Myofiber regeneration in a denervated human muscle 3.5 years after post-traumatic free flap reconstruction

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Abstract

A 47-years-old man had a severe loss of soft tissue in a lower limb caused by a traumatic accident. In January 2003 the conspicuous loss of soft tissue was covered with a fasciacutaneous flap, transferred from vastus lateralis. 3.5 years after surgery, during a surgical remodeling of the flap, the receiving area was biopsied and then analyzed. Beside a large tissue area presenting patent vessels surrounded by adipose tissue, a layer of atrophic muscle is present. Among very small myofibers, MHCemb-positive fibers are demonstrated by the specific monoclonal antibody. The observation of muscle regeneration in this likely-traumatic denervated muscle is in full agreement with the results described in paretic muscles after complete spinal cord injury involving the lower motoneurons. The present case supports long term viability of denervated myofibers, to which myofiber regeneration seems to provide an important contribution.

Key words: denervation, embryonic myosin, free flap, muscle regeneration, NCAM.

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We analyzed the case of a patient (male, 43 years) of the Plastic Surgery Unit of the University of Padua. After a traumatic accident in January 2003 he had a severe loss of soft tissue in a lower limb and he was submitted to free flap reconstruction. A flap is a slice of tissue transplanted from one area to another. Unlike a graft, a flap contains skin, fascia, fat, muscle and blood vessels. Free flap consists of the transposition of a tissue from its natural site to a new one, with total vascular and nervous deconnection [2]. In the reported case a fasciocutaneus flap from vastus lateralis was transferred to a lower limb to cover the traumatic area. After 3.5 years a full-thickening biopsy of the tissues in the receiving area was harvested during a surgical remodeling of the flap.

Analyses of human muscle biopsy

The biopsy was performed in the Unit of Plastic Surgery of University of Padua, and then it was transported to the Laboratory of Applied Myology of the Interdepartmental Research Center of Myology, University of Padua, where it was frozen in liquid nitrogen and cryosectioned. Histological, immuno-histochemical and morphometric analyses were then made. Cryosections (10 μm-thick sections) of frozen biopsies were stained with hematoxilyn-eosin (HE) according to conventional techniques [9]. Cryosections were also labeled with antibodies anti the embryonic MHC (NCL-MHCd Novocastra, diluted 1:80). The slides then were washed twice with TBS (5 min each) and incubated with FICT-conjugated anti-mouse Ig (from Sigma, F-2266 diluted 1:200) for 1 hour at room temperature. This was followed by a second 5 minute washing of the slides with TBS and nuclei counter – stained by Hoechst 33258. In the negative control, the primary antibody was omitted. Following the same methods, cryosections were labeled also with antibodies anti MHC–fast and –slow and with antibodies anti NCAM (from Chemicon International, diluted 1:200).

Images were acquired with a Zeiss microscope connected to a Leica DC 300F camera at low magnification under the same conditions that were used to acquire a reference ruler. Total area of the section and percentual content of adipose tissue, fibrous and loose connective and muscle area were determined. In section stained with HE, the minimum trasverse diameter of each myofiber was measured against a reference ruler and the myofiber size spectrum was plotted. Morphometric analyses were performed with Scion Images for Windows, version Beta 4.0.2 (By 2000 Scion Corporation).

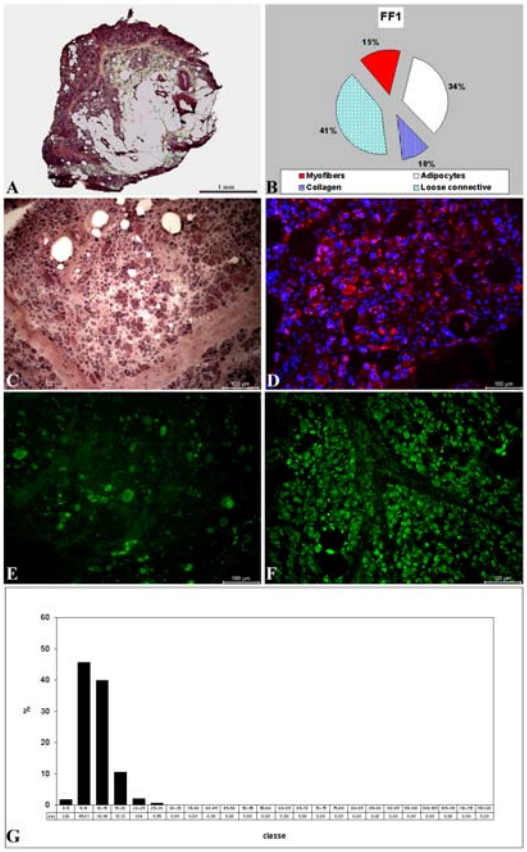

***Figure 1**. Biopsy observed after 42 months from surgery. A) and C) HE stainings. In A we note the division in two areas: one with very little few fibers, another with adipocytes. In C we note little fibers in loose connective tissue. B) Cryosection area: 41% of the section is covered by loose connective tissue, 34% by fatty tissue, only 15% by myofibers. D) anti-NCAM: mostly of the fibers are positive. E) anti-MHC embryonic staining: more than 100 fibers are positive. F) anti-MHC-fast staining: mostly of the fibers are fast. G) minimum transverse diameter distribution: 90% of myofibers have less than 20 µm diameter.*

Results

The histological pattern after HE stain (Fig. 1 A) shows that a large area of the cryosection is fatty tissue, among which two large patent vessels are visible. Panel B report the percentual area of fat (34%), loose connective (41%), collagen (10%), while myofibers represent the 15% of the cryosection. In the left upper corner of the cryosection, a thin bended layer of dense connective tissue limits fat from the parenchymal tissue, which is shown to be, at large magnification, skeletal muscle in panel C, i.e., small centrally nucleated myofibers surrounded by large layers of loose connective tissue (Figure 1, A and C). This interpretation is confirmed by figure 1, F and D, where the myofibers are labeled with anti-MHCfast and anti-NCAM, respectively. The distribution of minimum transverse diameter shows that more than 90% of the observed myofibers have a diameter lower than 20 µm, and therefore that they are severely atrophic due to permanent denervation as also indicated by their NCAM positivity (Figure 1, G). A surprising high number of small myofibers are also positive after anti-MHCemb labeling: more than 100 fibers are therefore the result of regenerative events (figure 1, E).

Discussion

Denervation of skeletal muscle causes immediate loss of function, which is followed by a decrease in contractile force when elicited by direct electrical stimulation. This is accompanied by several structural, biochemical and physiological changes of muscle atrophy. Denervated muscle becomes atrophic in different degrees, but after long term denervation, the tissue response is much more complex than expected, with atrophic and degenerative processes occurring simultaneously with some regenerative events. In rodents it is since long known that in denervated muscle there is "spontaneous" activation of satellite cells producing several generation of myoblasts, which after fusion generates new muscle fibers, expressing the embryonic isoforms of muscle-specific genes [1-2-3-4]. Recently, Carraro and Kern extended these observations to human muscles [4].

In this study, we report a case of post-traumatic denervation: a severe trauma of lower limb caused large loss of soft tissue and likely denervation of the injured muscles. This tissue loss is repaired with free flap by plastic surgery. Different free flap may be used, according to entity of damage and residual function of the traumatic area. In the present case a fasciocutaneus flap was transferred from vastus lateralis area to the lower limb. During a surgical remodeling of the flap, 3.5 years later, a biopsy of the transferred tissues was performed. The presence of a large area of the biopsy covered by adipocytes and fibrous connective tissue probably reflects the site of biopsy harvesting, that is, the deep board between the flap and the receiving muscle tissue. In any case, the characteristics of the myofibers included in the biopsy have all the features of a 3-year long permanent denervation. Though the trauma may be the determining factor in this case, a peripheral neuropathy may also contribute. A part from this, the biopsy shows similarities with paretic muscles after complete spinal cord injury involving the lower motoneurons, as is the case of *Cauda Equina* permanent lesion [8].

The absence of spontaneous reinnervation, which is known to occur seldom, could be the consequence of fibrosis, which plays a major role in preventing proper reinnervation of a long-term denervated muscle, acting as a mechanical barrier or by providing a poor substrate for axonal elongation [5]. In case of severe trauma both

muscle permanent denervation and myofiber necrosis occur (in fact a few small myofibers are found).

On the other hand, among severely atrophic myofibers a large number of "early" regenerated myofibers are present. Indeed embryonic myosin positivity (and NCAM positivity likely, 3.5 years after muscle damage) indicates regenerative events occurring during the last 2 weeks, since in aneural muscle regeneration the embryonic isoforms are soon substituted by adult fast types of contractile proteins [3]. A useful method to maintain myofibers trophism after denervation could be Functional Electrical Stimulation (FES), because it may mimic the neural activity. Electrical stimulation of permanent denervated muscle increases the mean size of the myofibers, maintains the sarcomeres and possibly prevents secondary degeneration and apoptosis/necrosis of the muscle fibers. Pilot studies in humans showed that standing up with denervated muscles using electrical stimulation is a reality [6, 7].

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