IGF-I restores satellite cell proliferative potential in immobilized old skeletal muscle

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Chakravarty, Manu V., Bradley S. Davis, and Frank W. Booth. IGF-I restores satellite cell proliferative potential in immobilized old skeletal muscle. J Appl Physiol 89: 1365–1379, 2000.—One of the key factors responsible for the age-associated reduction in muscle mass may be that satellite cell proliferation potential (number of doublings contained within each cell) could become rate limiting to old muscle regrowth. No studies have tested whether repeated cycles of atrophy-regrowth in aged animals deplete the remaining capacity of satellite cells to replicate or what measures can be taken to prevent this from happening. We hypothesized that there would be a pronounced loss of satellite cell proliferative potential in gastrocnemius muscles of aged rats (25- to 30-mo-old FBN rats) subjected to three cycles of atrophy by hindlimb immobilization (plaster casts) with intervening recovery periods. Our results indicated that there was a significant loss in gastrocnemius muscle mass and in the proliferative potential of the resident satellite cells after just one bout of immobilization. Neither the muscle mass nor the satellite cell proliferation potential recovered from their atrophied values after either the first 3-wk or later 9-wk recovery period. Remarkably, application of insulin-like growth factor I onto the atrophied gastrocnemius muscle for an additional 2 wk after this 9-wk recovery period rescued ~46% of the lost muscle mass and dramatically increased proliferation potential of the satellite cells from this muscle.

I. Introduction

The human body is invested with more than 650 skeletal muscles, which thereby form a massive support system. Therefore it is not surprising that loss of skeletal muscle can lead to devastating consequences. One example is institutionalized living because of physical frailty. Weakened skeletal muscles contribute to a loss in the activities of daily living and consequently lead to a diminished quality of life (51). Despite these obvious facts, all factors contributing to the age-associated reduction in muscle mass (sarcopenia) have yet to be elucidated.

One candidate contributing to sarcopenia may be an age-related decrease in skeletal muscle regeneration (14, 15). Elegant studies have directly implicated satellite cells (mononuclear myogenic precursor “stem” cells; for reviews, see Refs. 10, 44, 45) as crucial players for muscle growth and repair in postnatal life, as the obliteration of satellite cell proliferative capacity by irradiation resulted in an inability to hypertrophy skeletal muscles in young rats (35, 38). Interestingly, decreased satellite cell proliferation has been shown with aging in humans (20) and rodents (22, 43), as well as with unloading-induced atrophy of skeletal muscle in juvenile rats (18, 41). Given the complex and sequential process of sarcopenia, it has been postulated that, at some point in this multistep process, a limited satellite cell proliferation potential could become rate limiting to the regrowth of old muscles, especially if the satellite cell’s proliferative potential were to have been used up during a long lifespan by repeated cycles of atrophy and regrowth. Such cases could be imagined in which there are repeated bouts of immobility secondary to recurrent debilitating illnesses and/or injuries.

There is ample evidence in the literature to suggest that satellite cell proliferation can become rate limiting in certain disease states, such as Duchenne muscular dystrophy (DMD). Studies by Webster and Blau (50) and Wright (52) suggest that the reduced replicative capacity of DMD satellite cells in culture reflects the number of cell divisions already expended in vivo during the course of fiber regeneration. As a consequence of the increased degeneration-regeneration cycles that characterize this disease, the reserve proliferative capacity of satellite cell populations is used up such that skeletal muscles eventually lose the ability to regenerate, consequently leading to muscle wasting. Indeed, Bischoff (10) stated that these studies were consistent with the hypothesis that progressive loss of muscle in DMD resulted from replicative senescence of satellite cells, although he cautioned that this had not been critically evaluated. Another set of supporting information was given by Schultz and Jaryszak (42), who reported that, with three cycles of regeneration induced by the myotoxin bupivacaine, the cumulative proliferation potential of the satellite cells in young animals was significantly reduced, with the magnitude
of the reduction being proportional to the number of regeneration cycles in which the cells had participated. Furthermore, Moore (32) suggested that, although age by itself is not a factor resulting in exhaustion of stem cells in general, other factors such as cytotoxic drugs, cachexia, and repeated immobility superimposed on aging can indeed exhaust the replicative potential of the stem cells. In support of this, a common clinical observation is that in many nursing homes there are mobile and functioning aged individuals who, on being subjected to one or more periods of immobility due to illness or injury, are unable to return back to mobility (51). Even with extensive rehabilitative therapy, some of these individuals are unable to recover to preinjury functioning levels. Could this be due satellite cells’ proliferative reserves being exhausted by cycles of atrophy-regrowth in these aged individuals?

Nevertheless, to our knowledge, no studies have directly tested whether repeated cycles of atrophy-regrowth in aged animals deplete the remaining capacity of satellite cells to replicate, and, if so, what measures can be taken to prevent this from happening. Hence, we hypothesized that there would be a pronounced loss of satellite cell proliferation potential in gastrocnemius muscles of 25- to 30-mo-old Fischer 344/Brown Norway F1 hybrid (FBN) aged rats subjected to three cycles of atrophy by hindlimb immobilization (plaster casts) with intervening recovery periods. Moreover, on the basis of Barton-Davis et al.‘s reported demonstration (8) of insulin-like growth factor’s (IGF-I) rescue of skeletal muscle from sarcopenia in 27-mo-old aged mice and of their resultant untested hypothesis that satellite cells were involved in this rescue, we further hypothesized that IGF-I would rescue the muscle mass failing to regrow after such repeated cycles of atrophy-regrowth in aged rats, as well as rescue the diminished proliferative potential of satellite cells.

MATERIALS AND METHODS

Experimental Design and Methods to Determine Effect of Normal Aging on Satellite Cells

Animals. Thirty pathogen-free FBN male rats in three different age groups (3, 18, and 31 mo old) were obtained from the NIH aging program from the National Institute on Aging (i.e., 10 rats in each age group). Pairs of animals were housed in previously autoclaved bedding in microisolator cages and provided with autoclaved food and water ad libitum, in a room maintained at 21°C with a 12:12-h light-dark cycle. The animals were allowed to acclimatize to their new surroundings for ~2 wk before any experiments were done.

Satellite cell isolation. Satellite cells were isolated from gastrocnemius muscles of young (3-mo-old), adult (18-mo-old), and old (31-mo-old) FBN rats to establish an age-associated decrease in satellite cell proliferation potential. The muscle was removed from both legs, trimmed of excess connective tissue and fat, and quickly weighed on an electronic analytical balance to record the wet weight. Gastrocnemius muscles from five animals from each group were pooled to ensure adequate amount of tissue for satellite cell isolation. The methods for the isolation were as previously described by Allen et al. (5). Briefly, the trimmed muscles were finely minced with iris scissors in 1× sterile PBS, pH 7.4, and subjected to enzymatic digestion by incubation at 37°C in 1.25 mg/ml Pronase for 1 h. The protease was removed by centrifugation at 1,500 g, followed by decantation of the supernatant. Pellets were then resuspended in sterile PBS, mixed with a vortex mixer for 20 s, and centrifuged for 10 min at 500 g. The supernatant containing the cell fraction was removed, and the pellet was resuspended in PBS, mixed, and centrifuged as before. This process was repeated a third time, and all the supernatant fractions were pooled. Cells were separated from tissue debris by filtration through sterile nylon mesh (Sigma 200), followed by centrifugation at 1,500 g for 3 min. Cell pellets were pooled and resuspended in sterile DMEM containing 10% horse serum and 1% penicillin-streptomycin antibiotic solution. This suspension was then passed through sterile 40-μm cell filters (Falcon), and the filtered suspension was centrifuged again at 1,500 g. The resultant pellet was then resuspended in 10 volumes of DMEM containing 10% horse serum and 1% penicillin-streptomycin and plated onto uncoated 150-mm tissue culture dishes for 3 h in a 37°C incubator humidified with 5% CO2-air atmosphere. The unattached cells floating in the medium after the 3-h incubation were then transferred onto a fresh 150-mm uncoated tissue culture dish, and this procedure was repeated twice. As reported by Richler and Yaffe (37), this preplating technique helps to further enrich the myogenic population because most of the attached cells are fibroblasts and endothelial cells, whereas the floating cells are satellite cells. After the third preplating, cells were collected by centrifugation at 500 g for 4 min, and the resultant pellet was resuspended in DMEM containing 20% fetal bovine serum and 10% dimethyl sulfoxide and stored in liquid nitrogen until needed. As reported previously (5, 27), there is no noticeable deterioration in cell viability after freezing.

Satellite cell culture conditions. Frozen satellite cells from each age group were thawed and initiated into culture in proliferation media (Ham’s F-10 nutrient mix containing 20% fetal bovine serum, 1.0% chicken embryo extract, with 1% penicillin-streptomycin antibiotic mix, and 1% l-glutamine) on rat-tail collagen-coated tissue culture plates, as described by Rando and Blau (36). High-serum conditions were chosen to ensure no loss of proliferative cells due to differentiation, and cells were passaged before they reached confluence. All cultures were maintained at 37°C in a humid air atmosphere containing 5% CO2. Before the isolated satellite cells were used for any experiments, they were routinely monitored for myogenic purity by immunocytochemistry with a mouse monoclonal antibody specific for desmin (D3, in hybridoma supernatant form, obtained from the Developmental Studies Hybridoma Bank at the University of Iowa), an intermediate filament protein that is expressed only in myogenic cells (9, 30), by using the methods of Allen et al. (4). During the lifespan of the cultures, we also ensured that there was no loss of proliferative cells either by cell death (apoptosis) or by differentiation in growth medium. Apoptotic cells that could be identified by a compacted and fragmented chromatin after a Hoechst 33342 staining (20) (1/1000, Molecular Probes, Eugene, OR) were not detected in cultures maintained in high-serum media. Differentiated cells were detected with the mouse monoclonal antibody MP20 (hybridoma supernatant from Developmental Studies Hybridoma Bank at the University of Iowa) against sarcosmin myosin (a marker of the differentiated state) by using the methods of Yablonka-Reuveni and Rivera (53), and these routinely represented <3–5% of the cells in serum-rich medium.

Colony size distribution assays. Given the inherent heterogeneous nature of primary cell cultures, it was vitally important to examine the proliferative behavior of such cells at
the level of the individual, single cell. This was accomplished by utilizing colony size distribution assays as described by Smith et al. (46) and Pendergrass et al. (34). In brief, non-confluent satellite cells from each of the three different age groups were trypsinized and counted by use of a hemacytometer to calculate cell number as well as to ensure a single-cell suspension. About 100 cells from each group were inoculated into each of several collagen-coated 100-mm tissue culture dishes and left for 5 wk at 37°C, 5% CO2-95% air atmosphere at 98–100% humidity in serum-rich proliferation medium. Fresh medium was given to the cells weekly. This extended period was chosen to ensure that all the cells would have enough time to reach their maximum proliferative potential and thus to reduce variation between experiments due to differences in the clonal growth rates between groups. Pilot experiments using satellite cells from the hindlimb muscles of control 31-mo-old FBN rats, grown in the presence of 10 μM bromodeoxyuridine (BrdU) for the last 72 h of the 5-wk growth period, revealed that <3% of the cells had incorporated the label, thereby indicating that a great majority of cells had completed their last doubling (5 wk) and no longer in the proliferative phase of the cell cycle. Cells were then fixed in 1% glutaraldehyde and stained with 0.5% crystal violet. The number of cells in each colony (defined as one or more attached cells) was determined (up to a maximum of 256 cells) with a dissection microscope at ×20–40 magnification.

A total of 200–250 colonies was scored for each group (which amounted to ten to fifteen 100-mm tissue culture dishes per group). Colony size was designated by the number of doublings necessary for the initial single cell to reach the observed cell number in that colony and was expressed as the percent of colonies that had obtained a specified number of doublings in the 5-wk period. Hence, the larger the colony size, the greater the proliferation potential of the individual cell that gave rise to the progeny in that colony. Each of the groups was cloned in triplicate in three separate experiments. Parallel sets of cultures at the end of the 5-wk growth period were also stained with Hoechst 33342 and MF20 antibody, as described above, to ensure that the lack of proliferation was not due to increased apoptosis or due to differentiation. Parallel sets of plates were also stained with desmin, as described under Satellite cell culture conditions, to ensure that the cells constituting the colonies were myogenic.

Determination of CPD. Documentation of the decreased proliferative capacity of satellite cells with age in this strain of rats was also accomplished by quantitating the cumulative number of population doublings (CPD) attained over the entire course of their in vitro culture lifespan. Satellite cells obtained from the gastrocnemius muscles of 3- and 31-mo-old FBN rats were initiated into culture immediately after being harvested from the animal and were subcultured until the end of their in vitro lifespan. When the cells were subconfluent, they were harvested with trypsin and counted. At each passage, cell viability was monitored by trypsin blue dye exclusion (trypan blue stain 0.4%, GIBCO) to ensure >98% viability of the cells. Cells in early passage were replated in triplicate at a density of 1.5 × 10^3 cells/cm²; in late passage, cells were replated at a density of 3.5–4.0 × 10^3 cells/cm² in serum-rich proliferation medium. The culture medium was changed three times a week. At the time of cell isolation from the animal, cell populations from both young and old groups were considered to be at 1 population doubling. The number of doublings at every passage thereafter was calculated as log N/log 2, where N is the number of cells harvested divided by the number of cells inoculated, based on the methods of Smith et al. (46). Cell number was determined in triplicate by use of a Coulter counter. At each passage, we ensured that cells were not lost due to apoptosis or differentiation by monitoring cultures stained with Hoechst 33342 and MF20, respectively. Cultures were deemed to have reached the end of their in vitro lifespan when they failed to reach subconfluence after 3 wk of refeeding (20), at which point they were reassessed with Hoechst 33342 and MF20 to ensure that the loss of proliferation was not due to apoptosis or differentiation. A lack of proliferation was also verified by pulsing these late-passage cells with 10 μM BrdU (Sigma) for 3 days, and the labeling index was determined by quantitating the percentage of cells labeled with BrdU using a mouse monoclonal anti-BrdU antibody (BMC9318; Boehringer-Mannheim) utilizing the procedures provided by the company. Less than 1% of cells were labeled with BrdU in late-passage satellite cells from 31-mo-old FBN rats, indicating that a lack of proliferation in these cells was most likely due to replicative senescence.

Experimental Design and Methods to Determine Effect of Repeated Bouts of Atrophy-Recovery on Muscle Mass and Satellite Cells

Animals. Thirty-six FBN male rats, free of specific pathogens, were obtained from the NIH aging program (Harlan, Indianapolis, IN) at the ages of 25–26 mo, weighing 523 ± 10 g. Pairs of animals were housed in previously autoclaved bedding in microisolator cages and provided with autoclaved food and water ad libitum, in a room maintained at 21°C with a 12:12-h light-dark cycle. The animals were allowed to acclimatize to their new surroundings for ~2 wk before any experiments were done.

Overview of experimental design. One group of rats (N = 4) was killed as a control group (25-mo-old) at the start of the experiment. Body weight-matched pairs of rats were then assigned to one of two groups: a control group (N = 5) that was allowed normal caged activity for the entire duration (150 days) of the experiment and an experimental group (N = 30) that underwent three repeated cycles of hindlimb immobilization via casting, with each casting bout followed by a recovery period (see Fig. 1). In each group, four or five animals were killed to determine gastrocnemius muscle mass and to obtain satellite cells from those muscles at the end of hindlimb immobilizations 1 and 3 (groups C1 and C3 in Fig. 1) and after recovery periods 1 and 3 (groups R1 and R3 in Fig. 1). Animals in group R3 were further subdivided into two subgroups after the 9-wk recovery period: one of the subgroups (group R3 + I in Fig. 1; N = 6) was perfused with recombinant human IGF-I (hIGF-I, Austral Biologicals) onto its right gastrocnemius muscle via a catheter attached to a miniosmotic pump, whereas the other subgroup (N = 5) was perfused with normal saline solution (group R3 + S in Fig. 1), for an additional period of 2 wk to extend the last recovery period for a total of 11 wk. The gastrocnemius muscle was chosen as a target for hindlimb immobilization, as well as for pump implantation, because this muscle is not a postural muscle and has been shown to undergo significant atrophy (~30%) with age in this strain of rats (26). Also, because the gastrocnemius muscle is composed of ~57% of type IIb fibers (21), the fiber subtype that preferentially atrophies with increasing age (16), we wanted to study the effect of repeated immobilization on this muscle and its satellite cells. All animal protocols used were reviewed and approved by the Animal Welfare Committee of the University of Texas Health Science Center, Houston.

Hindlimb immobilization. Both hindlimbs of rats were immobilized with plaster of Paris for 10 days using the
Infusion of either saline or hIGF-I was accomplished via a catheter attached to a miniosmotic pump [Alzet model 2002 (14 days), Alza, Palo Alto, CA]. The catheters consisted of ~12 cm of highly flexible implantable tubing (0.048 in. OD, 0.02 in. ID; Cole Parmer), which was fenestrated at one of the ends using a microtipped soldering iron (Antex). The entire tubing was then gas sterilized with ethylene oxide for 48 h. The miniosmotic pumps were filled under aseptic conditions according to the manufacturer's instructions, either with sterile-filtered hIGF-I (Austral Biologicals) at a concentration of 0.16 μg/μl in saline or with sterile saline alone. The unfenestrated end of the catheter was mated with the osmotic pump to allow priming for optimal delivery on implantation by preincubation in sterile saline at 37°C for 24 h.

For pump implantation, the rats were anesthetized via an intraperitoneal injection with anesthetic cocktail consisting of 0.84 mg/kg ketamine, 0.54 mg/kg xylazine, and 2.2 mg/kg acepromazine. Incisions were made in the skin on the back ~4 cm caudal from the neck in the midscapular region. The pump was placed under the skin and secured by 2.0 Ethicon nylon sutures. The catheter was then tunneled under the skin to the small (~1 cm) incision near the proximal end overlying the gastrocnemius muscle on the lateral side of the right leg. After two more small cuts (one near the proximal end and the other near the distal end) were made in the fascial layers overlying the gastrocnemius muscle, the distal end of the catheter (fenestrated portion) was tunneled under the fascia of the gastrocnemius and secured in place by 2.0 Ethicon nylon sutures. A similar procedure was performed on the contralateral (left) leg of each animal, with the catheter in place, but without it being attached to an osmotic pump, serving as a “sham” operation control. All surgical manipulations were performed under a sterile field, and standard postoperative care was administered to the animals. At the end of the infusion period (14 days), the osmotic pumps were removed, and any remaining solutions within the pumps were aspirated via a syringe to verify that the pumps had functioned properly. On the basis of the osmotic pump specifications, the total volume delivered per day onto the gastrocnemius muscles was 12.0 ± 0.2 μl (~0.5 μl/h).

Fig. 1. Schematic representation of experimental design to illustrate repeated cycles of atrophy-recovery and the different treatments. A total of 8 experimental groups were employed, as indicated by the circled numbers 1–8. Group 1 (25-mo Ctrl) was an untreated control group killed at 25 mo of age, before any immobilization, serving as a reference for the percentage of atrophy occurring during immobilization. Group 2 (C1) was killed after the first 10-day bout of hindlimb immobilization (cast 1) at the age of 25 mo. Group 3 (R1) was killed after the first 3-wk recovery period following cast 1 at the age of 26 mo. Group 4 (C3) was killed after the third 10-day period of hindlimb immobilization (cast 3) at 27 mo of age. Group 5 (R3) was killed after a 9-wk recovery period following cast 3 at the age of 29 mo. Group 6 (R3+S) and Group 7 (R3+I) were killed at 30 mo of age after receiving saline or insulin-like growth factor I (IGF-I) perfusion, respectively, on the gastrocnemius muscle for an additional 2 wk after the 9-wk recovery period (10th–11th week of recovery after cast 3). Group 8 (30-mo Ctrl) was an untreated control group maintained from the start of the experiment with normal caged activity for the entire duration of the experiment (~150 days) to serve as normal aging control animals. Numbers in parenthesis indicate number of rats for each group. At each time point (indicated by the circled numbers), both the gastrocnemius muscles were obtained, and satellite cells were harvested from those muscles for the various analyses.
Assessment of muscle atrophy-hypertrophy. Tissue collection. At the indicated time points designated by the circled numbers shown in Fig. 1, the rats were killed via an overdose of anesthetic cocktail (see above), and both gastrocnemius muscles were removed. After careful dissection and trimming of excess connective tissue and fat, the muscles were quickly weighed on an electronic analytical balance before further processing. To normalize muscle masses to account for any age and/or body weight differences between groups, tibia length from each leg was measured via slide calipers, and muscle mass was expressed as a ratio of the gastrocnemius mass to its corresponding tibia length. The plantaris muscles from each leg, as well as the heart for groups receiving either saline or IGF-I infusion, were also removed and weighed to document absence of systemic effects of IGF-I. A small portion (~50–100 mg) of the muscle from the midbelly of the gastrocnemius was quickly cut, flash frozen in liquid N$_2$, and stored at −80°C for protein determination (see Total Muscle Protein Determination). The remaining muscle was immediately used for satellite cell isolation after pooling muscles from each group (see Satellite Cell Isolation and Culture Conditions). In the groups that received saline or IGF-I infusion, the gastrocnemius muscles of both the contralateral sham (left) and infused (right) legs were cut into two parts (~50 mg portions) in the midbelly of the muscle near the region approximating the fenestrated portions of the catheter, flash frozen in liquid N$_2$, and saved at −80°C for determination of total protein and IGF-I peptide concentration (see Total Muscle Protein Determination and IGF-I Peptide Determination). The remaining portion from the midbelly of the gastrocnemius muscle was immediately used for satellite cell isolation after pooling muscles within each group.

Total Muscle Protein Determination. Muscle protein was determined from whole muscle homogenates using methods modified from Fluck et al. (25). Briefly, frozen gastrocnemius muscles from each of the eight different groups (Fig. 1) were homogenized for 3 × 30 s at 4°C in buffer (50 mM HEPES, pH 7.4, 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM Na$_2$P$_2$O$_7$, 10 mM H$_2$O$_2$, 100 mM β-glycerophosphate, 25 mM NaF, 50 μg/ml leupeptin, 50 μg/ml pepstatin, and 33 μg/ml aprotonin) using 2.5 ml of buffer per 100 mg of tissue with a Polytron mixer. Protein concentration was estimated by using the Bio-Rad DC protein kit according to the manufacturer’s instructions, with bovine serum albumin used as a standard. Coomassie blue staining of proteins analyzed by SDS-PAGE verified protein concentration and integrity of the extracted proteins. Total protein per whole muscle was calculated from the product of the concentration and the original wet weight of the whole muscle.

IGF-I Peptide Determination. The concentration of hIGF-I peptide within the gastrocnemius muscles in contralateral sham and infused legs from both the saline- and IGF-I-infused groups was determined using a two-site immunoradiometric assay (IRMA) commercial kit specific for hIGF-I (Diagnostic Systems Laboratories, Webster, TX), as described previously (17). Briefly, an ~50-mg portion of the muscle was cut out from the midbelly of the gastrocnemius muscle and homogenized on ice in 7 volumes of PBS (pH 7.4) containing protease inhibitors (in mM: 0.06 leupeptin, 0.01 aprotonin, 0.04 antipain, and 2.0 phenylmethylsulfonyl fluoride). Homogenates were then extracted in 3 volumes of acid-alcohol extraction solution for 30 min at room temperature to dissociate the IGF binding proteins and were subsequently neutralized with 4 volumes of neutralization solution. The neutralized samples were frozen overnight at −80°C, after which they were thawed and microfuged at 13,000 g for 10 min, and the supernatant was used for the IRMA assay. The IRMA assay was performed in duplicate on hIGF-I standards and positive controls, which were provided in the kit, and on the extracted/neutralized samples, according to the manufacturer’s instructions. Gastrocnemius muscle samples from separate 31-mo-old aged rats were used as negative controls to verify the absence of cross-reactivity between the hIGF-I and endogenous rat IGF-I in this assay. All samples were analyzed on the same day with the same reagents. The intra-assay coefficient of variation for this assay was ~10%.

To document levels of endogenous rat IGF-I peptide concentration in gastrocnemius muscles and plasma (from blood samples obtained from the aorta before death of the animal) of saline-infused and IGF-I-infused rats, a dual-antibody radioimmunoassay kit (Diagnostic Systems Laboratories, Webster, TX) specific for rat IGF-I was utilized. Samples were extracted and prepared as described for the hIGF-I measurements. Additionally, porcine muscle samples were included as negative controls to verify the absence of cross-reactivity with hIGF-I (because porcine and hIGF-I have identical amino acid sequences), and rat liver samples were used as positive controls. All samples were assayed on the same day using the same reagents. The intra-assay coefficient of variation was ~14%.

Satellite cell isolation and culture conditions. Satellite cells were isolated from the remaining portions of the midregion of the gastrocnemius muscles from saline- or IGF-I-infused legs (groups 6 and 7; Fig. 1) or from the midregions of the gastrocnemius muscles from animals in the other groups (groups 1–5 and 8; Fig. 1) immediately after the muscle was removed from the animal, and parts of it were cut and frozen for further analysis as described under Tissue Collection. The gastrocnemius muscles from four to six animals from each group were pooled to ensure an adequate amount of tissue for satellite cell isolation. The methods for the isolation were as described above, after modifications from Allen et al. (5). The cells were then cultivated in serum-rich proliferation media and assessed for myogenic purity with D3. Cell loss due to apoptosis and/or differentiation was also monitored by using Hoechst 33342 staining and anti-sarcomeric myosin antibody as detailed under Satellite cell culture conditions in the normal aging part of our study.

Colony size distribution assays. To determine the effects of the different treatments on the proliferative potential of satellite cells isolated from gastrocnemius muscles from animals in each of the eight groups (see Fig. 1), the cells were initiated into culture at clonal density after one passage (as detailed above) for clonogenicity assays to estimate colony size, which is a reflection of the remaining in vitro proliferation potential of individual cells. Approximately 100 cells from each group were plated per 100-mm dish, and a total of 150–200 colonies were scored for each of the eight groups (which amounted to ten to fifteen 100-mm tissue culture dishes per group). Each of the groups was cloned in triplicate, and the colony sizes attained were averaged for all three. In total, three such separate experiments were done for each of the groups. After 5 wk of growth in proliferation-rich media, cells were fixed with 1% glutaraldehyde and stained with 0.5% crystal violet. Parallel sets of cultures were also stained with anti-desmin to ensure that the cells were myogenic and with Hoechst 33342 and MPF20 antibody, as described under Satellite cell culture conditions, to ensure that lack of proliferation was not due to increased apoptosis or to differentiation, respectively. Additionally, we also ensured that the 5-wk growth period was indeed sufficient to allow cells to reach their full proliferative potential by pulsing another set of parallel plates with 10 μM BrdU for the last third of the
and cultivated for 5–6 days in low serum conditions, the satellite cells from 31-mo-old animals readily formed characteristic myotubes, as shown in a representative photomicrograph in Fig. 2B, in a fashion similar to those from 3-mo-old young rats (data not shown), consistent with the observations made by Allen et al. (6).

Replicative Capacity of Satellite Cells From FBN Rats

Before proceeding with the experiment to determine whether repeated atrophy-recovery cycles diminish satellite cell proliferative potential in FBN, it was necessary first to establish an age-dependent decrease in proliferative potential of satellite cells isolated from the aged gastrocnemius muscles in this particular strain. The FBN rats were specifically chosen for study because their average lifespan is ~34 mo, and, due to this longer lifespan, they show a more pronounced muscle atrophy, especially of their hindlimb muscles, and therefore may provide a better model for the atrophy seen in aged humans (especially >85 yr). Although Gomes and Booth (26) reported an age-associated atrophy of the gastrocnemius muscles (~30% loss of muscle mass in 31-mo-old compared with 3-mo-old FBN rats), no studies have looked at proliferation potential of satellite cells from this strain of rats. Schultz and Lipton (43) and Dodson and Allen (22) showed an age-related decrease in satellite cell proliferative capacity using Sprague-Dawley and Fischer 344 rats, respectively. However, their experimental methods did not account for the inherent heterogeneity of primary cultures to get a more accurate representation of the replicative potential of individual cells in the whole population, which we have addressed by using clonogenicity assays. In the current study, cells were isolated from each of the three different age groups [young (3 mo), adult (18 mo), and old (31 mo)] and initiated into culture at clonal density to allow each individual cell to replicate to its fullest potential. If the cell has replicative reserves, the parent cell would replicate to give progeny, which manifests itself as a colony, the colony size being proportional to the proliferative potential contained within the parent cell. Satellite cells from young FBN rats show a greater preponderance to form large-sized colonies compared with corresponding cells from older animals, as illustrated with a representative set of culture plates after staining the colonies with 0.5% crystal violet (Fig. 3A, top). The number of such large-sized colonies was then quantitated using a colony size distribution assay by determining the percent of colonies that are able to form a specified size in the 5-wk-growth period. This is portrayed in Fig. 3A (bottom) by a dramatic shift in the colony size values to the right (i.e., colonies with ≥256 cells or more than eight cell doublings) in young (3 mo) compared with old (31 mo) FBN rats. Profiles with more than eight cell doublings accounted for 20–25% of the total of 200–250 colonies analyzed in triplicate in

% of colonies with

Statistical analysis. All values are means ± SE. Differences between the groups for muscle weights, total protein, and IGF-I peptide levels were analyzed by ANOVA with post hoc testing (Scheffé’s test) using the Statistica software package. Differences in colony size distributions between saline- and IGF-I-infused groups were accomplished by using a two-tailed Student’s t-test. For all statistical tests, the 0.05 level was used as statistical significance.

RESULTS

Morphological Analysis of Primary Cultures of Satellite Cells From 31-Mo-Old Aged FBN Rats

A major aim of this study was to determine the effects of multiple cycles of atrophy induced by hindlimb immobilization with intervening recovery periods on skeletal muscle mass and proliferative capacity of the satellite cells from those muscles. To ensure the quality of the satellite cells isolated from these aged muscles, we stained the cultured satellite cell preparations with an antibody specific to the muscle-specific intermediate filament protein, desmin (4, 9, 28). We were able to routinely harvest relatively pure (>90–95%) myogenic populations, as illustrated in a representative photomicrograph in Fig. 2A. When the serum-rich proliferation medium was changed to differentiation medium consisting of DMEM, 2% horse serum, and 1% mixture of insulin-transferrin-selenium and avidin-biotin-peroxidase complex (Vector), both diluted 1:100 in PBS, with 3-amino-9-ethyl carbazole as the chromogen (Sigma). Cells were photographed in phase-contrast at ×10 magnification. More than 95% of cultures (obtained after harvesting and isolation using the methods described) stained with desmin, indicating high myogenic purity in these cultures. Arrow indicates a fibroblast; these cells readily form myotubes as shown.

Fig. 2. Morphological analysis of primary cultures of satellite cells obtained from gastrocnemius muscles of 31-mo-old Fischer 344/Brown Norway F1 hybrid (FBN) rats. A: representative photomicrograph of satellite cells stained with a monoclonal antibody against the muscle-specific marker desmin (D3, 1.5 dilution in PBS, pH 7.4) and visualized by using biotinylated horse anti-mouse antibodies and avidin-biotin-peroxidase complex (Vector), both diluted 1:100 in PBS, with 3-amino-9-ethyl carbazole as the chromogen (Sigma). Cells were photographed in phase-contrast at ×10 magnification. More than 95% of cultures (obtained after harvesting and isolation using the methods described) stained with desmin, indicating high myogenic purity in these cultures. Arrow indicates a fibroblast; these are routinely present (albeit in significantly lower numbers) in primary cultures. B: representative photomicrograph of myotubes stained with a monoclonal antibody specific for sarcomeric myosin (MF20, 1.5 dilution in PBS), an indicator of differentiation, visualized using the same methods as for desmin, and photographed at ×4 magnification. When the cultures are allowed to reach confluence and the medium is changed to low-serum conditions (DMEM with 2% horse serum, and 1% insulin-transferrin-selenium mixture), the satellite cells readily form myotubes as shown.
satellite cells from the 3-mo group, in contrast to 3–5% for the 31-mo group.

We further validated these findings of decreased proliferation potential in aged satellite cells by determining the number of CPD attained in mass cultures over the entire duration of their in vitro culture life-span, as illustrated in Fig. 3B. Freshly isolated satellite cells from both young and old FBN rats were serially passaged in triplicate (passaged before cells became confluent) until the cells senesced. Satellite cells derived from young (3 mo) rats started to proliferate earlier (2 days) and in greater numbers than those from older rats (31 mo) (4–5 days). Both populations underwent a period of rapid proliferation, after which the growth rates slowed and finally ceased. Satellite cells isolated from young rats had a significantly greater ($P < 0.01$) proliferative capacity than those isolated from older donors, as reflected by the higher CPD of satellite cells from young donors (CPD = 20) than of those from old donors (CPD = 12). Overall, these results demonstrate for the first time in FBN rats that the proliferative potential of satellite cells from these aged muscles is markedly diminished compared with that of cells from young animals and concur well with previous observations in other strains of aged rats (22, 43).

Effects on Body Mass With Multiple Cycles of Hindlimb Immobilization and Recovery

Body weights from each of the eight groups of aged FBN rats were recorded at specific time points, as detailed in MATERIALS AND METHODS and in the legend to Table 1. There was a significant ($P < 0.05$) decrease of ~14% in body mass after the first bout of hindlimb immobilization (C1) relative to 25-mo-old untreated control rats that persisted through the first 3-wk recovery period (R1) (Table 1). Indeed the body mass was significantly lower ($P < 0.05$) by ~9%, even during the second 3-wk recovery period after the second bout of hindlimb immobilization compared with untreated 25-mo-old control rats. Body weights recovered back to the 25-mo untreated control weights only after an ex-

Fig. 3. Proliferation potential of skeletal muscle satellite cells decreases with age. A: colony size distribution of satellite cells derived from young (3 mo), adult (18 mo), and old (31 mo) FBN. Satellite cells from each of the three age groups were seeded at clonal density of ~100 cells per 100-mm tissue culture dish and grown in proliferation media for 5 wk at 37°C in 5% CO$_2$-95% air mixture. Plates were then fixed and stained in 0.5% crystal violet and photographed. Top shows a representative set of plates for each of the 3 age groups. Colony size was then estimated by counting the number of cells per colony for up to 256 cells. The data represent the percentage of the total colonies (y-axis) that are able to attain a specified size (x-axis) (numbers represent the number of cell doublings attained during the continuous 5-wk culture period during which the cells were not passaged). Results are representative of 3 separate experiments. B: cumulative population doublings (y-axis) of satellite cells derived from 3-mo-old and 31-mo-old FBN rats attained in culture from the time of isolation from the animal (time 0) over the duration of their lifespan (x-axis). Each point represents the time at which cells were passaged when they were subconfluent and were routinely assessed for viability and myogenic purity with trypan blue staining and D3. These cells were also monitored with Hoechst 33342 and anti-sarcomeric myosin antibody staining to ensure no loss of proliferative cells due to apoptosis and/or differentiation, respectively. Results are representative of 3 separate experiments. Total cumulative population doublings attained was significantly increased by 67% in satellite cells derived from 3-mo-old rats compared with those from 31-mo-old rats.
tended recovery period of 9 wk (R3) after the third bout of hindlimb immobilization (C3). During this extended recovery phase, body weights were recorded weekly, and the body mass approached 25-mo untreated control weights during the seventh week of the 9-wk recovery period (519 ± 15 g). By the end of the ninth week of recovery, body weights were similar to those of untreated age-matched (30-mo-old) control animals. A 2-wk infusion of either saline or recombinant hIGF-I onto the gastrocnemius muscles after the ninth week of recovery did not affect body weights significantly, either between the saline and IGF-I groups or compared with the subgroup of rats killed immediately after the ninth week of recovery (R3). Their body mass was similar to age-matched untreated control (30-mo-old) animals as well. Careful attention to food and water intake was monitored by weighing out the food pellets and also by monitoring fecal output during the entire course of this experiment. We observed no significant decrease in either food intake or fecal output compared with untreated age-matched control animals.

Effects of IGF-I Infusion on Gastrocnemius Muscle Mass and Protein Content From Hindlimbs Subjected to Repeated Cycles of Atrophy-Recovery

The gastrocnemius muscles atrophied 11% ($P < 0.05$) from 25 to 30 mo of age in untreated control animals, indicating that age-associated atrophy was occurring (Fig. 4). In the hindlimb-immobilized groups, there was a 30.8% and 40.4% loss ($P < 0.05$) of gastrocnemius muscle mass (normalized to the corresponding tibia length) after the first (C1) and third (C3) 10-day bout of hindlimb immobilization, respectively (Fig. 4), compared with untreated control (25-mo-old) animals. The tibial lengths only varied by 3.7%. These results demonstrate not only that immobilization per se produces significant atrophy in gastrocnemius muscles, but also that repeated bouts of immobilization further accentuate this atrophy, as shown by a further loss of 13.8% in mass between groups C1 and C3 (Fig. 4). Interestingly, there was no significant regain of muscle mass after the first 3-wk recovery period (R1) or after the final 9-wk recovery period (R3), denoting an impaired muscle regrowth. Remarkably, 46% ($P < 0.05$) of this atrophy was rescued by 2 wk of IGF-I infusion during the tenth and eleventh weeks of recovery (R3+IGF-I, Fig. 4) from the third immobilization, compared with saline-infused control animals (R3+S, Fig. 4), hence reflecting significant muscle regrowth due to IGF-I application. IGF-I, by itself, produced a 12% and 13% ($P < 0.05$) increase in whole muscle mass relative to either the contralateral gastrocnemius muscle (R3+IGF-I) or with those of the saline-infused muscles (R3+S), respectively (Fig. 4).

The total muscle protein content (mg/whole muscle) essentially paralleled the changes observed in muscle mass in each of the eight groups (Fig. 5). Important comparisons among the eight different groups revealed the following: 1) total protein per whole gastrocnemius muscle decreased 12% ($P < 0.05$) from 25 to 30 mo of age in untreated control animals, substantiating further the age-associated atrophy that is commonly observed in aged mammals. 2) There was a 39.5% and 50.7% ($P < 0.05$) reduction in total protein after a single 10-day bout of hindlimb immobilization (C1) and after the third 10-day bout (C3), respectively. As seen with muscle mass, repeated bouts of hindlimb immobilization further exacerbated this loss in total protein

### Table 1. Body weights before immobilization, after repeated bouts of immobilization, and after recovery following immobilization, with and without IGF-I

<table>
<thead>
<tr>
<th>Group</th>
<th>Group Designation</th>
<th>Never Immobilized</th>
<th>Day Immobilized</th>
<th>Day Casts Removed/Start of Recovery Period</th>
<th>End of Recovery Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25-mo</td>
<td>523 ± 9</td>
<td>523 ± 9</td>
<td>452 ± 9a</td>
<td>470 ± 10b</td>
</tr>
<tr>
<td>2</td>
<td>C1</td>
<td>523 ± 9</td>
<td>476 ± 13c,d,e</td>
<td>447 ± 14a</td>
<td>536 ± 11b</td>
</tr>
<tr>
<td>3</td>
<td>R1</td>
<td>523 ± 9</td>
<td>476 ± 13c,d,e</td>
<td>447 ± 14a</td>
<td>531 ± 12c</td>
</tr>
<tr>
<td>4</td>
<td>C3</td>
<td>523 ± 9</td>
<td>476 ± 13c,d,e</td>
<td>447 ± 14a</td>
<td>531 ± 12c</td>
</tr>
<tr>
<td>5</td>
<td>R3</td>
<td>523 ± 9</td>
<td>476 ± 13c,d,e</td>
<td>447 ± 14a</td>
<td>531 ± 12c</td>
</tr>
<tr>
<td>6</td>
<td>R3 + Saline</td>
<td>523 ± 9</td>
<td>476 ± 13c,d,e</td>
<td>447 ± 14a</td>
<td>531 ± 12c</td>
</tr>
<tr>
<td>7</td>
<td>R3 + IGF-I</td>
<td>523 ± 9</td>
<td>476 ± 13c,d,e</td>
<td>447 ± 14a</td>
<td>531 ± 12c</td>
</tr>
<tr>
<td>8</td>
<td>30-mo</td>
<td>573 ± 16</td>
<td>573 ± 16</td>
<td>573 ± 16</td>
<td>573 ± 16</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4–6 animals per group (in grams). The experiment was initiated with a total of 36 Fischer 344/Brown Norway F1 hybrid rats. Four untreated control animals were immediately killed at the very beginning of the experiment, before any immobilization (25 mo). Five animals were then separated out and allowed normal caged activity for the entire duration (~150 days) of the experiment to serve as normal aged control animals (30 mo). The remaining 22 animals were then hindlimb immobilized for 10 days, and body weights were obtained on the 10th day after the casts were removed, and 4 animals (group C1) were killed to obtain gastrocnemius muscle for the various analyses. The remaining 23 animals were allowed a 3-wk recovery period, at the end of which body weights were recorded before the second cycle of hindlimb immobilization, and 4 animals were killed at this point (group R1) to obtain gastrocnemius muscles. The remaining 19 animals were then subjected to the second 10-day bout of hindlimb immobilization, followed by a second 3-wk recovery period, and subsequently a final bout of 10-day hindlimb immobilization. Body weights were recorded at the end of each of these time points as well (data not shown), and 4 animals were killed at the end of the third immobilization bout (group C3), after body weights were recorded. The remaining 15 animals were then allowed a recovery period of 9 wk, and their body weights were recorded weekly. At the end of the 9-wk period, 4 animals were killed after body weights were recorded (group R2), and the remaining 11 rats were divided into 2 groups (group R3 + saline; N = 5) and (group R3 + IGF-I; N = 6) to be infused with either saline or IGF-I, respectively, for an additional 2 wk after the 9-wk recovery period. Body weights were recorded at the end of 11 wk of recovery for each of these 2 latter groups before the gastrocnemius muscles were removed. a, b and c Recovery period of 3, 9, and 11 weeks, respectively. *Body weight at the end of the second 3-wk recovery period, just before the third 10-day hindlimb immobilization bout. $P < 0.05$ compared to 25-mo-old untreated control animals.
by 18.4% \((P < 0.05)\) (compare C1, 354.3 ± 10 mg vs. C3, 289.2 ± 14 mg; Fig. 5), 3) Neither the first 3 wk (R1) nor the last 9 wk (R3) of recovery had any significant effect on total protein content, substantiating that multiple cycles of atrophy-regrowth impaired muscle regrowth in this age group. 4) Finally, IGF-I infusion during the tenth and eleventh weeks of recovery from the third immobilization increased total protein content by 27% and 26% \((P < 0.05)\) compared with contralateral (R3+IGF-I animals, Fig. 5) and saline-infused (R3+S animals, Fig. 5) gastrocnemius muscles, respectively. Furthermore, IGF-I infusion was able to rescue ~76% of the decreased total protein content, hence bringing the values similar to that seen in 30-mo-old untreated control rats. One of the reasons for this apparent disproportionate increase in total muscle pro-
tein content compared with the muscle wet weight (Fig. 5 vs. Fig. 4) with IGF-I infusion may be the fact that the infusion protocol used produced a gradient effect in the amount of IGF-I that diffused into the muscle, as originally reported by Adams and McCue (1). Also, we observed a trend (albeit nonsignificant) toward an increase in the total protein concentration in the IGF-I-infused muscle samples, similar to that observed by Adams and McCue as well. Thus these results not only confirm, but also extend on, the observations they had made in young rats.

**IGF-I Infusion Results in a Localized Increase in IGF-I Concentration and Has No Systemic Effects**

IGF-I infusion resulted in increases in hIGF-I concentrations only in the infused muscle [195 ± 58 (SE) ng/g], as evidenced by a lack of detectable hIGF-I levels in either the contralateral gastrocnemius muscle or the saline-infused control muscle. Also, there was no statistically significant difference in the endogenous rat IGF-I level in gastrocnemius muscles between saline-infused [81 ± 12 (SE) ng/g] and IGF-I-infused [75 ± 17 (SE) ng/g] groups, suggesting that exogenous IGF-I application was not affecting endogenous rat IGF-I levels. At the doses and duration used in this study, IGF-I infusion did not appear to have systemic effects based on the following observations. 1) There was no evidence of increased body weight gain in the control saline-infused groups (531 ± 6 g in saline-infused vs. 528 ± 15 g in IGF-I-infused). 2) The heart weights were similar between the saline- and IGF-I-infused groups (506 ± 9 mg with saline infusion vs. 510 ± 12 mg with IGF-I infusion). 3) Weights of the underlying plantaris muscle, which lies directly deep to the gastrocnemius muscle, were similar in both the saline- and IGF-I-infused groups (680 ± 16 mg in saline-infused vs. 684 ± 22 mg in IGF-I-infused), suggesting that the infused IGF-I affected only the target muscle. 4) Muscle weights, total protein content, and hIGF-I concentration of the contralateral (sham) gastrocnemius in the IGF-I-infused group (R3+IGF-I) were similar in all these respects to those of the saline-infused (R3+S) gastrocnemius muscle (Figs. 4–5). 5) Finally, infusion of recombinant hIGF-I had no effect on the endogenous rat plasma IGF-I concentrations, because the plasma IGF-I levels were similar to those found in saline-infused control rats (458 ± 70 ng/ml in saline-infused rats vs. 471 ± 39 ng/ml in IGF-I-infused rats). Also, no hIGF-I could be detected in rat plasma.

**Effect of IGF-I on the Proliferative Potential of Satellite Cells From Gastrocnemius Muscles Subjected to Repeated Bouts of Hindlimb Immobilization**

Satellite cells isolated from the gastrocnemius muscles at the end of each time point (C1 through R3+IGF-I) and the two untreated control groups (25- and 30-mo-old) were then subjected to clonogenicity assays to determine their proliferation potential in culture. Typical colony formation, as seen after the different treatments after a 5-wk growth period in culture, is illustrated in Fig. 6. Qualitatively, there is a dramatic reduction in colony size after the first 10-day bout of hindlimb immobilization (C1), which persists up to after 11 wk of recovery (see Fig. 6, B–F). Only after IGF-I infusion for two additional weeks after the ninth week of recovery does the colony size increase. Indeed, the colony sizes of satellite cells from IGF-I-infused gastrocnemius muscles are significantly larger than from either 25- or 30-mo-old untreated control groups (compare Fig. 6G to Fig. 6, A and H).

The colony sizes were then quantitated by determining the distribution of the colonies of different sizes as the percentage of colonies containing at least a specified number of cells 5 wk after seeding (Fig. 7). The number of colonies per dish did not change from week 1 to week 5, indicating that the migration of cells or reseeding of clones did not alter the colony size distributions. There was an age-associated decrease of ~33% in the ability of satellite cells to produce colonies of 256 or more cells, reflecting eight or more cell population doublings at 30 mo (Fig. 7) compared with 25-mo-old untreated control rats (Fig. 7). Overall, there was a greater preponderance of small-sized colonies (distribution shifted to the left) with either one (C1) or three bouts (C3) of hindlimb immobilization (Fig. 7). Moreover, the recovery periods (R1 and R3) were unable to shift the colony distributions back to the right to 25-mo-old untreated control levels (Fig. 7), suggesting a diminished proliferative capacity, possibly due to a potential exhaustion of the replicative reserves of the satellite cells that were subjected to repeated bouts of atrophy by hindlimb immobilization. The ability to produce colonies of more than eight cell population doublings was significantly reduced by ~70% with multiple atrophy-recovery cycles. Compared with satellite cells from 25-mo-old rats (Fig. 7), in which 9% of colonies were able to produce colonies of more than eight cell doublings, only 2–4% of cells cloned from groups C1 through R3+S (Fig. 7) were able to reach this state of proliferation. However, if one examines the percentage of colonies able to yield colonies of ≥8–16 cells, reflecting 3–4 cells doublings in groups C1 through R3+S, it appears that these cells still possess a modest proliferative capacity that is similar to the levels seen in untreated controls at 25 and 30 mo (15–17% in groups C1 through R3+S vs. 15% in 25-mo-old and 14% in 30-mo-old control rats). These results therefore appear to indicate that satellite cells with the higher replicative potentials are lost with multiple atrophy-recovery cycles. Remarkably, 2 wk of IGF-I infusion in the tenth and eleventh weeks of recovery from the third hindlimb immobilization resulted in a shift of the colony size distribution to the right and produced ~13% of colonies that were able to attain more than eight cell doublings. This represents an increase of ~31 and 54% above those formed in 25- and 30-mo-old untreated control groups, respectively, thereby suggesting that IGF-I restores the apparently depleted proliferative capacity that multiple cycles of atrophy-regrowth had produced.
DISCUSSION

In this study, we have evaluated the effects of multiple cycles of atrophy induced by hindlimb immobilization, with intervening periods of recovery, on gastrocnemius muscle mass and the proliferative potential of satellite cells from this muscle in old rats. Several lines of evidence have implicated physical inactivity to play a vital role in the loss of muscle mass in many clinical situations (39). Immobilization as a consequence either of the condition itself (e.g., cancer or femoral fractures) or the treatment for the condition (e.g., chemotherapy, radiation, surgery) starts the loss of skeletal muscle mass, which then leads to more immobility and consequently exacerbates the muscle mass loss (39). Therefore, we utilized the model of hindlimb immobilization to produce inactivity of skeletal muscle without the direct effects of the disease on muscle mass. More importantly, this model, for the most part, looks at muscle regrowth from atrophy, rather than muscle regeneration, as seen with myotoxin treatments such as bupivacaine. Therefore, this model may more closely replicate limb immobilization in humans (12), especially in those conditions in which individuals are confined to wheelchairs or beds, and, consequently, may be closer to the physiological regrowth seen in humans after bouts of atrophy induced

Fig. 6. Representative clonogenicity assays using satellite cells harvested from gastrocnemius muscles from the two control groups (25 and 30 mo) and each of the six experimental groups. Group designations are as described in the legend to Table 1. Colonies of satellite cells harvested from gastrocnemius muscles of FBN of untreated 25-mo-old control rats (A), from 25-mo-old rats after a single bout of casting for 10 days (B), from 26-mo-old rats at the end of the first 3-wk recovery period (C), from 27-mo-old rats after the third 10-day period of hindlimb immobilization (D), and from 29-mo-old rats after the third recovery period of 9 wk (E). F and G: colonies from satellite cells derived from gastrocnemius muscles of 30-mo-old rats perfused for 2 additional weeks with saline or IGF-I, respectively, after the 9-wk recovery period. H: colonies of satellite cells from the gastrocnemius muscle of untreated 30-mo-old control rats. Each group (comprising satellite cells pooled from 4–5 animals) was cloned in triplicate using the corresponding satellite cells after the first passage in culture. Cells were grown in serum-rich proliferation media for 5 wk, at the end of which the cells were fixed in 1% glutaraldehyde and subsequently stained in 0.5% crystal violet and photographed.

Fig. 7. Colony size distribution of colonies represented in Fig. 6. Colonies were counted using a dissection microscope at ×10 magnification. A colony is defined as any attached cell, and the colony size is determined by the number of cells within each colony, counted up to a maximum of 256 cells. The percentage of total colonies (y-axis) that were able to attain a specified size, denoted by the number of cell doublings attained in culture over the 5-wk growth period (x-axis), is shown in the representative panels. A total of 150–200 colonies were counted for each group, and the results are representative of 3 separate experiments. Group designations are as described in Fig. 6 and in the legend to Table 1.
by a sequence of clinical events, such as major surgeries, rehabilitation from falls, and injuries. From a functional aspect, this model causes ~50% atrophy of the fast-twitch type II fibers (11), the fiber type that is preferentially atrophied with aging (16), in contrast to the hindlimb unloading (tail suspension) model, which only causes ~15% atrophy in the fast-twitch fibers (2).

When old skeletal muscles were subjected to repeated bouts of atrophy-recovery, we anticipated that recovery of skeletal muscle mass would be complete, associated with an increased satellite cell proliferation response (above and beyond that of normal untreated control animals) to compensate for the preceding atrophy after the very first bout of hindlimb immobilization. With subsequent cycles of atrophy followed by recovery, we expected that, after the third bout of hindlimb immobilization, muscle mass would recover slowly compared with the first recovery period and would be associated with an inability of satellite cells to proliferate sufficiently, secondary to the forced depletion of their proliferative reserves due to the previous cycles of regrowth of the muscle. Such an interpretation was reasonable considering the previously reported studies of repeated bouts of skeletal muscle regeneration (either with a myotoxin (42) or in DMD (50, 52)), which consequently subjected the satellite cells to multiple rounds of replication, forcing on them extra doublings to compensate for the loss of myonuclei from the damaged fibers and ultimately resulting in a marked diminishment of their proliferative reserves, as evidenced by a decreased cloning capacity when cultured in vitro. We envisioned that this would uncover a contribution of defective satellite cell proliferation to the muscle weakness in physical frailty.

On the basis of the results of this study, we could not directly test this hypothesis, because the muscles did not regrow from their atrophied state, even during the first 3-wk recovery period. The lack of any regrowth, even after the first recovery cycle, was surprising, given that our laboratory had previously published (12) a complete recovery of skeletal muscle mass in adult rats on the third week after ending 10 days of hindlimb immobilization. Therefore, these results suggest that, in aged 25- to 30-mo-old FBN rats, when subjected to periods of inactivity-induced atrophy, there is impaired regrowth of their lost muscle mass even after extended periods of recovery.

Examination of satellite cell proliferative potential after hindlimb immobilization in 25- to 30-mo-old FBN rats revealed the unexpected finding of a failure to recover in the in vitro proliferative potential (after the first bout of atrophy), which remained depressed even after an 11-wk recovery period (Fig. 6, B–F). Given the failure to regrow the atrophied muscle mass to control levels after the first bout of hindlimb immobilization, it is unlikely that the satellite cell’s proliferative reserves were depleted enough to explain the persistently diminished proliferative potential seen after 11 wk of recovery (Fig. 6F). Indeed, the decrease in satellite cell proliferative potential seen after just one bout of hindlimb immobilization (Fig. 6B) is in direct contrast to what one might expect in light of the previous results by Schultz et al. (41), who showed that in vivo satellite cell mitotic activity is decreased as early as 24 h after hindlimb unweighting by tail suspension in juvenile rats. If the same were to be assumed in aged rats, then in vivo proliferations would have been conserved and, consequently, would have resulted in an increase in the in vitro proliferative potential during the recovery period. Because our results are opposite of what one might expect (i.e., decreased in vitro proliferative potential), and, moreover, because our techniques did not measure satellite cell mitotic activity directly, the question of whether the decreased in vitro proliferative potential seen in the hindlimb-immobilized aged rats is due to an in vivo increase in satellite cell mitotic activity (potentially as a compensation for the unloading in the aged muscle) remains to be critically evaluated.

Based on the findings of Donoghue et al. (23), who suggested that satellite cells retain a memory of the environment from which they were derived, one may speculate that the decreased proliferative potential seen in our culture dishes, even after the first bout of hindlimb immobilization, could be a manifestation of the combined aged-physical inactivity environment from which these cells were derived. For instance, it is well known that the regenerative abilities (15), concentrations of hormonal factors such as IGF-I (19), growth hormone (48), and testosterone (33), as well as satellite cell proliferative potential (20, 22, 43), are decreased with age. Hence, it is conceivable that, when satellite cells isolated from such an environment are plated in culture, they may be manifesting the “memory” of that environment from which they were isolated. Such speculation is partly supported by the recent studies of Johnson et al. (29), who showed that satellite cells isolated from steers implanted with Revalor-S (120 mg trenbolone acetate + 24 mg estradiol) demonstrated a more robust increase in their in vitro proliferation immediately after their isolation, compared with control steers, implying a memory of the in vivo Revalor-S-enriched environment. Indeed, a novel observation of the present study is the dramatic restoration of the proliferative potential of satellite cells from those muscles infused with IGF-I (Fig. 6G), which had been previously subjected to repeated bouts of atrophy. In other words, given the documented decrease in serum IGF-I concentration with age (19), it is possible that this may partly contribute to the failure of aged muscles to recover from periods of atrophy. Provision of an IGF-I-enriched environment to old muscle in the current study restored, in part, some of the lost muscle mass, as well as the in vitro proliferative potential of the resident satellite cells.

One of the questions raised by increasing the in vitro proliferative potential of aged satellite cells with IGF-I application, as shown by the results of this study, is whether IGF-I delays replicative senescence of these cells in vivo. Hayflick and Moorhead (28) were the first to document that human diploid fibroblasts in culture had a finite replicative lifespan, after which they reach...
a state called replicative senescence. Given that the proliferative potential of normal diploid cells is inversely related to age and that cells from older individuals undergo fewer divisions in vitro before they senesce (46), it has been predicted that the proliferative potential of cells is reduced by the use of cell divisions in vivo. This is reflected in our studies by the significantly reduced number of large-sized colonies containing ≥256 cells, reflecting more than eight cell population doublings in aged (31-mo-old) rats compared with those from young (3-mo-old) rats. Therefore, satellite cells from FBN rats, like other normal diploid cells, undergo replicative senescence as well. Hence, the IGF-I-induced increase in the proliferative potential seen in satellite cells after the 9-wk recovery period does not mean that satellite cells become immortal and therefore bypass senescence. Instead, giving exogenous IGF-I perhaps reconstituted this decreased growth factor in aged muscles and in turn stimulated the satellite cells to proliferate. The resident satellite cells in the muscles subjected to multiple cycles of atrophy-regrowth have plenty of replicative reserves left, as they were never depleted (and hence likely not senescent) because the aged muscle never underwent periods of increased regrowth after bouts of atrophy.

Consequently, these results also raise the yet-unanswered question of whether IGF-I application increased the in vivo satellite cell mitotic activity (which was not measured in this study) and, if so, would it not then lead to a decrease in the in vitro proliferative potential, rather than the observed increase. The data from Johnson et al. (29) suggest that activating satellite cells in vivo results in an increased in vitro proliferation, as shown by increased incorporation of [3H]-thymidine in satellite cells isolated from steers implanted with Revalor-S. Also, data from Barton-Davis et al. (8) clearly implied that IGF-I application increases satellite cell proliferation because centralized nuclei were observed. However, neither their data nor ours have looked at the effects of long-term IGF-I infusion by directly following the proliferative history of these cells. Hence, it remains to be critically evaluated what the long-term effects of IGF-I are on satellite cell proliferative potential.

Nevertheless, the results of the present study have provided us with novel insights into the role of exogenous growth factors on rescuing senile skeletal muscles from atrophy and extend the results of many investigators. The age-related decrease in the restorative capacity of senile skeletal muscles has been largely attributed to limitations in the aged environmental milieu that impairs the aged skeletal muscle’s ability to completely regenerate existing muscle fibers. Cross-transplantation studies by Carlson and Faulkner (14), as well as the studies by Sadeh (40) and Marshall et al. (31), indicate an important role for environmental determinants during regeneration of senile muscles, such as age-associated changes in neuromuscular junction, as well as in the extracellular matrix composition. The observed rescue of regrowth with IGF-I infusion after multiple bouts of atrophy by hindlimb immobilization (~12% increase in gastrocnemius wet weight and ~26% increase in total protein content in IGF-I-infused muscles, relative to saline-infused atrophied muscles) seen in this study further extends the notion of the importance of the environmental factors as key determinants of muscle regrowth in old animals.

Although the effects of IGF-I in cells are clearly pleiotropic (47), one of its well-documented roles is as a potent mitogen for satellite cells in culture (3, 24). Elegant work by Barton-Davis et al. (8) showed that overexpression of IGF-I in the extensor digitorum longus muscles of 27-mo-old mice prevented their age-associated skeletal muscle atrophy by enhancing muscle regeneration, in turn implying an activation of the resident satellite cells. However, they never directly addressed satellite cell proliferation. This study provides the first documentation of an associated involvement of IGF-I on the proliferative potential of skeletal muscle satellite cells in aged rats by infusion of IGF-I. Although Adams and McCue (1) were the first to use the IGF-I infusion technique to document a hypertrophy in skeletal muscle, their data, however, were from young rats, and they did not examine the effects of IGF-I infusion on in vitro satellite cell proliferative potential. Therefore, the results in the present study further extend the observations of both Barton-Davis et al. (8) and Adams and McCue (1) and show that IGF-I infusion into aged muscles, even after being subjected to repeated bouts of atrophy-recovery, can still respond and regrow with an associated increase in the proliferative potential of satellite cells. It is also likely that, given the multifaceted functions of IGF-I, the observed regrowth of the atrophied muscles with IGF-I infusion could also be due to multiple IGF-I mediated effects, such as enhancement of protein synthesis (7) or increased nerve sprouting (49), which we did not directly address in this study.

In summary, we did not test our original hypothesis of whether multiple cycles of atrophy-recovery would deplete satellite cell proliferative reserves, because even one bout of atrophy was sufficient to result in a failure of skeletal muscle regrowth. However, the remarkable findings of the study were that the decreased proliferative potential of satellite cells, as well as the impaired regrowth of gastrocnemius muscle from repeatedly atrophied aged muscle, were able to be rescued by IGF-I infusion. These results suggest that, first, rather than the numbers of satellite cells per se in these old rats, it is the total inherent proliferative potential contained within each cell that appears to be more critical for the restorative processes required for skeletal muscle growth. Second, these results also suggest that the effects of the environment, such as increased IGF-I, can modulate proliferative potential. Previously published work (14, 31, 33), as well as the results from this study, allows us to conclude that, in aged skeletal muscle alterations in the nature of the microenvironmental factors, rather than an intrinsic defect in the cellular capacity of skeletal muscle to respond to atro-
phy, may be largely responsible for the impaired restorative ability of senile muscles.

The ability to alter the environmental factors may be advantageous from a therapeutic perspective, because localized supplementation of IGF-I (or other exogenous factors) to activate satellite cells in old atrophied muscles seems to be a more feasible first step than the injection of young satellite cells into an allogenic old skeletal muscle. These results will have profound implications in our understanding of skeletal muscle recovery from atrophy and injuries, especially in the increasing elderly population, and, consequently, for developing effective molecular countermeasures against physical frailty to ultimately prevent increases in nursing home admissions.

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REFERENCES


29. Marshall PA, Williams PE, and Goldspink PE. Accumulation of collagen and altered fiber-type ratios as indicators of
abnormal muscle gene expression in the mdx dystrophic mouse. 


