoma	1
Mammary hyperplasia	1
Endocardial schwannoma	1
Leiomyosarcoma of cecum	1
Hypertrophic osteopathy	1
Congestive heart failure	1
Granulomatous pneumonia	1
Pulmonary adenoma	1
Hemangioma of lymph nodes	1
Osteomyelitis	1
Harderian gland metaplasia	1
Osteosarcoma	1
Prostatitis	1
Cystitis	1
Gastric fibrosis	1

old WI/HicksCar rats, old BN rats were used in a parallel experiment involving Marcaine injections. One experiment involved WI/HicksCar rats. Eight young (4-month-old) and two old (32-month-old) rats survived the 41-day experimental period. In the second experiment involving BN rats, six young (4-month-old) and five old (34-month-old) animals survived the 41-day experimental period. Four old BN rats died during the study.

Rats of both strains were aged two per cage under SPF conditions at Harlan–Sprague–Dawley in Indianapolis, Indiana, and the ages stated above refer to the ages of the animals at the beginning of the experiments. All operations were carried out 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 each operation, the rats were placed on oral terramycin for 5 days. At the termination of the experiments, the rats were euthanized by an overdose of the anesthetic.

Contractile Properties

Rats were anesthetized with ether until they were unresponsive to tactile stimuli. Each control and experimental EDL muscle was exposed in anesthetized rats and dissected free of other tissue. Then 3-0 silk sutures were placed around the proximal and distal tendons, and the tendons were subsequently severed. The muscle to be tested was removed from the rat and secured at resting length in a tissue bath with a Krebs–Ringer bicarbonate solution containing 137 mM of NaCl, 5 mM of KCl, 2 mM of $CaCl_2$, 1 mM of $MgSO_4$, 1 mM of NaH_2PO4, 24 mM of $NaHCO_3$, and 0.025 mM of tubocurarine chloride. The solution was maintained at 25 ± 0.2°C and gassed with 95% oxygen and 5% carbon dioxide. At 25°C, 86 mg of EDL muscles of young rats remain completely stable and viable in structure and function even when required to make maximum contractions every 15 minutes (22). In contrast, the grafts and muscles in the present study were only required to make a single maximum contraction and experiments were completed within 15 minutes. One tendon of the muscle was tied to a fixed post and the other end to a Cambridge Technology servomotor–force transducer (Model 305, Cambridge, MA). 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 that were 0.2 milliseconds in duration. The voltage was adjusted to produce a maximum isometric twitch contraction and then increased slightly. The length of the muscle was adjusted to the optimum length for the development of twitch force. The fiber length–muscle length (L_f/L_o) ratio has been determined for control EDL muscles of 4-month-old male WI/Hicks/Car rats, L_f/L_o = 0.40 (20), 2-month-old male Sprague–Dawley rats, L_f/L_o = 0.40 ± 0.05 (22), and 8-month-old male Lewis rats, L_f/L_o = 0.35 ± 0.03 (23). The determination of fiber length is a laborious procedure that requires the total muscle (24), and consequently data are not available specifically on muscles of old rats or on grafts because the valuable muscle tissue is required for more important assays. The L_f/L_o ratio does not vary greatly even among species, and a value of 0.40 was used throughout this study for all groups. The fiber lengths are slightly longer for muscles of old rats and for grafts; this explains to some degree, but not completely, the lower values for specific force for these groups. The maximum isometric tetanic force (P_o) is also developed with voltages and fiber length developed by these procedures. The frequency of stimulation was increased until the force plateaued, and this value was defined as the P_o. The frequency of stimulation was increased from 80 Hz in increments of 20 Hz until the force plateaued at a maximum value. This value for maximum isometric tetanic force was defined as P_o. The P_o was achieved usually at frequencies of stimulation between 100 Hz and 140 Hz. Although considerable overlap among the groups occurred, the muscles in which fibers had regenerated either through transplantation or Marcaine treatment tended to be in the lower range of frequencies and control muscles in the upper range. After the measurement of contractile properties, the muscles were removed from the bath and weighed. With a fiber length (L_f/L_o) ratio of 0.40 for control muscle, the total muscle fiber cross-sectional area (CSA) was calculated from CSA = muscle mass (grams)/L_f (centimeters) × 1.06 g/cm^2 (muscle density). The specific P_o (kilonewtons per square meter) was calculated by dividing the P_o (kilonewtons) by the CSA (square meter).

Morphological Analysis

Following the measuring of contractile properties and the weighing of each muscle, the muscles were subdivided into three parts and were fixed for subsequent histological, immunohistochemical, or electron microscopic analysis. For a histological analysis, distal segments of the muscles were fixed in Bouin's solution, embedded in paraffin, sectioned at 7 μm, and stained with Ehrlich's hematoxylin and eosin.

For immunohistochemical analysis, muscle segments cut transversely across the midbelly region were fixed in 2% paraformaldehyde in 0.1 M of phosphate-buffered saline (PBS) at pH of 7.4 for 24 hours and then washed overnight in 0.1 M of PBS. To prevent tissue dehydration and the formation of ice crystals during freezing, the muscles were cryoprotected with sucrose. After they were washed in PBS, the muscle pieces were immersed in 0.25 M of sucrose in PBS for 1 hour, transferred to 0.5 M of sucrose in PBS for 45 minutes, and finally kept for 30 minutes in 1.5 M of sucrose in PBS. After cryoprotection was completed, the muscle samples were placed in specimen molds containing TBS Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC) and were quick frozen by immersing the molds in 2-methylbutane (isopentane) that had been cooled in dry ice. Transverse serial 9-μm sections from the midbelly of each muscle were cut on a Shandon cryostat (Life Science International Ltd., England) at −28°C, mounted on warm uncoated glass slides, and placed in a freezer at −20°C for storage. Before staining, the sections were washed in doubly distilled water for 3 minutes at room temperature in order to remove the cryoprotective medium. They were then fixed in cooled methanol at −20°C for 10 minutes, allowed to air dry, and then washed in 0.1 M of PBS for 4 minutes at room temperature.

The sections were doubly labeled with a mixture of primary antibodies—mouse antislow muscle myosin mono-

clonal antibody (Chemicon International, Inc., Temecula, CA) and a primary rabbit antilaminin polyclonal antibody (Sigma, St. Louis, MO). Incubation with the primary antibodies was carried out at room temperature for 3 hours, followed by three 3-minute rinses in 0.1 M of PBS. Goat anti-mouse labeled with Rhodamine (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used as a secondary antibody against the antislow muscle myosin primary antibody, whereas Fluorescein-labeled goat anti rabbit (Jackson ImmunoResearch) was used as the secondary antibody against the antilaminin primary antibody. Incubation with a mixture of the secondary antibodies was carried out at room temperature for 45 minutes and was followed by three 4-minute rinses in 0.1 M of PBS. After staining, the sections were mounted with Vectashield Mounting Medium for Fluorescence with DAPI (4,6-diamidino-2-phenylindole) for counterstaining nuclei (Vector Laboratories, Burlingame, CA) and coverslipped. The sections were examined and photographed with Zeiss Axioplan 2 and Axiophot 2 universal microscopes (Carl Zeiss Inc., Jena, Germany).

For an electron microscopic analysis, small pieces from the proximal regions of the muscles were fixed in a mixture of formaldehyde–glutaraldehyde buffer (2.5% each in 0.1 M of Na cacodylate buffer at pH 7.4) at 4°C for 4 hours, washed with 0.25 M of sucrose in 0.1 M of PBS, and post-fixed in 2% OsO_4 in 0.1 M of PBS at 4°C for one hour. The muscles were then washed with 0.25 M of sucrose in 0.1 M of PBS, dehydrated in graded ethanol, and embedded in a mixture of Epon–Araldite (Eponate12-Araldite 502 kit from Ted Pella, Inc., Redding, CA). Sections were cut with a Reichert–Jung Ultracut E ultramicrotome at 0.5–1.0 μm thick and mounted on glass slides. For basic muscle structures to be localized at the light microscopic level, the sections were stained with Toluidine Blue dye. Ultrathin sections were collected on Formvar-coated (Electron Microscopy Sciences, Fort Washington, PA) slot grids and stained with uranyl acetate and lead citrate. The sections were examined under a Philips CM-100 transmission electron microscope (Philips, Mahwah, NJ).

Quantitative Morphological Studies

Immunohistochemically stained sections of both control and Marcaine-treated EDL muscles were examined with a Leitz Diaplan microscope and the images were captured onto a Power Macintosh 8500/120 computer, under the same magnification, using a Pixera camera 1.2.4. (Pixera Corporation, Los Gatos, CA). Two images of the same region of the section were captured through the fluorescence microscope by using different filters for the Fluorescein- and Rhodamine-labeled secondary antibodies. The double images of one microscopic field, stained by secondary antibodies with two different colors, were transformed into a single color image by using Adobe Photoshop 4.0. As a result, it was possible to accurately recognize the type I (slow) fibers, which were stained red by Rhodamine, and the non-type I (presumably type II fast) muscle fibers, which were not stained by the slow myosin heavy chain (MHC) antibody, but were sharply outlined by the Fluorescein-stained laminin in the basal laminae that surrounded all the muscle fibers. This technique permitted the two major types of muscle fibers to be distinguished within the same section and greatly facilitated quantitative analysis. The circumferences of both fiber types, delineated by the Fluorescein staining of the secondary antibody to laminin that extended along the edge of all muscle fibers, were then electronically traced by using an ArtPad II and a graphics tablet with an Erasing UltraPen (Wacom Technology Co., Vancouver, WA). For each type of muscle fiber the CSAs were calculated with the help of NIH Image 1.62 software. For each animal examined, muscle fiber profiles were taken from 15 microscopic fields of transverse sections of the EDL muscles. In total, 780 muscle fibers from young rats and 640 muscle fibers from old rats were examined in this manner. The distribution by CSA of both fast and slow muscle fibers was laid out in histograms.

Satellite cells from two control EDL muscles from 32-month-old Wistar rats used in the cross-age transplantation study were counted under the electron microscope. So that the same satellite cell was not counted twice, single sections from different blocks taken from the same muscles were examined. Initially, a chart of the selected section was drawn at low magnification, and then myonuclei and satellite cell nuclei in different fields of each section were counted at high magnification. As fibers were examined, their positions were marked on the chart. Satellite cells were expressed as a percentage of the total nuclei counted beneath the basal laminae of the muscle fibers.

Statistical Analysis

The means and standard errors were determined for each of the three variables, muscle mass, absolute P_o, and specific P_o, for experimental and control EDL muscles in young and old rats. The experimental muscles included either transplants or Marcaine-treated muscles and their respective control EDL muscles. For the cross-age transplantation study only WI/HicksCar rats were used, but the Marcaine treatments involved both young and old WI/HicksCar and BN rats. Differences between the body masses of each of the two experimental groups and between BN and WI/HicksCar rats were determined by *t* tests. In the cross-age transplant experiment, differences between the muscle masses and values for absolute P_o and specific P_o of each of the two transplant groups and the young control group were determined by a three-way analysis of variance (ANOVA; young-into-young transplant vs old-into-young transplant vs young control) for each variable. Because the old control group consisted of only two rats, the old controls could not be included in the statistical analysis. For the two Marcaine-treatment groups of young and BN rats, and their respective control EDL muscles in young and old rats, each of the three variables was determined by a 2×2 ANOVA for age differences and treatment differences. The method of least squares was used to fit a general linear model. In circumstances of a significant overall *F* ratio for the ANOVA, differences between individual group means were sought by the use of a post hoc pairwise *t* comparison of least-squares means with an appropriate correction of the significance level to account for multiple comparisons. This analysis permitted the assessment of whether these differences were significant between the EDL muscles: (a) transplanted

young-to-young or old-to-young and differences between transplants and (b) in the BN rats, control EDL muscles in old compared with young rats and between the EDL muscles in young or old rats treated with Marcaine compared with control EDL muscles in age matched rats. The significance for each statistical analysis was set a priori at $p \leq .05$.

Results

Cross-Age Transplantation Experiment

At the time of removal for the transplantation of the muscles, the 32-month-old donor WI/HicksCar rats were sluggish in their movements and appeared to be in a premorbid condition. The EDL muscles, when isolated, were very small and thin. Light microscopic observation on control EDL muscles from old rats of the same cohort as the old-into-young muscle donors showed considerable evidence of muscle fiber atrophy, grouping of type I muscle fibers (Figure 2) and increased deposition of connective tissue. At the electron microscopic level, changes indicative of partial denervation were seen. These included resorption of neuromuscular junctions (Figures 3A and 3B), empty nerve channels with surviving Schwann cells (Figure 3C) and extensive fibrosis within nerve trunks (Figure 3D). In addition, an abundance of satellite cells was noted. Some satellite cells were quiescent (Figure 4A); others showed morphological evidence of activation, such as the presence of cellular processes and the movement of the satellite cell away from the muscle fiber (Figure 4B). In the control muscles from the 32-month-old WI/HicksCar rats, which showed gross signs of hind-limb neuropathy, satellite cells constituted 8.1% of the total nuclei beneath the basal lamina of the muscle fibers.

Two months after transplantation, as reported previously (2,3,25), the young-into-young autotransplants had muscle masses, absolute P_o, and specific P_o that were 67%, 43%, and 58% of their respective control values (See Table 3). In contrast, compared with the control values for the old rats, the old-into-young cross-age transplants had a transplant mass of 144%, an absolute P_o of 216%, and a specific P_o of 120%. In the comparison of old-into-young transplants with the young-into-young transplants, no differences were observed for muscle mass, absolute P_o, or specific P_o (Table 3). Although two of the old-into-young transplants were removed from an old donor rat that had severe hind-limb neuropathy, the absolute P_o for each of the transplants was 938 mN and 1272 mN, which did not differ from values for other transplants regardless of donor age.

Old-into-young transplants had a similar histological appearance to young-into-young transplants. In both types of transplants, the bulk of the muscle consisted of large regenerated muscle fibers, with small areas of atrophic fibers that, presumably, were not penetrated by regenerating nerve fibers.

Marcaine-Treated Muscles

Direct injection of EDL muscles with Marcaine results in massive muscle fiber damage and reduces the mean P_o of the muscles to approximately 10% of control values at 2 days post injection (20). In the present experiments, Marcaine was originally injected into the right EDL muscles of 4-month-old and 32-month-old WI/HicksCar rats, but because only two of the old rats survived the procedure, the experiment was repeated on a group of young (4-month-old) and old (34-month-old) BN rats. In both series, the experiments were terminated 41 days after Marcaine injection rather than the standard 60 days because of the high mortality rate of the old BN rats.

In the young BN rats, the mean mass of the 41-day regenerated EDL muscles was 131% that of the contralateral control muscles, whereas in the old rats, the masses of regenerated and control muscles were not different (Table 4). The mean mass of the old control muscles was 69% that of the young control muscles.

The absolute P_o of the Marcaine-treated muscles in the young rats was significantly greater than that of the contralateral control muscles, whereas in the old rats the mean absolute P_o was not different from than that of control muscles (Table 4). Despite their high level of return relative to

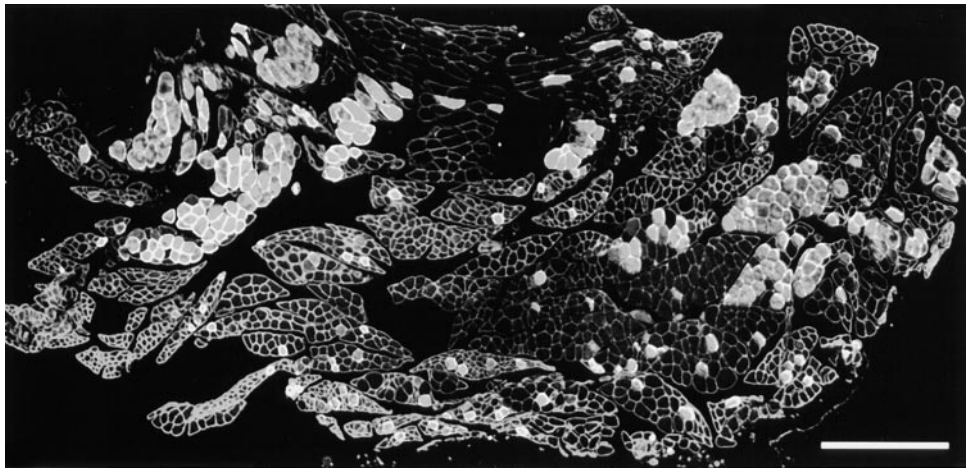

Figure 2. Cross section through control extensor digitorum longus muscle from a 32-month-old WI/HicksCar rat, reacted with antibodies against slow myosin and laminin. Note the groupings of type I fibers (lightly stained in this photomicrograph). Type II fibers appear black and are surrounded by a light antilaminin stain. Bar = 500 μm.

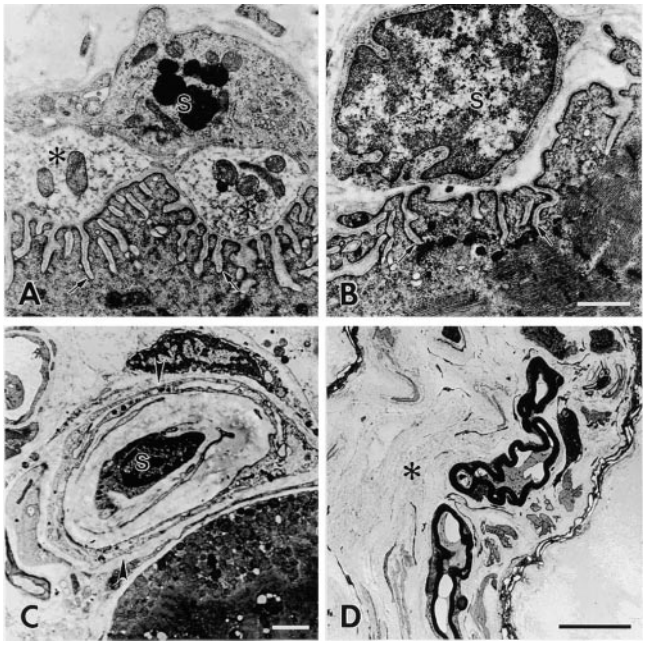

Figure 3. Electron micrographs of control tissue from 32-month-old WI/HicksCar rats. **A**, a normally innervated neuromuscular junction (asterisks = axon terminals, arrows = postsynaptic folds. S = Schwann cell, and bar = 1 μm); **B**, empty neuromuscular junction lacking axon terminals between the Schwann cell (S) and the postsynaptic folds (arrows; bar = 1 μm); **C**, empty nerve channel (arrowheads) with persisting Schwann cell (S; bar = 2 μm); **D**, section through intramuscular nerve, showing extensive fibrosis (asterisks; bar = 10 μm).

control muscles, the absolute P_o of the regenerated old EDL muscles was only 37% that of the young regenerates. In both young and old rats the specific P_o (kilonewtons per square meter) of the regenerated muscles was significantly less than that of the control muscles.

In the WI/HicksCar group the results of Marcaine injections into the EDL muscles were very similar to those of the BN group. The absolute P_o of young regenerates exceeded those of the contralateral controls (2807 mN for regenerates vs 2573 mN for controls), and in the two surviving old WI/HicksCar rats the mean P_o of the regenerates was 471 mN versus 450 mN in the control EDL muscles. Also similar to

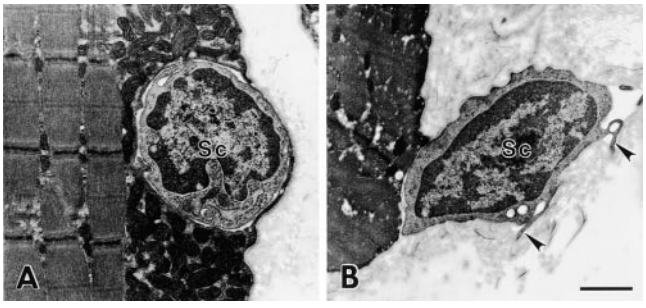

Figure 4. Satellite cells (Scs) from extensor digitorum longus muscles of 32-month-old WI/HicksCar rats: **A**, quiescent, characteristically deeply embedded in the muscle fiber; **B**, activated, showing cytoplasmic projections (arrowheads) and evidence of separation from the muscle fiber. Bar = 1 μm.

Table 3. Muscle Mass and Contractile Properties of Control EDL Muscles From Young and Old Rats and of Young and Old Transplants Both Into Young Rats

Parameter	Young Into Young (n = 8)	Old Into Young (n = 7)	Young Control (n = 8)	Old Control (n = 2)
Body mass (g)	354 ± 3.1*	354 ± 3.1*	339 ± 7.0	361
Muscle mass (mg)	117 ± 6.3*	127 ± 6.1*	174 ± 4.0	88
Absolute P_o (mN)	1098 ± 58.6*	970 ± 108.1*	2573 ± 132	450
Specific P_o (kN/m^2)	146 ± 7.2*	116 ± 12.2*	250 ± 9.2	97

Notes: Muscle mass and contractile properties are mean ± 1 standard error of the mean. Data were obtained on only two old (32-month-old) control WI/HicksCar rats because of the high mortality rate of these rats at this age. These data were also used for the experiments on Marcaine treatment reported in Table 2. No statistical treatment comparisons could be performed with the data on extensor digitorum longus (EDL) muscles of the old rats because of the sample size. No differences were observed at the $p \leq .05$ level of significance between old-into-young transplants and young-into-young transplants for any of the four variables. The young rats were 4 months old at the start and 6 months old at the end of the experiment. The old rats were 32 months old at the start and 34 months old at the end of the experiment.

*Significant differences at the $p \leq .05$ level of significance between data on both young-into-young and old-into-young transplants with data on the young control EDL muscles are indicated.

the BN rats, the specific P_o of the young WI/HicksCar regenerates (204 kN/m^2) were not different from those of control muscles (219 kN/m^2), and those of the old regenerates (85 kN/m^2) were similar to control values (93 kN/m^2).

Like those of the old WI/HicksCar rats, the control EDL muscles of the old BN rats showed both muscle fiber type grouping and differential atrophy, with the type II muscle fibers having smaller CSAs than the type I muscle fibers (Figure 5C). Fiber type grouping was not seen in the young EDL muscles (Figure 5A). Regenerated muscle fibers of both age groups showed large amounts of central nucleation (Figures 5B and 5D). In the very old rats, grouping of type I muscle fibers was prominent in the regenerates, whereas type grouping was minimal in the young muscle regenerates. Another prominent feature of the muscle regenerates in young rats was the presence of small numbers of hypertrophic muscle fibers (Figure 5B).

Morphometric studies on EDL muscles of BN rats showed absolute decreases with age in the size distribution and mean CSA of the type II muscle fibers, whereas the mean CSA of type I fibers actually increased in the old rats (Figure 6 and Table 5). In young rats, the size distribution of type II fibers was skewed to the right in regenerates as compared with controls, but in old rats, the differences were less pronounced (Figure 5). Not only was the relative CSA of the type II fibers less in old than in young rats, but the proportion of type II fibers in relation to that of type I fibers was also lower. The ratio of type II to type I fibers was 5.4 in the old rats versus 8.8 in the young rats. In the 41-day regenerates, the ratios were reduced from control values in both very old (4.1) and young (5.2) rats. Especially in old rats, the number of small diameter muscle fibers in regenerates was greater than in controls (Figures 6A and 6C).

At the electron microscopic level, both control and Marcaine-treated EDL muscles of the old rats showed evidence of partial denervation, such as heterogeneity in size of

Table 4. Muscle Mass and Contractile Properties of Old and Young Rat EDL Muscles 41 Days After Marcaine Injection

		Muscle Mass (mg)		Absolute P_o (mN)		Specific P_o (kN/m^2)	
Rat	Body Mass (g)	Control	Marcaine	Control	Marcaine	Control	Marcaine
Young BN (n = 7)	328 ± 9.2	167 ± 4.8	219 ± 9.6†	2629 ± 126.0	2972 ± 79.8†	266 ± 7.1	226 ± 6.1
Old BN (n = 5)	396 ± 11.0*	116 ± 4.8*	117 ± 9.9*	1211 ± 106.0*	1102 ± 157.0*	206 ± 17.5*	184 ± 16.0*
Young WI/HicksCar (n = 8)	339 ± 7.0	174 ± 4.0	206 ± 6.3	2573 ± 132.0	2807 ± 100.0	250 ± 9.2	233 ± 4.8
Old WI/HicksCar (n = 2)	361	88	83	450	471	85	93

Notes: Muscle mass and contractile properties are mean ± 1 standard error of the mean. No significant differences were observed between the Marcaine-treated and control extensor digitorum longus (EDL) muscles of young or old BN rats. Control data were obtained on only two old WI/HicksCar rats because of the high preoperative mortality rate. Statistics could not be performed relative to the data on the old control rats because of the small sample size.

*Significant differences between data on old compared with young control muscles and Marcaine-treated muscles in BN rats are indicated.

†Differences between Marcaine-treated EDL muscles and control muscles are indicated.

muscle fibers (Figures 7A and 7B), interstitial fibrosis, resorption of some neuromuscular junctions (Figures 7C and 7D), and fibrotic changes in nerve trunks (Figures 7E and 7F). However, unlike control muscles, the presence of numerous activated satellite cells was a prominent feature of the very old Marcaine-treated muscles (Figures 8A and 8B), and continuing myogenesis was still seen at 41 days of regeneration (Figures 8C and 8D).

Discussion

The principal finding of these experiments is that even near the end of the life span, rat EDL muscles still possess a normal capacity for regeneration, but this capacity can only be expressed if conditions for regeneration are favorable. In the experiments reported here, the specific favorable conditions were cross-age transplantation into young hosts or muscle fiber damage without significant damage to the motor nerve supply.

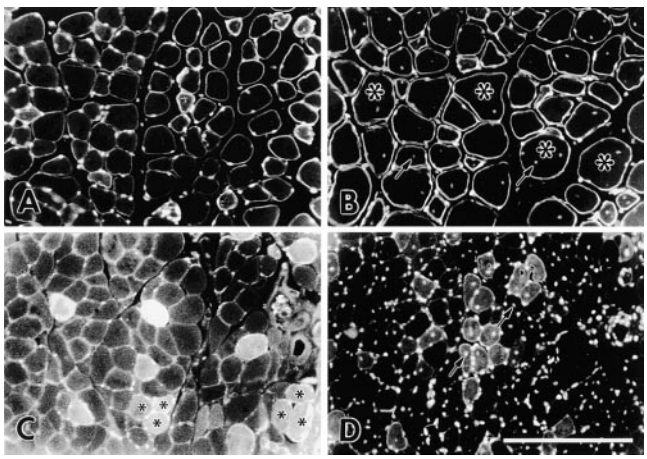

Figure 5. Cross sections through control and Marcaine-treated extensor digitorum longus muscles from 5- and 35-month-old BN rats reacted with antibodies against slow myosin and laminin: **A**, young control; **B**, young Marcaine-treated; **C**, old control; **D**, old Marcaine-treated (bar = 250 μm). Fiber-type grouping (small asterisks) is seen in old but not in young control muscle. Central nuclei (arrows) are prominent in regenerated muscle fibers in both old and young rats. In old control muscle, the type II fibers (dark) are smaller than the type I fibers (light). Hypertrophic muscle fibers (asterisks) are seen in the young regenerated muscle.

In the cross-age transplantation experiment, the old donor rats at the start were 32 months of age, an age corresponding to the 20–25th percentile point on the survival curve. Even though the very old donor muscles were very thin and at least one rat showed evidence of significant hind-limb neuropathy, the regenerated muscles produced a mean absolute P_o that was only 10% less (not statistically significant) than that of young-into-young host autografts in the contralateral limbs. This level of return (970 mN) is higher than that reported earlier (3) for cross-age grafts from 24-month old EDL muscles grafted into limbs of 4-month-old hosts (less than 800 mN), and it is more than twice the value of P_o from unoperated EDL muscles in two 32-month-old control rats.

One explanation for the somewhat better regeneration of EDL muscles from 32-month-old donors than from 24-month-old donors (3) could be the presence of increased numbers of activated satellite cells in the muscles of the extremely old Wistar rats, stemming from partial denervation caused by the normal aging process or from the hind-limb neuropathy. Normal EDL muscles from 6-month-old WI/HicksCar rats had 2.8% satellite cells (26). Gibson and Schultz (27,28) found 2.9% satellite cells in 12-month-old and 1.9% satellite cells in 24-month-old Sprague–Dawley rats. The 8.1% satellite cells that we found in the 32-month-old muscle differ little from the 9.1% satellite cells that Viguie and colleagues (26) reported in the EDL muscle 2 months after denervation. This could be interpreted as indirect evidence of prior denervation in the cross-age transplanted EDL muscles used in this study. Schultz (29), however, left open the question of whether or not an increased pool of satellite cells actually improves regeneration in muscle grafts. What is not known is the minimum number of satellite cells that will support full regeneration of the EDL muscle. Experiments involving myoblast therapy for dystrophic muscle (30) suggest that only a small number of satellite cells can account for massive amounts of regeneration.

The Marcaine-injection experiment, conducted on 34-month-old BN rats and 32-month old WI/HicksCar rats, showed that in each case, the 41-day regenerates had regained control values for absolute P_o. However, in the WI/HicksCar group, the absolute P_o of the Marcaine-induced regenerates in situ was only half that of the old-into-young cross-age regenerates, suggesting that the overall conditions for regeneration in the very old hosts did not elicit the maxi-

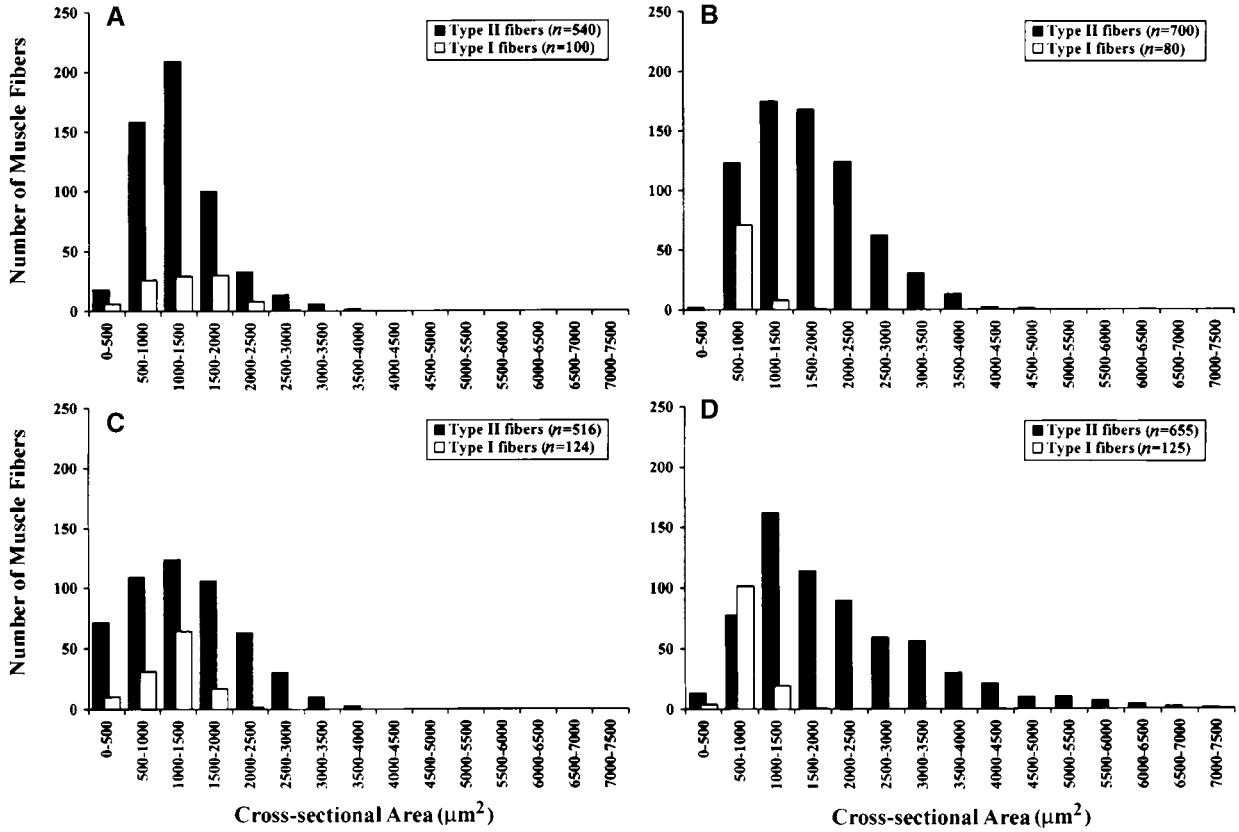

Figure 6. Cross-sectional areas of type I and type II muscle fibers in control and regenerated extensor digitorum longus muscles of 5- and 35-month-old BN rats: **A**, old control muscle; **B**, young control muscle; **C**, old regenerated muscle; **D**, young regenerated muscle.

mum possible regenerative response from the injured muscle fibers.

One of the most significant questions arising from this study is the maximum level of regeneration of which very old muscle is capable. Even in the oldest of rats, EDL muscles can regenerate back to control levels of mass and force. From the limited strain-specific comparative data on cross-age muscle transplantation in WI/HicksCar rats, a very old muscle graft in a young host can regenerate to better than very old control levels. However, by necessity, cross-age muscle grafts must involve transection of the nerve to the EDL muscle and the regeneration of host axons into the regenerating muscle. Even with nerve-implant EDL autografts in young rats, the maximum return is typically only ~50% of control values (3). Therefore the nerve-implant cross-age transplant model does not provide conditions suitable for full expression of the regenerative capacity of the very old grafted muscle.

Another significant question is why the muscles of very old rats have lost so much mass and function and how the mechanisms underlying this affect the success of the in situ regenerative process. Certainly, the loss of neural support plays a significant role in the aging of muscle. There is a documented loss of motor units, especially fast-fatigable motor units, in aging muscle (8,31) as well as the reinnervation of type II muscle fibers by sprouts of slow axons, thus enlarging the existing slow motor units (8,32). Other factors that could lead to a loss of muscle mass and the attendant loss of function are reduced activity of the animal, a loss of trophic support from hormones, such as growth hormone (33,34), or growth factors, such as fibroblast growth factor (35) or insulin-like growth factor (36), or some combination of factors.

In both the young BN and young WI/HicksCar rats the mean mass and P_o of the 41-day Marcaine-induced regenerates were greater than those of the contralateral control

Table 5. Morphometric Data on Muscle Fibers of Control and Marcaine-Treated EDL Muscles of Young and Very Old BN Rats

Rat	Control Mean CSA (μm²)				Marcaine-Treated Mean CSA (μm²)			
	No. of Examined Fibers	Type I	Type II	No. Type II/ Type I Ratio	No. of Examined Fibers	Type I	Type II	No. Type II/ Type I Ratio
Young	780	823 ± 17.1	1746 ± 28.1	8.75	780	855 ± 17.4	2138 ± 48.2	5.24
Old	640	1280 ± 51.01	1292 ± 24.1	5.40	640	1108 ± 36.2	1361 ± 33.1	4.16

Note: EDL = extensor digitorum longus; CSA = cross-sectional area.

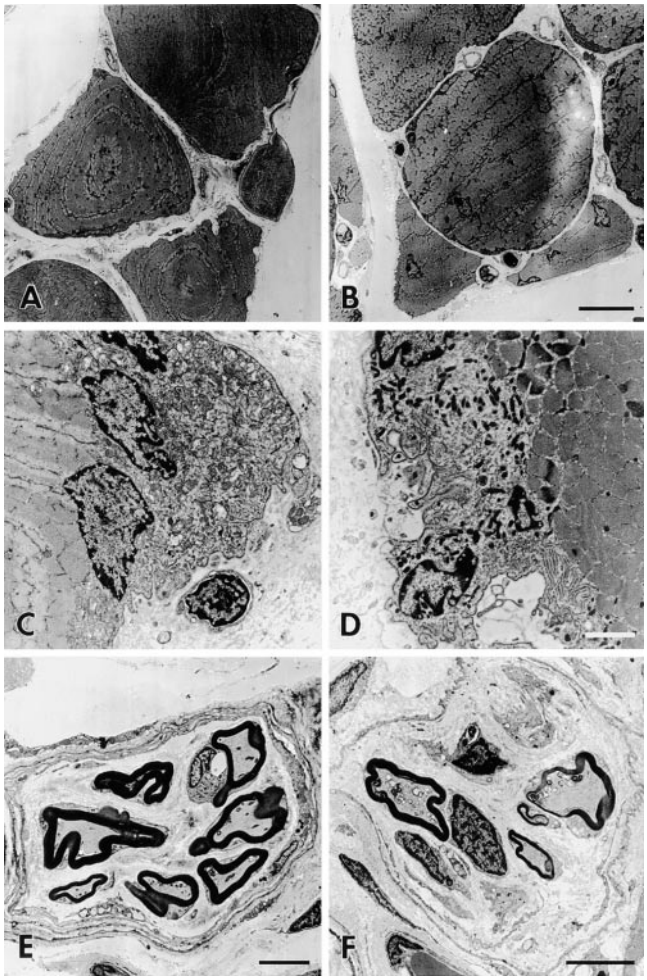

Figure 7. Electron micrographs of control (**A**, **C**, **E**) and regenerated (**B**, **D**, **F**) extensor digitorum longus muscle from 35-month-old BN rats: **A**, **B**, heterogeneity in size of the muscle fibers (bar = 10 μm); **C**, **D**, resorption of neuromuscular junctions (bar = 2 μm); **E**, **F**, aging changes in intramuscular nerves, including fibrosis (bar = 5 μm).

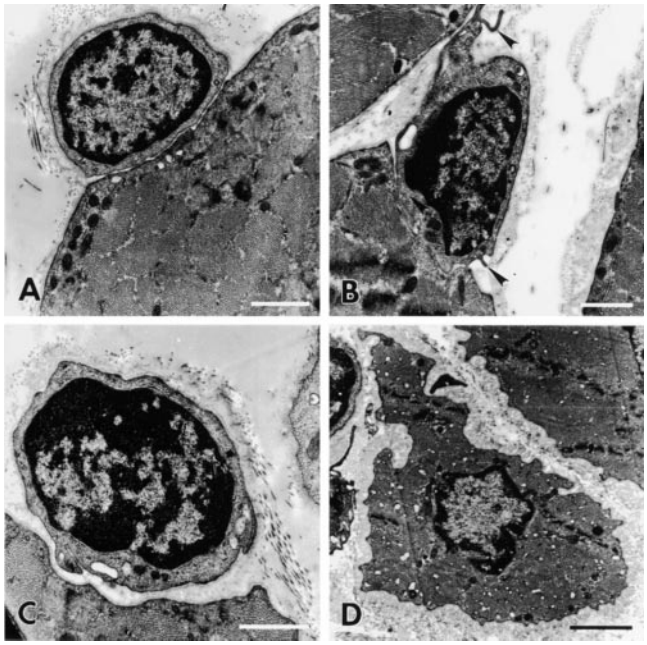

Figure 8. Electron micrographs showing stages in the activation of satellite cells and new muscle fiber formation in regenerated extensor digitorum longus muscles of 35-month-old BN rats: **A**, satellite cell beginning to pull away from the associated muscle fiber; **B**, cytoplasmic processes forming on an activated satellite cell (arrowheads); **C**, satellite cell completely separated from the muscle fiber (bar = 1 μm for **A**, **B**, **C**); **D**, new muscle fiber, showing the presence of organized contractile filaments (bar = 2 μm).

muscles. In the very old individuals, the mass and P_o of control and regenerated muscles were approximately equal. A mass for regenerates greater than that of the control value has been noted before, especially for longer periods after the initial Marcaine injection (20,37). There is no obvious reason why the P_o of a 41-day regenerate should be relatively greater than that of a 60-day regenerate unless there is a temporary rebound effect that is muted later in the regenerative process.

Because satellite cells are considered to be the main, if not sole, source of regenerating muscle fibers, these results indicate that, as in 24-month-old rats, the satellite cell populations of 32- and 34-month-old rats is still sufficient to support very good muscle regeneration. The regenerative product from any population of satellite cells is a function of the total population of cells and the proliferative capacity of the individual satellite cells. The design of this study did not allow a level of analysis that would distinguish the relative contributions of these two factors. Although a number of studies have suggested a decline in the total number of muscle fibers with increasing age in rats (38,39), the number of satellite cells does not significantly decrease, even up to extreme old age (28; E. Dedkov, unpublished). Even though satellite cells from senescent muscles retain their myogenic properties under culture conditions (40), some investigators have noted a decrease in proliferative potential of cultured satellite cells with increasing age (41). Given the results of the physiological studies and the background data present in the literature on aging, a reasonable conclusion appears to be that despite some potential losses in both numbers and in proliferative potential, there remains sufficient cellular reserve in extremely old muscle to allow regeneration to occur equally well as young muscle in vivo, as long as the satellite cells are regenerating in similar environments.

In conclusion, advanced age in itself does not appear to be a factor that limits the regenerative potential of rat muscle fibers. The best evidence for this is the cross-age transplantation experiment, in which 32-month-old and 4-month-old EDL muscles regenerated equally well in the same hosts. Despite the fact that in the Marcaine-injection experiments damaged EDL muscles in very old rats regenerated to normal control levels of mass and force, in absolute terms neither the control or regenerated muscles were as large or as strong as EDL muscles in younger rats. This finding raises the question of whether the factors that limit muscle regeneration in old rats are the same as those that account for the overall deterioration of muscles in these animals.

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