Myosin heavy-chain composition in striated muscle after tenotomy

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The myosin heavy-chain (MHC) isoform pattern was studied by biochemical methods in the slow-twitch (soleus) and fast-twitch (gastrocnemius) muscles of adult rats during atrophy after tenotomy and recovery after tendon regeneration. The tenotomized slow muscle atrophied more than the tenotomized fast muscle. During the 12 days after tenotomy the total MHC content decreased by about 85 % in the slow muscle, and only by about 35 % in the fast muscle. In the slow muscle the ratio of MHC-1 to MHC-2A(2S) remained almost unchanged, showing that similar diminution of both isoforms occurs. In the fast muscle the MHC-2A/MHC-2B ratio decreased, showing the loss of MHC-2A mainly. After tendon regeneration, the slow muscle recovered earlier than the fast muscle. Full recovery of the muscles was not observed until up to 4 months later. The embryonic MHC, which seems to be expressed in denervated adult muscle fibres, was not detected by immunoblotting in the tenotomized muscles during either atrophy or recovery after tendon regeneration. The influence of tenotomy and denervation on expression of the MHC isoforms is compared. The results show that: (a) MHC-1 and MHC-2A(2S) are very sensitive to tenotomy, whereas MHC-2B is much less sensitive; (b) expression of the embryonic MHC in adult muscle seems to be inhibited by the intact neuromuscular junction.

INTRODUCTION

Myosin is known to be a major muscle-specific protein. It is composed of six subunits: two heavy chains and four light chains. The myosin heavy chain (MHC) has many isoforms which are specific for different muscle or fibre types. Maximum velocity and power of muscle contraction are correlated with the MHC type [1,2]. Expression of the myosin subunit genes is dependent on muscle innervation, function and hormone influence; it also changes with muscle development and aging (for reviews see [3,4]). The MHC of embryonic type is expressed in embryonic and fetal muscles of mammals. It disappears from rat skeletal muscle during the first days after birth and is absent from the mature muscle [5–7]. In denervated mature skeletal muscle the embryonic MHC seems to be re-expressed [8,9]. In such muscles expression of the ‘adult’ isoforms of MHC is also disturbed: the proportions of MHC type 1 and type 2B decrease in the denervated slow and fast muscle respectively, whereas the proportion of MHC type 2A increases in both muscles. In the denervated muscles the other type-defining characteristics of fibres change in correspondence with the MHC subunits [9,10]. In the present work the MHCs were examined in the tenotomized muscle. The study was performed to compare these two atrophying muscles, denervated and tenotomized, in order to obtain some information about whether lack of neuromuscular connection or some other reasons are responsible for changed expression of the MHC genes. After tenotomy a muscle–nerve junction remains intact, but activity of the motoneuron is decreased following decreased activity of the afferent system [11–13]. Additionally the tenotomized muscle is shortened and unloaded. The shortened position of the muscle brings about elimination of part of the contractile structure and its reorganization with a decrease in sarcomere number [14–16]. Both shortening and unloading cause muscle atrophy [17,18].

MATERIALS AND METHODS

Experiments on rats

Female albino Wistar rats (WAG inbred strain, bred in the Nencki Institute of Experimental Biology in Warsaw), 2.5–3 months old (150–190 g) were used. Animals were fed on the Polish standard Murigran diet; they were allowed access to food and water *ad libitum*. Tenotomy of the soleus and gastrocnemius (GAST) muscles was done (under ether anaesthesia) by cutting the Achilles tendon. After tenotomy the animals were left to move freely in cages. Soleus and GAST of the same animals were examined. Some experiments were performed on the fast extensor digitorum longus (EDL) muscle. Tenotomy of this muscle was performed by cutting its distal tendon. In all cases controls were used: (1) the muscles from the contralateral leg; (2) the muscles from non-experimental animals from the same population. In selected experiments controls were used: (3) leg muscles of 3-day-old rats; (4) soleus and EDL muscles either denervated or recovering from denervation atrophy after self-reinnervation, respectively after cutting or crushing the sciatic nerve (as described previously [9]). Muscles from two to four animals were used in each experiment. Results from some experiments were pooled.

Preparation of myofibrils

At 4–120 days after the operation animals were killed by decapitation, and the muscles were immediately removed, measured, weighed and transferred to ice. Tendons and fasciae were removed before homogenization of the muscles. Myofibrils, either crude or purified, were separated as described in detail [9,19]. The total content of protein in myofibrils was estimated by the method of Lowry *et al.* [20].

Abbreviations used: MHC, myosin heavy chain; GAST, gastrocnemius muscle; EDL, extensor digitorum longus muscle.

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Fig. 1. Evaluation of linearity between the amount of protein subjected to electrophoresis and the intensity of protein staining eluted from gels

Purified myosin (●) and BSA (Serva; ■) were used as standards. Electrophoresis was performed on 6%-acrylamide gels, as described by Weber & Osborn [22]. After staining with Coomassie Blue, individual bands were cut out and eluted from the gels with 2 ml of 1% SDS at 37 °C. Intensity of staining was measured colorimetrically at 600 nm. The MHC band contained 76% of the myosin protein loaded on gels; the 67 kDa band contained 90% of the BSA loaded on gels.

Estimation of the total MHC content

The total MHC content in the muscle was evaluated by estimating the total MHC in crude myofibrils, as described previously in detail [9,21]. Myofibrillar proteins were separated by SDS/PAGE as described by Weber & Osborn [22]; samples (20–40 μg of protein) were applied to 6–8%-acrylamide gels. After separation and staining with Coomassie Blue, individual bands were cut out and eluted from gels; the amount of protein was estimated colorimetrically. The intensity of protein staining was proportional up to 30 μg of protein within one band (Fig. 1). The content of the total MHC was referred to albumin (added to crude myofibrils as an internal quantitative standard [9]) eluted from the same gel, and then compared with the MHC content from other gels. Gels from the same electrophoresis run, and stained and destained together, were exclusively used for comparison. The data from each experiment was derived from analysis of at least six electrophoresis runs.

Analysis of MHC isoforms

MHC isoforms were separated from isolated myofibrils by SDS/PAGE (6% gel) with high glycerol concentration (37%, v/v) as described by Carraro & Catani [23], as modified by Danieli Betto *et al.* [24]. Individual isoforms of MHC (1, 2A and 2B) were identified: (1) according to their electrophoretic mobility and relative abundance in fast and slow muscles [23,25,26] and in histochemically characterized cryostat sections [27,28]; (2) by immunochemical staining [24]; (3) by co-migration with individually purified MHC markers, which were isolated by electroendosmotic preparative-gel electrophoresis and characterized by peptide mapping [29,30]. Relative amounts of MHC isoforms were determined by comparing the degree of intensity of staining with Coomassie Blue of the electrophoretic bands by using a Shimadzu densitometer model CS-930. A linear response is obtained on densitometry when 0.1–2.0 μg of individual MHC are analysed [31].

Analysis of the embryonic MHC isoform

This was performed by the procedure of Towbin *et al.* [32] as described previously [9]. The MHCs after separation by SDS/PAGE were electrophoretically transferred to nitrocellulose paper. The blots were stained with 0.2% Ponceau Red and photographed. The blots were incubated with purified monoclonal antibodies BFG6 for embryonic MHC [33] (1:20000), then with the affinity-purified anti-mouse IgG antibodies (1:2000), conjugated with alkaline phosphatase. Blots were developed for 40 min [34], and a photograph was taken every 5 min during the phosphatase reaction.

Materials

The purified monoclonal antibodies BFG6 for embryonic MHC was a gift from Professor S. Schaffino (Institute of General Pathology, University of Padova). The affinity-purified anti-mouse IgG conjugated with alkaline phosphatase was obtained from Kirkegard and Perry Laboratories (Milano, Italy). The Hybond TM-C hybridization-transfer membrane was obtained from Amersham. All chemicals were of analytical grade, obtained from Serva, Sigma, Merck or POCH (Gliwice, Poland). The myosin used as standard was purified basically by the Perry [35] method.

RESULTS

The Achilles tendon

After cutting the Achilles tendon, the muscles shortened and a

Table 1. Length and mass of tenotomized muscles

Results (% of contralateral) are expressed as means ± S.E.M. for the numbers of measurements in parentheses.

Time after operation (days)	Soleus		GAST	
	Muscle length	Muscle mass	Muscle length	Muscle mass
4	69 ± 2 (6)	76 ± 3 (7)	90 ± 3 (6)	90 ± 2 (3)
12	58 ± 3 (8)	49 ± 2 (6)	84 ± 2 (5)	84 ± 3 (9)
22	68 ± 2 (6)	54 ± 3 (8)	87 ± 2 (4)	80 ± 2 (4)
28	71 ± 1 (3)	61 ± 2 (4)	82 ± 2 (5)	77 ± 2 (4)
47	73 ± 3 (5)	62 ± 1 (5)	84 ± 1 (3)	76 ± 4 (5)
70	80 ± 4 (5)	68 ± 4 (9)	91 ± 5 (7)	89 ± 2 (5)
120	75 ± 5 (8)	67 ± 6 (10)	92 ± 1 (2)	88 ± 4 (7)
Controls	18 ± 0.5 mm	77 ± 1.6 mg	24 ± 0.6 mm	994 ± 56 mg

Fig. 2. Changes in total MHC content in tenotomized muscles

The muscle content of the total MHC was evaluated as described in the Materials and methods section and in Fig. 1. Each point represents the results from at least four pooled muscles, and from at least three independent estimates. Values are means $\pm$ S.E.M. (bars).

gap appeared between the two stumps of tendon. Some increase of the gap was observed during the next few days. On days 7–11 after the operation, the tendon began to be restored by way of a connective-tissue bridge: the gap between the stumps was filled with a gelatinous substance, which became solid and hard during the following days. Then, during the following weeks it was transformed to a massive tendon, longer and thicker than the normal Achilles tendon. At 4 months after the operation, some involution of that massive tendon was observed.

Muscle length, mass and total MHC content

The changes in length and mass of the muscles studied were taken as criteria of muscle atrophy and recovery. After cutting the Achilles tendon, the length of muscles diminished progressively and muscles atrophied. The degrees of length and mass diminution were not the same for the soleus and GAST muscles. In the soleus the length decreased by about 40 % and the mass by about 50 % during 12 days, whereas in the GAST both of them decreased by only 16 % (Table 1). Simultaneously the total MHC content decreased by about 85 % in the soleus, and by about 35 % in the GAST (Fig. 2). The decrease in the parameters described in the soleus was greatest on day 12 after tenotomy. Then, owing to recovery of the tendon, they increased. The dynamics of recovery were highest on days 15–30 after tenotomy. Then all the parameters studied remained at similar levels, much lower than those in the contralateral legs, for at least 4 months (Table 1, Fig. 2). The soleus muscle belly remained shortened to approximately two-thirds of the original length. In the GAST muscle all these parameters recovered later and with much smaller intensity than in the soleus. At 4 months after operation they were still lower than those of the contralateral legs (Table 1, Fig. 2). The data reported here are in general in agreement with the previous preliminary ones [36].

MHC isoforms

The MHC isoforms were separated by SDS/PAGE as described in the Materials and methods section (examples are presented in Fig. 3). The MHC of the control soleus muscle contained about 15 % of type 2A isoform and about 85 % of type 1. After tenotomy some slight changes in the proportion of the

Fig. 3. Electrophoretograms of MHC isoforms

(a) Tenotomized soleus, GAST and EDL, about 3 or 6 μ g of myofibrillar protein, loaded on gels; MHC of myofibrils from: soleus, 4 days after tenotomy (lane 1); soleus, 7 days after tenotomy (lane 2); soleus, 12 days after tenotomy (lane 3); soleus, 28 days after tenotomy (lane 4); soleus, 47 days after tenotomy (lane 5); soleus, 70 days after tenotomy (lane 6); control soleus (lane 7); control GAST (lane 8); control EDL (lane 9); leg muscles of 3-day-old rats (lane 10); EDL, 7 days after tenotomy (lane 11); GAST, 7 days after tenotomy (lane 12); GAST, 12 days after tenotomy (lane 13); GAST, 28 days after tenotomy (lane 14); GAST, 47 days after tenotomy (lane 15); GAST, 70 days after tenotomy (lane 16). (b) Tenotomized soleus and GAST, overloaded gels (about 10 μ g of myofibrillar protein). MHC of myofibrils from: soleus, 4 days after tenotomy (lane 1); GAST, 4 days after tenotomy, Expt. 1 (lane 2), Expt. 2 (lane 3); soleus, 12 days after tenotomy (lane 4); GAST, 12 days after tenotomy (lane 5); soleus, 21 days after tenotomy (lane 6); GAST, 21 days after tenotomy, Expt. 1 (lane 7), Expt. 2 (lane 8); control soleus (lane 9); control GAST (lane 10).

Fig. 4. Changes in the MHC isoform pattern in the tenotomized muscles

(a) Slow muscle; (b) fast muscle. Electrophoretograms of the MHC isoforms were scanned as described in the Materials and methods section. The amounts of individual MHC isoforms are shown. The sum of all MHCs in the sample was taken as 100%: ○, MHC-1; □, MHC-2A; △, MHC-2B. Each point represents results from at least four pooled muscles, and from at least three independent estimates. Values are means $\pm$ S.E.M. (bars).

isoforms appeared within a few days: MHC-1 decreased, whereas MHC-2A increased (Fig. 4a). They recovered to the control pattern within some days. The MHC-2B band was not detected in the tenotomized atrophying or recovering soleus.

The MHC of the control GAST contained about 35% of isoform 2A and about 65% of 2B. After tenotomy the proportion of isoform 2A decreased, whereas that of isoform 2B increased within about 12 days (Figs. 3 and 4b). Recovery of the normal MHC pattern took several weeks. Similar changes in proportion of the MHCs were observed in the EDL muscle during the first days after tenotomy.

Embryonic MHC

A search was made for embryonic MHC by immunoblotting. The embryonic isoform of MHC was not detected in either soleus or GAST muscle, both atrophying after tenotomy and recovering after tendon regeneration (Fig. 3, lanes 4–9). The reaction, which was also not detectable with MHC of control muscles of adult animals (Fig. 5, lanes 1, 2), was very intensive with MHC of muscle of 3-day-old rats (Fig. 5, lanes 3, 10). For comparison, the denervated muscles and those recovering from denervation atrophy after self-reinnervation were examined in parallel (Fig. 5, lanes 11–14). The results confirmed the presence of the embryonic isoform of MHC in the denervated and recovering muscles of adult rats [8,9].

DISCUSSION

Atrophy and recovery of the tenotomized muscles

In the present study, tenotomy of the soleus and GAST muscles was performed by cutting the same tendon. However, owing to their anatomical insertions and their different lengths (Table 1), the relative decrease in length was not the same in the two muscles studied: it was much larger in soleus than in GAST muscle (Table 1). Thus experimental conditions applied were not the same for the two muscles, and differences in the progress of atrophy appeared: the degree of atrophy was higher in the soleus than in the GAST muscle (Table 1). Such a difference could also be caused by the greater sensitivity of the slow muscle than the fast one to tenotomy [37–39]. After tendon regeneration the recovery of these two muscles was not synchronous. It was much earlier and more rapid in soleus than in GAST (Table 1, Fig. 3). This could occur as effect of the increased activity of the soleus after the function of the afferent system has resumed. However,

Fig. 5. Immunoblots of MHC from experimental muscles with a monoclonal anti-embryonic MHC antibody

(a) Blot stained with Ponceau Red immediately after transfer. (b, c) Blots after incubation with monoclonal antibody BFG6 for embryonic MHC, then with anti-mouse IgG antibodies conjugated with alkaline phosphatase. The photographs were taken after 5 min (b) and 40 min (c) of development. The MHC of myofibrils from: control soleus (lane 1); control GAST (lane 2); leg muscles of the 3-day-old rats (lane 3); soleus, 12 days after tenotomy (lane 4); soleus, 22 days after tenotomy (lane 5); soleus, 70 days after tenotomy (lane 6); GAST, 12 days after tenotomy (lane 7); GAST, 22 days after tenotomy (lane 8); GAST, 70 days after tenotomy (lane 9); leg muscles of the 3-day-old rats (lane 10); EDL, some days recovering from denervation atrophy, 21 days after crushing the sciatic nerve (lane 11); denervated EDL, 21 days after cutting the sciatic nerve (lane 12); soleus, some days recovering from denervation atrophy, 21 days after crushing the sciatic nerve (lane 13); denervated soleus, 21 days after cutting the sciatic nerve (lane 14).

in neither soleus nor GAST was full recovery observed. It could perhaps be explained by the increased length of the regenerated tendon, and by the presumed new steady state, different from that in the normal muscle.

Table 2. Sensitivity of the individual types of MHCs to tenotomy

Data presented in Figs. 2 and 4 were re-calculated and expressed relative to 100 mg of the total MHC muscle content.

		Total MHC		MHC-1		MHC-2A		MHC-2B	
		Content (mg)	Loss (%)	Content (mg)	Loss (%)	Content (mg)	Loss (%)	Content (mg)	Loss (%)
Soleus	Controls	100		84		16			
	12 days tenotomized	15	85	12.5	85	2.5	84		
GAST	Controls	100				35		65	
	12 days tenotomized	66	34			18	49	48	26

Identification of the MHC isoforms

Under the electrophoretic conditions used, the MHC-2S isoform [30] has very similar mobility to the MHC-2A isoform. These two isoforms are also called 2A and 2X [40] or IId and IIa [41]. Thus the MHC-2A band can also contain the MHC-2S isoform. In the soleus muscle this band contained exclusively MHC-2S, which is the only fast component of the soleus muscle [30]. The embryonic and neonatal MHC isoforms, on the other hand, have the same mobility as the MHC-2B isoform. Thus the MHC-2B band can also contain the embryonic or neonatal MHC. In tenotomized muscles the presence of embryonic MHC may be excluded by immunoblot analysis (Fig. 5).

The MHC 1 and MHC 2B isoforms are known to be present in very low amounts in rat GAST and soleus muscle respectively. They can be detected [9], especially when gels are over-loaded. They were not detected in the electrophoretograms here shown (except traces of MHC-1 in the over-loaded gels of GAST; Fig. 3b). Taking into account the sensitivity of the staining procedure used, it may be concluded that the proportion of these isoforms in the muscles studied was lower than 5%. The very low content of the 2B isoform in the soleus may be connected with the female sex of the animals (type 2 is dependent on androgenic hormones [42]).

Differences between MHC isoforms in reaction to tenotomy

To explain the reaction of the particular MHC isoforms on tenotomy, changes in amount of total MHC (Fig. 2) and in proportions of MHC isoforms (Fig. 4) were analysed. These speculations were based only on the results from day 12 after the operation, when atrophy was most advanced but the muscle recovery was not yet observed. In tenotomized soleus muscle the MHC-1 and MHC-2A-band isoforms show very similar decreases (Table 2). On the other hand, re-calculation and comparison performed in the tenotomized GAST points out the larger loss of the 2A isoform content than of the MHC-2B (Table 2). The above results allow the conclusions that: (1) reaction on tenotomy of MHC-1 and MHC-2S is similar; (2) tenotomy affects MHC-1, MHC-2A and MHC-2S, isoforms characteristic of fatigue-resistant fibres, much more than the MHC-2B isoform.

Comparison of muscle reactions to tenotomy and denervation

As mentioned in the Introduction, atrophy after tenotomy is caused by at least three 'factors': decreased activity, shortening and unloading of the muscle, while denervated muscle is inactive and devoid of a connection with the nervous system. In tenotomized muscle atrophy is known to be more rapid, and regularity of the contractile structure is known to be more disturbed than in denervated muscle. Additionally, the rapid atrophy of tenotomized muscle depends on its intact innervation [43].

Comparison of the results of this paper with previous ones concerning the denervated muscle [9] allows for some speculations and conclusions. The MHC-1 was found to be sensitive to both tenotomy (Table 2) and denervation [9]. Thus it seems that its content is mainly dependent on function, since electrical stimulation can induce an expression of this isoform in the denervated fast muscle [44]. On the other hand, it is possible that the MHC-1 is sensitive to any other of the 'factors' of the experimental models applied: for example, the fibres of type 1 atrophy after muscle unloading or shortening [17,18]. The MHC-2A(2S) is very sensitive to tenotomy, but it is relatively insensitive to the lack of innervation: in the denervated muscle the 2A type replaces both the 2B and 1 types [9]. Fibres of the 2A type were found to be sensitive also to unloading and shortening [17,18]. The MHC-2B is relatively more sensitive to denervation [9] than to tenotomy (Fig. 4, Table 2). On the other hand, it is suppressed by the increased muscle activity, as observed in reinnervated-recovering muscle [9] and in muscle stimulated electrically [45]. It seems that both lack of a muscle-nerve junction and increased muscle activity are the factors which decrease the content of the MHC-2B isoform. The same rules seem to apply also to the muscle fibre types.

The embryonic isoform of MHC, which is expressed in denervated slow and fast muscle [8,9], was not detected in the tenotomized muscles and in those recovering after tendon regeneration (Fig. 5). Nor was the embryonic MHC found in 4-day-tenotomized muscle (results not shown), when fibres recover from segmental necrosis, appearing in the tendon region [46], and the embryonic isoforms of myosin subunits could be expected [47-49]. It seems that the expression of the embryonic MHC is suppressed by the preserved neuromuscular junction, similarly to what was found in the innervated muscle regenerating after injury [50].

The mechanism regulating the proportion of individual MHC isoforms in the striated muscle (and those of muscle fibre types) are far from being elucidated. Changes in function and in innervation undoubtedly influence expression of the genes of particular MHC isoforms. If, and how, they regulate the degradation processes of these proteins is unclear at present. The results in this paper also suggest that selective degradation of the MHC isoforms could be expected.

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