The Regenerating Muscle as an Experimental Model for the Study of Factors which Affect Muscle Differentiation or Adaptation

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Abstract

Bupivacaine-induced regeneration was studied in the rat soleus muscle in the presence or absence of innervation, in the presence of tetrodotoxin (TTX)-induced block of nerve impulse conduction, and/or in the presence of vinblastine-induced block of nerve axoplasmic flow. Part of experiments were carried out on tenotomized muscles. Regenerated muscles were analysed for myosin heavy chain (MHC) composition 14 days after bupivacaine injection. In TTX-paralysed-regenerated muscles type 1 and type 2A MHC isoforms were not expressed. In denervated-regenerated muscles type 1 isoform was lacking, while all fast isoforms (2A, 2B, 2X) were expressed. Tenotomy alone increased type 2A fibres, but did not modify the effects of surgical or functional denervation. Vinblastine-block caused up-regulation of 2A isoform expression in non-tenotomized muscles. The results confirm the essential role played by neuromotor impulses for type 1 and type 2A isoform expression. They also support the hypothesis that axoplasmic flow carries some chemical factor inhibiting 2A isoform expression.

Key words: neurotrophic factors, regeneration, skeletal muscle, tenotomy, tetrototoxin, vinblastine.

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The muscle regenerating after an acute degeneration is an useful experimental model for studying factors which direct or affect muscle differentiation, and muscle plastic adaptation to functional demand. Regeneration starts from undifferentiated (satellite) cells, and through the myoblast and myotubes stages it ends with the adult, differentiated cells. Then, a sequence of events occurs which is similar to foetal myogenesis, with the advantage that the regenerating muscle allows greater experimental manipulations than the foetal muscle. Moreover, it is possible to affect the phenotype expression in the very initial steps of muscle differentiation.

The interest of our laboratory is mainly focused on the role of innervation on muscle differentiation. Innervation can affect muscle properties by the pattern and/or the frequency of motor impulses and the release of chemical factors by nerve fibres (for references see [7]). In our investigation, we tried to dissociate these two possible components of nerve influence by selectively blocking impulse conduction or axoplasmic flow in the nerve during muscle regeneration, and by comparing the effect of the blocks with that of surgical denervation of regenerating muscle. Since it is known that the terminal phenotype of regenerating muscle is changed in the absence of normal postural load [3], we also investigated the interactions between a particular kind of muscle postural unloading, such as caused by tenotomy, and surgical or functional denervation on the pattern of regeneration.

Materials and Methods

All experiments were carried out according to the Helsinki Accords for Human Treatment of Animals during Experimentation. The study was approved by the Ethics Committee of the Medical Faculty of the University of Padova.

All surgical procedures were performed under general ether anaesthesia. Acute degeneration was induced in the soleus (slow) muscle of the rat by injecting 0.5-0.7 ml of 0.5% bupivacaine solution [1] in the muscle exposed through a small cutaneous incision. Surgical denervation was performed by cutting the sciatic nerve near the trochanter. Chronic block of impulse conduction was achieved by superfusion of sciatic nerve with tetrototoxin (TTX, Sankyo, Japan, 500 µg/ml), released from a miniosmotic pump (Alzet 2002, 200 µl volume, 0.5 µl/h release rate, 14 days discharge time)[2]. The pump was implanted in the interscapular region and connected by means of a polyethylene catheter to a silicon cuff surrounding the nerve [5, 7]. The axoplasmic flow was blocked by applying around the nerve, for 20 min, a cotton wool soaked with 0.15 mM vinblastine

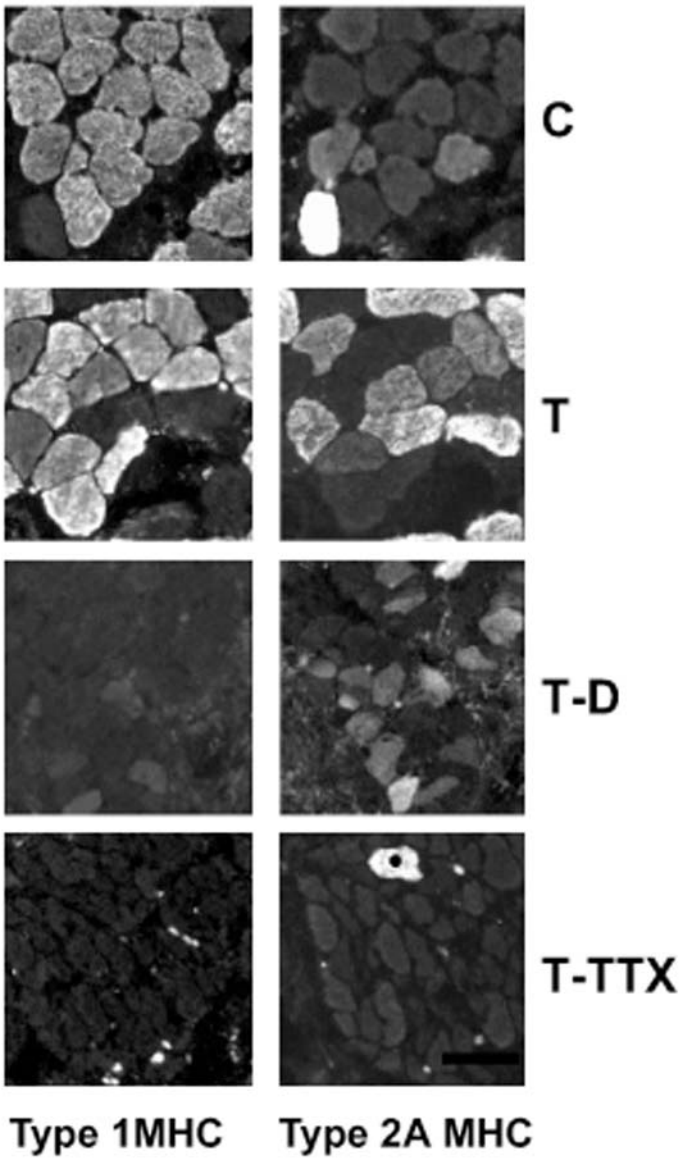

Figure 1. Immunofluorescence staining of serial cross-section from control innervated (C), denervated (D), tenotomized-innervated (T), tenotomized-denervated-regenerated (T-D), tenotomized-TTX-paralysed (T-TTX) soleus muscles. In the T-TTX specimen, black dot indicates a preexisting adult fibre. Scale bar = 50 µm.

(Sigma, USA) in 0.9% saline, according to the technique described by Kashiba et al. [4]. This procedure ensure an axonal flow blockade without neuronal damage (for references see Megighian et al. [5]). Tenotomy was performed by cutting the tendo calcaneus.

The patency of TTX-nerve block was checked daily by controlling the presence of paralysis of the leg, and the absence of withdrawal and toe-spreading reflexes in the experiments with TTX [8]. The absence of paralysis was also checked in the experiments with vinblastine.

Fourteen days after bupivacaine injection, the regenerated muscles were excised and frozen in a stretched position. Serial cross cryosections (8µm thick) were analysed by immunofluorescence. Primary monoclonal antibodies (mAbs) specific against type 1 (BA-D5), type 2A (SC-71), type 2B (BF-F3), type 2B-2X (RT-D9) and embryonic (BF-G6) MHC isoforms were used (kindly donated by prof. S. Schiaffino) [9]. A TRITC-conjugated rabbit antimouse immunoglobulins (Dako, Denmark) was used as secondary antibody.

Results and Discussion

As previously reported [5, 7], after 14 days regeneration the normal fibre type composition of innervated soleus muscle is almost reconstituted. In the denervated-regenerated muscle, type 1 myosin heavy chain (MHC)

isoform is lacking, whereas type 2A is diffusely expressed. In the TTX-blocked preparations both type 1 and type 2A MHC isoforms are lacking, and only type 2X and type 2B isoforms are expressed. In vinblastine-blocked preparations the 2A isoform expression is up-regulated. In TTX-vinblastine nerve-blocked preparations, type 1 isoform expression is absent, but type 2A is present in about 50% of regenerated fibres. These results show that expression of type 1 and type 2A MHC isoforms in the innervated-regenerating muscle is dependent on neuromotor impulses. The fact that type 2A isoform is lacking in the presence of TTX-block, while it is diffusely expressed in the denervated muscles and up-regulated in the presence of vinblastine-block, suggests that axoplasmic flow carries some chemical factor which inhibits 2A MHC isoform expression [5].

In the tenotomized-innervated-regenerated soleus muscle we found an increase of fibres expressing type 2A isoform (Fig. 1 T) with respect to the innervated-regenerated controls (Fig. 1 C). However, this isoform was again lacking, or almost lacking, in TTX-paralysed preparations (Fig. 1 T-TTX), where only 2B-2X isoforms were expressed. Vinblastine block had no effect on the pattern of regeneration. In the tenotomized-denervated-regenerated muscles type 2A isoform was expressed (Fig 1 T-D) along with 2B-2X isoforms.

The effects of tenotomy on regeneration are rather similar to those observed by Bigard et al. [3] on regenerating soleus muscle in hindlimb suspended rats, and confirm the "speeding" effect of disuse on slow muscle (for references see Midrio et al. [6]). The results show that also when it is enhanced - as in the case of tenotomized-innervated-regenerating muscle - the expression of type 2A MHC isoform is dependent on neuromotor discharge and is inhibited by the silent nerve. The lack of effects of vinblastine-block in tenotomized muscles may be due to the fact that the high expression of 2A isoform in this experimental condition mask the disinhibiting effect of axoplasmic flow blockade.

Perspectives

Our immediate research program is aimed to extend the study to fast muscle, and to investigate whether neuromotor and/or neurotrophic influences are of importance for other aspects of muscle differentiation/specialization, such as sarcoplasmic reticulum properties. Another line of investigation will be on the effects of chronic release on regenerating muscles of selected substances known to be involved in muscle differentiation.

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