

To stage and quantify regenerative myogenesis in FES-Induced Functional Recovery of Human Long-Term Permanent Denervated Muscle

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Abstract

Following denervation, skeletal muscle undergoes rapid loss in both mass and contractile force, with an accompanying series of changes in structure, biochemistry and physiology. Morphologic features of the long-term denervated muscle suggest that the original fibers are lost and those seen are the results of repeated cycles of cell death and regeneration. Markers of myogenic events in adult muscles are activated satellite cells, presence of embryonic myosin and myogenic transcription factors. Electrical stimulation of permanent denervated muscle increases the mean size of the myofibers, maintains the sarcomeres and possibly prevents apoptosis/necrosis and secondary degeneration.

We here describe histological, immunohistochemical and molecular methods, which identify markers of damage, regeneration, and repair on a muscle sample of just a few milligrams. A suitable source is a small biopsy, whose cryostat sections allow fiber typing and histopathology by immunochemical analyses. A few additional serial cryostat sections are used for molecular analyses. By light morphometry percent fat and/or interstitial tissue area, and fiber size distribution are determined. Myosin ATPases and immunohistochemistry are used to quantify fiber types, including early or late regenerated myofibers. By electron microscopy we identify abnormalities of sarcomeric organization, nuclear distribution, mitochondrial content, and activated satellite cells. Myosin heavy chain profiles are indicators of regenerative events and of muscle adaptation to changing demands. Total protein content, myosin: total protein and myosin: actin ratios are determined by SDS-PAGE to reliably complement or substitute morphometry in quantifying muscle trophism in skeletal muscle biopsies.

Taken together the results of morphometry and molecular analyses solidly demonstrate that two-year-long FES substantially reverses the severe atrophy expected in four-year denervated human muscles, and that generative/regenerative myogenic events significantly contribute to the functional recovery of long-term paralyzed human muscles.

Key words: atrophy, FESS, long-term permanent denervation, morphometry, regeneration, SDS-PAGE, skeletal muscle, therapeutic functional electrical stimulation.

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The high dynamic state of skeletal muscle is well exemplified by its ability to accommodate to different amounts of contractile activity and/or hormonal stimuli by: i) continuously adjusting total mass (atrophy, eutrophy and hypertrophy); ii) responding to damage with fiber regeneration; and iii) switching among a relatively broad range of genes' pools, so that myofibers tune their metabolic and contractile characteristics to changing demands, pathology included. Reduction of neuromuscular activity and/or unloading by denervation, hind limb sus-

pension or zero gravity during space flight, results in rapid and substantial atrophy of skeletal muscle fibers [54]. Furthermore conditions of elevated catabolism as starvation, cancer and aging cachexia, burn injury, iperthyroidism present a profound muscle involvement with protein loss and reduction of myofiber size [48].

Long-term permanent denervation (LT-PD) induces severe atrophy of skeletal muscle accompanied by apoptotic loss of myonuclei [10, 49]. Preferential atrophy of fast fibers followed by atrophy of slow fibers appears to

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be the typical feature of the early phases of denervation, producing only a small unbalance in fiber typing. However during several months of permanent denervation there is an almost complete transformation of rat mixed muscles into almost pure fast muscles [14, 16], the residual slow myosin being present with fast myosin in single myofibers [16, 55].

Morphologic characteristics of the long-term denervated muscle suggest that the original fibers are lost and those seen are the results of repeated cycles of cell death [1, 5, 10, 16, 42, 52] and regeneration. Markers of myogenic events in adult muscles are activated satellite cells and presence of developmental myosin. Satellite cells are small mononucleated skeletal muscle stem cells located between the basal lamina of the muscle and the sarcolemma of myofibers [61]. Satellite cells are mobilized in response to injury of the myofibers or increased loading conditions (see for review [1, 46]). The initial event after satellite cell activation is a proliferative response in which some or all of the activated satellite cells undergo at least one mitotic cycle. Some of the progeny of the activated cells differentiate into myoblast-like cells, then the myoblasts fuse with each other to form new myofibers or become incorporated into existing myofibers. Since myoblasts and myotubes express peculiar isomyosin genes, light and heavy chains of embryonic myosin are sensitive indicators of myogenic events in adult muscles.

In LT-PD necrotic and apoptotic cell death is known to occur at significant rate [4, 5, 18, 34, 36, 56, 57]. Permanent denervation does not prevent induced muscle regeneration and a long-term retention after denervation of this capability has been also demonstrated [3, 13, 15, 17, 20-22, 27, 31, 38, 60]. Spontaneous myofiber regeneration in LT-PD has been quantified and shown to be non-compensatory [1, 5, 17, 22, 34] and to result in reduction of satellite cell pools [20, 31].

Electrical stimulation of permanent denervated muscle increases the mean size of the myofibers, maintains the sarcomeres and possibly prevents secondary degeneration and apoptosis/necrosis [23, 32, 59]. Satellite cell proliferation and myofiber regeneration is enhanced in LT-PD muscles subjected to electrical stimulation [2, 11, 29, 30, 37, 39, 40, 47].

Since satellite cells activity is required for extreme hypertrophy of overloaded adult muscles [1, 6, 21, 43, 61] or eutrophy after severe atrophy in recovering muscles [1], positive regulation of activation, division and fusion of myoblasts could be an important tool to understand limits of recovery of neurogenic muscle myopathies by functional electrical stimulation (FES).

We will describe morphological features and scaled-down molecular procedures, based on protein chemistry, that are sensitive enough to allow several markers of plasticity, damage, regeneration, and repair to be determined on a muscle sample of just a few milligrams. A suitable source of material is a small biopsy specimen removed from muscles for fiber typing and histopathol-

ogy. A few additional serial cryostat sections cut from the block constitute a sample that is adequate for molecular analyses. Since such a small biopsy can be taken sequentially, it becomes feasible to follow responses of skeletal muscle to different demands in both experimental and clinical settings. Where indicated, these protocols may be combined with other analyses, such as identification of proteins with antibodies or in situ hybridization with specific nucleic acid markers. A variety of highly sensitive methods may be added: chemiluminescence and polymerase chain reaction techniques are capable of detection sensitivities that extend almost to the level of a single molecule [8].

We here describe our approaches to stage and quantify long-term denervation and related myogenic events in 4-year human flaccid paralysis after 2-year of functional electrical stimulation.

Material and Methods

Biopsies

Needle biopsies of tight muscles from 4-year human flaccid paralysis after 2-year of functional electrical stimulation were performed and treated for light and electron microscopy as described in [26].

Light microscopy

Serial sections are cryosectioned from samples obtained by needle biopsies frozen in isopentane cooled with liquid nitrogen. For histology or immunohistochemistry, sections of 10 μm thickness are collected on polylysinated glass slides. For molecular analyses, a precise number (usually two) of sections of 20 μm thickness are transferred to eppendorf test tubes. The slides and test tubes are stored at -80°C until use. Three 10 μm thick sections are collected on glass slides and stained using conventional techniques with Hematoxylin and eosin (H&E). Total area of the slide and percent areas covered by myofibers, interstitial and fat tissues are determined in H&E-stained sections as described below (morphometric analyses). Fiber counts to determine fiber type distribution are based on myofibers identifiable in H&E-stained sections, in which the smallest myofibers are hardly recognized. In long-term denervated muscle total myofiber counts and their fiber size distribution could be determined using semi-thin sections (see below).

Fiber typing

Myofibrillar ATPase. Serial sections (10 μm) are stained for myofibrillar ATPase according to [9]. The sections are preincubated at alkaline or acid pH and used to measure the size of type 1 and type 2 fibers. The percentage content of type 1 fibers is determined as area of ATPase-stained sections as described below.

Immunohistochemistry. Cryo-sections are labeled for the presence of MHC-emb (from Novocastra, NCL-MHCd diluted 1:20) or MHC slow (from Novocastra, NCL-MHCs diluted 1:20) for 1 hour at room tempera-

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ture. The slides are then washed twice with TBS (5 min each) and incubated with FITC-conjugated anti-mouse Ig (from Sigma, F-2266 diluted 1:200) for 1 h at room temperature. The slides are washed twice with TBS (5 min each) and nuclei counter-stained by Hoechst 33258. Negative controls are by omitting the primary antibody. Positive myofibers are counted and/or their collective fiber area determined as described below.

Myogenin construct, probes and in situ hybridization

The plasmid containing cDNA for myogenin was constructed as follows. The *Pfu* polymerase (Stratagene, La Jolla, USA) was used to amplify a region of the human myogenin mRNA (EMBL accession number X17651) from human skeletal muscle cDNA using the primer sequences 5'-GCA GGC TCA AGA AGG TGA AT-3' and 5'-ATG GAT GAG GAA GGG GAT AG-3'. The PCR product was blunt-end cloned into the *Sma*I site of the vector pBlueScript II SK(+) in the opposite direction of the lacZ gene. The plasmid was linearised with PstI before reverse transcription with T3 RNA polymerase and ³⁵S-UTP. In situ hybridization with the ³⁵S-labelled cRNAs for myogenin, MHC1 and MHC emb was performed as previously described [51].

Electron microscopy and size distribution of total myofibers

Samples obtained by biopsies are fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2, for 2 h on ice followed by buffer rinse and fixation for 1 h in 1% osmium tetroxide. The specimens are dehydrated in a graded series of ethanol solutions and embedded in epoxy resin before semithin (1 µm) and thin (300 Å) sectioning for light and electron microscope examination. Semithin sections, stained using conventional techniques with toluidine blue-fuxin, are used to plot fiber size distribution. The minimum transverse diameter of each myofiber is measured against a reference ruler as described below. Peripheral or central myonuclei and satellite cells are counted. On longitudinal sections random or clump distribution of myonuclei is determined.

After staining with uranium acetate and lead citrate, ultrathin sections were examined with a Philips M 301 electron microscope.

Morphometric analyses

Images are acquired by a Zeiss microscope connected to a Leica DC 300F camera at low magnification under the same conditions that are used to acquire a reference ruler.

Morphometric analyses are performed with Scion Image for Windows version Beta 4.0.2 (by 2000 Scion Corporation), a free software downloaded from the web site: www.scioncorp.com.

Molecular markers

Two serial sections are solubilized in 200 µl 2.3% sodium dodecyl sulphate (SDS), 10% (by volume) glycerol, 0.5% 2-mercaptoethanol, 62.5 mM Tris HCl, pH 6.8.

Test tubes closed with aluminum foil are 3-min warmed in boiling water to denature proteins. After boiling the volume is adjusted to 200 µl with bi-distilled water.

Total protein by 4% Stacking Gel - SDS PAGE

Total protein quantification is made using a very short run in SDS PAGE. By terminating electrophoresis while the protein front is still in the stacking gel, proteins are not resolved, but stacked in a thin band. SDS polyacrylamide gel electrophoresis (SDS-PAGE) is performed essentially according to Laemmli [28] in 0.75 x 130 x 80 mm (thick, large, high) polyacrylamide gel slab. To easier manipulate the slab after the run, the bottom gel (that is, the separating gel of standard SDS PAGE), is made of 35-mm 10% T acrylamide-Bis (36.5:1), 375 mM Tris HCl (pH 8.8), and 0.1% SDS. The upper (stacking) gel is composed of 20-mm high 4% T acrylamide-Bis (36.5:1), 125 mM Tris HCl (pH 6.8), and 0.1% SDS. To obtain clean wells over the stacking gel, the 25-mm well shoulders are made of high 5% T acrylamide-Bis (36.5:1), 125 mM Tris HCl (pH 6.8), and 0.1% SDS, and 20-mm high 4% T acrylamide-Bis (36.5:1), 125 mM Tris HCl (pH 6.8), and 0.1% SDS. The electrophoresis buffer consists of 25 mM Tris, 192 mM glycine pH 8.3, and 0.1% SDS. Aliquots of albumin and/or purified myosin (from 0.25 to 2 µg) are loaded in each SDS PAGE slab to obtain reference slopes, which are used to determine protein content of aliquots of soluted biopsy samples. Electrophoresis is performed in constant-current mode at a current of about 10 mA per slab, the actual current being set to a level that gives an initial voltage of 70 V. The run is ended after about 60 min when the dye front is 1 cm from the stacking/separating gel interface, i.e., after about 1 cm of run in the stacking gel. The voltage usually rises to 90 V by the end of the run. Protein bands are easily detected after shaking for 1 h with 200 ml of 0.1% Coomassie Brilliant Blue in 5% acetic acid, 40% methanol and rinsing in 6 changes (every 15 min) of 200 ml 40% methanol, 7% acetic acid. Slabs are stored in 1% acetic acid. Total protein content is determined by gel densitometry as described below.

Myosin: protein ratio by 5% Stacking Gel - SDS PAGE

If in the gel system described above the concentration of the stacking gel is raised to 5%, the myosin heavy chains separate from protein front during the stacking period of gel electrophoresis. Myosin heavy chains: soluble proteins ratio is determined by gel densitometry, as described below.

Myosin: actin ratio by 10% SDS - PAGE

Analytical SDS polyacrylamide gel electrophoresis (SDS-PAGE) is performed essentially according to Laemmli [28] in a discontinuous gel gradient system within a 0.75 x 130 x 130 mm polyacrylamide gel slab: this comprises a 10-mm stacking gel and a separating gel consisting of 90 mm of 10% polyacrylamide and 30 mm of 7% polyacrylamide. A major modification is that

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37.5% (by volume) glycerol is included in both separating and stacking gels [12, 19]. The electrophoresis buffer consists of 25 mM Tris, 192 mM glycine pH 8.3, and 0.1% SDS. Overnight separation is achieved in constant-current mode at a current of about 5 mA per slab, the actual current being set to a level that gives an initial voltage of 35 V. Gel electrophoresis is started late in the afternoon and the run is terminated after about 17 h when the dye front reaches the end of the slab. The voltage usually rises to 140-150 V by the end of the run. Normally 5 µg of total protein are loaded per well. MHC and actin bands are easily detected after shaking for 1 h with 200 ml of 0.1% Coomassie Brilliant Blue in 5% acetic acid, 40% methanol and rinsing in 6 changes (every 15 min) of 200 ml 40% methanol, 7% acetic acid. To avoid variability due to stain-destain procedure the MHC: actin ratio is checked against a standard muscle homogenate that is run in each gel. MHC and actin content are determined by gel densitometry, as described later.

Myosin heavy chain isoforms

MHCs are separated by analytical SDS-PAGE on 7% polyacrylamide gel slabs measuring 0.75x130x130 mm [15]. The slabs are prepared according to [45] with 37.5% (by volume) glycerol present in both separating and stacking gels [19]. The stacking gel is composed of 37.5% glycerol, 4% T acrylamide-Bis (36.5:1), 125 mM Tris HCl (pH 6.8), and 0.1% SDS. The separating gel is composed of 37.5% glycerol, 7% T acrylamide-Bis (36.5:1), 375 mM Tris HCl (pH 8.8), and 0.1% SDS. The running buffer consists of 50 mM Tris, 384 mM glycine pH 8.3, and 0.2% SDS (without correcting the pH with HCl). Usually the slab is prepared in the afternoon, and the samples are loaded early in the morning

the day after. Separation of MHC is achieved in the constant-current mode at 4 mA per slab, corresponding to a voltage of about 40 V. After 4-6 h the buffer is changed and electrophoresis is restarted with the same parameters. After 24 h running the voltage rises to 130-160 V and the electrophoresis is stopped. Gels containing 0.2 µg of protein per band are stained with 0.1% Coomassie Brilliant Blue in 5% acetic acid, 40% methanol and destained in 40% methanol, 7% acetic acid. Gels with less than 0.1 µg of protein per band are stained by the silver method [35].

Gel densitometry

After stain and destain as described in [50], an Epson Perfection 1650 scanner connected to a PC computer is used to scan gel slabs. Data are processed by Scion Image for Windows version Beta 4.0.2 (by 2000 Scion Corporation Scion Image software (Pc version). In these conditions, densitometry is linear between 0.20 and 1 µg of protein per band after Coomassie Blue stain, and between 0.02 and 0.20 µg after silver stain. Range of linearity could be improved by reversible silver staining [41].

Results and Discussion

Markers of myogenic events in long-term denervation

Embryonic myosin is a marker of early myogenic events in adult muscle: reinnervation of new myofibers induces at 5-7 days post-damage a transition of the gene expression from embryonic to either adult slow or fast type of myosins [15, 24], while in aneural regeneration of adult muscle a transition towards fast type isomyosins also occurs, but 15-30 days after damage. Fast-type myosins are lastly expressed, so that embryonic myosin light and heavy chains (MLC-emb and MHC-emb) are present in regenerated myofibers only up to 21 days post-myotoxic damage [15], as it occurs in denervation of developing muscles [12, 58].

Figure 1 shows that biopsies from two-year FES of four-year denervated human muscle display some myofibers positive to anti MHC-emb antibody staining, i.e., myofibers regenerated during the last month of FES. Figure 1, A shows that some of them have central nuclei

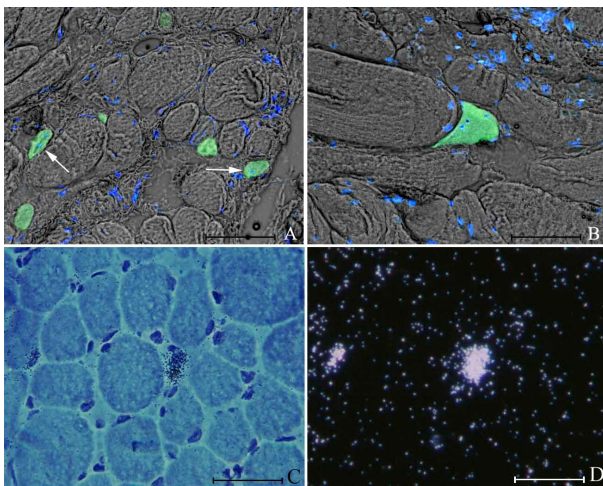


Figure 1. Markers of myogenic events in long-term denervation of human muscle. A and B, anti-MHCemb positive myofibers in skeletal muscle biopsies of 4-year human flaccid paralysis after 2-year FES. Arrows point to small centrally nucleated myofibers. C and D, Myogenin positive interstitial cells (myoblasts) [from 51].

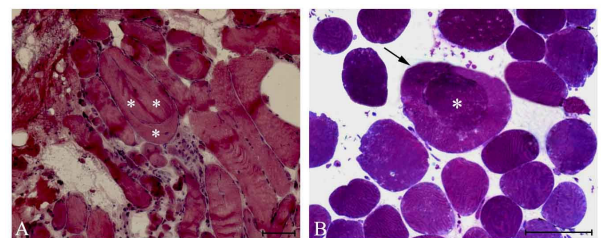


Figure 2. Muscle regeneration in skeletal muscle biopsies of 4-year human flaccid paralysis after 2-year FES. A, H&E stain. Stars label three unfused regenerated myofibers, one of which is centrally nucleated. B, Semi-thin section. A, arrow points to a large myofibers, which fully encircles a smaller one (star).

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(arrows), a feature suggesting early regeneration (one week). In figure 1, B the anti-MHCemb positive myofiber have 5-10 μm diameter and 4 near-sarcolemmal myonuclei, a feature present 2-4 weeks after damage/regeneration in myotoxin-induced muscle regeneration of permanent denervated muscles [38].

Figure 1, C and D show that the regenerative events could be identified even earlier by cryosection labeling for mRNA of myogenin, an early myogenic transcription factor. The method recognizes myoblasts present among the interstitial cells of the skeletal muscle.

Morphological features of muscle regeneration are also evident at light microscopy. Figure 2 shows that three myofibers (stars) co-exist into an old basal lamina. Note that one of these myofibers is centrally nucleated. Many authors take such a feature as evidence of fiber splitting, but it is now well known that the three myofibers are the result of incomplete fusion of regenerated myofibers being surrounded by two layers of basal lamina, the old one of the death fiber and the new ones secreted by the new myofiber(s) [5, 16, 20]. Panel B of figure 2 is a semi-thin section displaying a seemingly central-core myopathic, large myofiber (arrow). Indeed, two regenerated myofibers are contained, the larger fully embracing the smaller one (star), so establishing its regenerative nature.

By adding to MHCemb-positive myofibers those recognized by morphological features (unfused myofibers), occurrence of myogenic events in long-term denervated FES muscle may be estimated better (Table 1).

On the other hand, not all MHC-emb positive fibers seem to be related to myofiber death. Several of them are near to almost healthy large fibers, an occurrence that reminds second and third myogenic events during muscle development or extreme muscle hypertrophy

(Figure 1, A). We use “muscle generation” to describe these myogenic events, while we define “muscle regeneration” those events, which occur into old layer of basal lamina to replace death myofibers (Figure 2).

Fiber types

After two-year FES, in four-year denervated human muscle the majority of the myofibers recognized at light microscopy are large myofibers, which stain positive for either slow or fast myosins after myosin ATPase or anti-MHC stainings (Figure 3, A and B). They are the original denervated or reinnervated fibers re-grown to almost normal size in response to FES.

SDS-PAGE of MHC confirms the presence of adult fast and slow MHC in cryostat sections from biopsies of 4-year denervated human muscle after 2-year FES (Figure 3, C). Counts of different types of myofibers and of their percent area were determined in cryostat sections (Table 1). Results of morphologic and molecular analyses are in reasonable accord, suggesting that molecular analyses may complement or substitute tedious and labor intensive morphometry, when hundreds of samples ought to be analyzed.

Total fiber count and size distribution

In the 4-year denervated human muscle after 2-year of FES the true total number of myofibers and their fiber size distribution could only be established by analysis of muscle specimens in semi-thin sections (Figure 4, A and B). Indeed half of the myofibers have diameters of less than 10 μm , sizes that hardly could be recognized beyond doubt in cryo-sections stained with H&E.

Table 1. Trophism and fiber types in skeletal muscle biopsies of 4-year human flaccid paralysis after 2-year FES.

Area of the 20 μm cryosection	mm^2	10.4
Muscle tissue	% of total area	78.05
Interstitial tissue	% of total area	17.43
Fat	% of total area	4.52
Wet muscle weight of the 20 μm cryosection	μg	210
Total muscle protein	mg/g w.m.w.	100
MHC/total protein	%	37
Myosin/Actin	ratio	1.72
Myofibers in the cryosection (H-E stain)	n.	577
MHC emb by anti-MHCemb	% number of fiber	2.3
MHC 1 by anti-MHC 1	% number of fiber	43
MHC 2	% number of fiber	54.7
Type 1 by ATPase (pH 4.35)	% as fiber number	50.9
Type 2 by ATPase	% as fiber number	49.1
Type 1 by ATPase (pH 4.35)	collective % area	33.1
Type 2 by ATPase	collective % area	66.9
MHC 1 by SDS PAGE	%	36
MHC 2 by SDS PAGE	%	64

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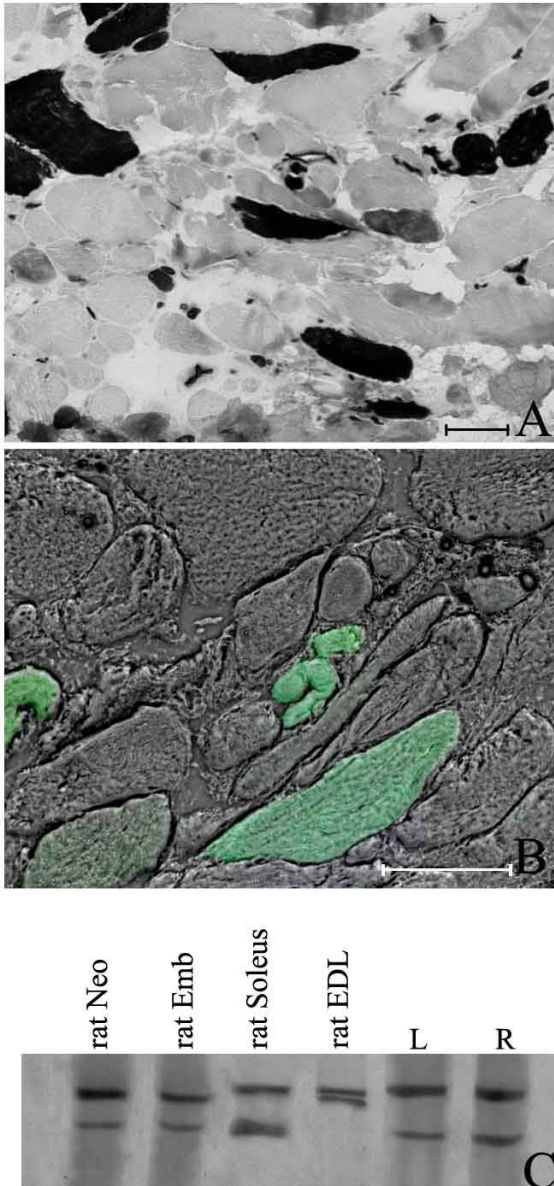


Figure 3. Fiber types in skeletal muscle biopsies of 4-year human flaccid paralysis after 2-year FES. *A*, ATPase staining. *B*, Myofibers positive with antibodies against MHC 1 (slow type). *C*, 7% SDS-PAGE of MHCs: L and R indicate left and right leg muscle biopsies of 4-year human flaccid paralysis after 2-year FES. F, fast type MHC, S, slow type MHC.

On the other hand, it is worth nothing that after two-year FES there are several large myofibers and that only few myofibers are angulated, a feature that is used by neuropathologists to identify denervated myofibers.

Severely atrophic myofibers and myotubes

Figure 5, A shows that in longitudinal semi-thin sections the majority of smaller myofibers (minimum diameter less than 10 μm) are long-term denervated, severely atrophic myofibers. In these very small fibers the myonu-

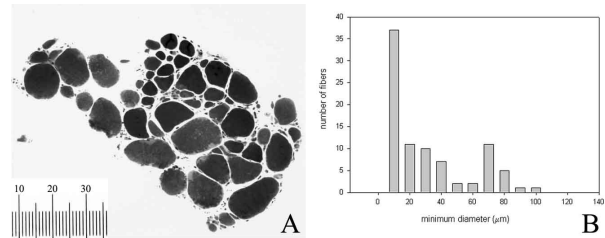


Figure 4. Semi-thin section of skeletal muscle biopsies of 4-year human flaccid paralysis after 2-year FES. *A*, muscle section shown at low-magnification (2.5 x). *B*, fiber size distribution (myofiber minimum diameter was determined, and myofibers were grouped in steps of 10 μm).

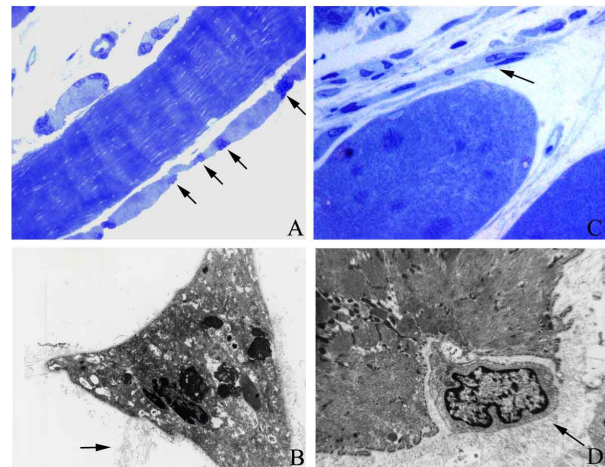


Figure 5. Longitudinal semi-thin sections and transverse section electron microscopy of small myofibers in skeletal muscle biopsies of 4-year human flaccid paralysis after 2-year FES. *A*, longitudinal semi-thin section of eutrophic and severely atrophic myofibers. Arrows point to clumps of myonuclei alternate with long stretches of anucleated sarcoplasm. *B*, cross-section of a very small myofiber, as shown by electron microscopy: the angulated aspect, which also displays overabundant folded layers of the original basal lamina, is the peculiar feature of severe atrophy. *C*, semi-thin section of an eutrophic and of a myotube-like structure (arrow). *D*, the myotube-like structure as shown by electron microscopy. The indented sarcoplasm is an evidence that the aneural regenerated myofiber is subjected to "denervation" atrophy.

clei are not randomly distributed, but clumps of myonuclei (arrows) alternate with long stretches of anucleated sarcoplasm. Figure 5, B is the cross-section of a very small myofiber, as shown by electron microscopy: the angulated aspect, which also displays overabundant folded layers of the original basal lamina, is the peculiar feature of severe atrophy. On the other hand, myotube-like structures are also seen among the trophic myofibers (Panels C and D). Though these are sparse observations, they are evidence of myofiber "generation" in long-term denervation of human skeletal muscle (see above). At the

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ultrastructural level evidence of long-term denervated myofibers are abundant. They displayed the typical features the muscle fibers atrophy (angulated or round, with central or peripheral clumps of myonuclei, loss or disorganization of myofibrils, numerous of lipid bodies, folds of basement membrane), which were often surrounded by increased amount of collagen fibers. Increased numbers of satellite cells are especially present in large myofibers. Not surprisingly, myotubes or early young myofibers are rare events in comparison to anti-MHCemb positive myofibers in cryostat sections.

Molecular analyses by SDS PAGE

Figures 6 shows the results of molecular analyses by SDS PAGE of proteins from one to three cryostat sections, serial to that presented in panel A of Figure 6. The ruler allows determine the section area. Since thickness and number of sections are known, volume of muscle tissue is computed. Therefore, by gel densitometry we determine total protein per wet muscle weight (Figure 6, C), myosin: total protein (Figure 6, D), and myosin: actin (Figure 6, B) ratios (Table 1). Molecular analyses data are consistent with morphometry results, and have the advantage of being performed in a much shorter time and with substantial less man-power, as we previously stressed describing SDS-PAGE of MHC in Figure 3, C.

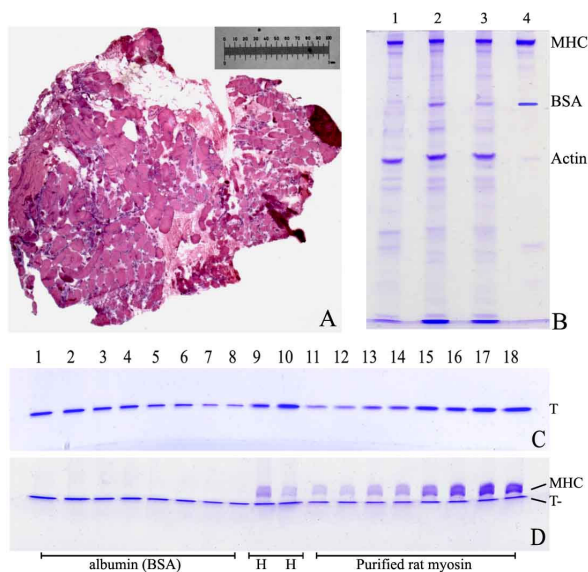


Figure 6. Proteomics of skeletal muscle biopsies of 4-year human flaccid paralysis after 2-year FES. A, H-E stain (2.5 x) cryostat section serial to those used to extract proteins. B, 10% SDS-PAGE of soluble proteins from: 1, rat muscle cryosection; 2 and 3, skeletal muscle biopsies of 4-year human flaccid paralysis after 2-year FES; 4, myosin and bovine serum albumin (BSA) markers. C, total protein (T) by 4% Stacking Gel - SDS PAGE. D, total protein: MHC ratio by 5% Stacking Gel - SDS PAGE. H, human muscle biopsy cryosection, MHCs, myosin heavy chains, T, total protein except MHCs.

Cryostat section total protein content by 4% Stacking Gel - SDS PAGE and total protein: MHC ratio by 5% Stacking Gel - SDS PAGE

To determine by standard colorimetric methods total protein content of cryostat sections, contamination with the medium used to embed frozen tissue specimen have to be carefully avoided, since it interferes with the assays, whether Lowry [33] or Bradford [7]. Our gel electrophoretic approach has the additional main advantage to need much less amount of protein, i.e., less than 1 µg by Coomassie blue stain, and ten time less if the slabs are silver stained (Figure 5, C). When the polyacrylamide concentration of the stacking gel is raised to 5%, the myosin heavy chains separate from protein front during the stacking period of gel electrophoresis (Figure 5, D). Bi-dimensional gel electrophoresis confirms that the retarded bands only contain MHC (manuscript in preparation). Results of densitometry is shown in Table 1, but discussed together with myosin: actin ratio reported below.

Myosin: actin ratio

Myosin and actin have different rates of metabolic turnover, and their ratio changes during muscle atrophy and hypertrophy, i.e., after denervation-reinnervation or tenotomy-repair [25]. Figure 6, B shows the patterns of contractile proteins on 10% SDS-PAGE. In previous experiments, the myosin: actin ratio obtained by densitometry of the gel slabs had the expected mean value of 2.2 in normal adult muscles of rat. In the human biopsy samples studied here the result is in between those of normal and severely atrophic fibers of long-term denervated rat muscles [44, 53].

Conclusions

The SDS-PAGE molecular assays of protein from few cryostat sections (proteomics, in the post-genomic era) can be carried out simply and reliably. Total protein, MHC: total protein and MHC: actin ratios were used as examples. Analyses can be extended when suitable antibodies are available for immunoblotting and immunohistochemistry. Molecular markers of inflammation or of any other normal or pathological event can be included in a procedure that can be carried out on a few cryostat sections. Clearly the approach is not confined to skeletal muscle tissue.

Taken together the results of morphometry and molecular analyses, as recapitulated in Table 1, solidly demonstrate that two-year-long FES substantially reverses the severe atrophy expected in four-year denervated human muscles, and that generative/regenerative myogenic events significantly contributes to the FES induced functional recovery of long-term paralyzed human muscles [26].

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