The Longitudinal Distribution of Isomyosins in Normal and Regenerating Extensor Digitorum Longus of the Rat

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Abstract

Extensor digitorum longus (EDL) muscle of rat was cut perpendicularly to their long axis in three parts. The proximal, middle and distal parts of the muscles were analyzed for their proportions in myosin heavy chains, isomyosins and type I fibres.

Proximal sections contain relatively more myosin heavy chains MHC2A, whilst the reverse is true for distal sections, which contain relatively more myosin heavy chains MHC2B.

The proportions of isomyosin FM_3 are highest proximally; those of FM_2 are highest distally; the proportions of FM_1 are constant along the lenght of the muscle. One may thus infer from our observations that the distal end is proportionally richer in LC_{3f} than in LC_{1f} when compared to the proximal end.

Intermediate isomyosin, IM, is localized proximally in the few EDL muscles where it is present.

Type I fibres are preferentially localized in a medio-anterior area. This area is larger at the proximal end than at the distal end, and consequently, the proportion of type I fibres is highest at the proximal end.

These observations indicate that EDL muscle contract faster at its distal end than at its proximal end.

EDL muscle regenerating from a mince up to 120 days have lost the spatial distribution of fibres observed in the control muscle.

Key words: muscle regeneration, isomyosin, myosin heavy chians.

BAM 1 (1): 55-61, 1991

Extensor digitorum longus is a striated skeletal muscle which in adult rat has multiple isoforms of myosin characterized by their heavy and light chains. Three myosin heavy chains are present in EDL muscle: MHC1, MHC2A and MHC2B, the former which is typical for slow-twitch muscle, and the latters which are typical for fast-twitch muscle. The distribution of the heavy chains (0.2% for MHC1, 26.8% for MHC2A and 73% for MHC2B [21, 34]) indicates that this muscle is of the fast-twitch variety.

Since the myosin heavy chains composition of muscle fibres determines their fibre type as evaluated by histochemical AT-Pase activity [35, 36], the relative proportions of fibre types should correspond to those of the myosin heavy chains. In agreement with this expectation, slow-twitch fibres (type I containing MHC1) occupy about 5% of the muscle area and fast-twitch fibres about 35% and 60% for type IIA (MHC2A) and IIB (MHC2B) respectively [23, 28]. The distribution of the slow-twitch fibres is not homogeneous in EDL muscle: it depends on the nerve branching pattern. EDL muscle is innervated by two distinct branches, the K branch (K for knee) and the F branch (F for foot). Most slow-twitch fibres (67%) are located in the anteromedial part of the muscle, a territory innervated by the K branch [1]. The fast twitch fibres (type IIA and type IIB) are distributed quite uniformly throughout the muscle section [31, 1]. All these observations were made on cross-sections obtained in the middle part of the muscle: they demonstrate the existence of a cross-sectional heterogeneity of EDL muscle. The question then arises whether EDL muscle also has a longitudinal heterogeneity: namely whether distal end of EDL has the same distribution in myosin isoforms than proximal end. We show in this paper that this is not the case: the proximal end has higher proportions of MHC2A and MHC1, whilst the distal end has more MHC2B.

We do not understand the functional significance of the unhomogeneous repartition of EDL muscle fibres which divides the muscle into at least two compartments. This arrangement arises during muscle ontogeny and it is probably controlled by the branching pattern of its nerve. It is an interesting question whether the factors which set this arrangement in embryo and foetus could also impose it when the muscle regenerate in adult animals. To investigate this point we have cut the nerve and destroyed the endomysium organization by mincing and we have induced the regeneration by grafting the mince orthotopically. This procedure first reported by Studitsky [38] and Carlson [9] strongly excites the satellite cells which proliferate, fuse and eventually regenerate normal muscle fibres (review in [30]). During the regeneration, embryonic and foetal isoforms of myosin are expressed [12, 24] and the functional recovery appears to be nearly completed in at least some cases [5]. We report that muscle regenerating from a mince has lost the spatial organization of isomyosins and muscle fibre types which is present in normal untreated muscle.

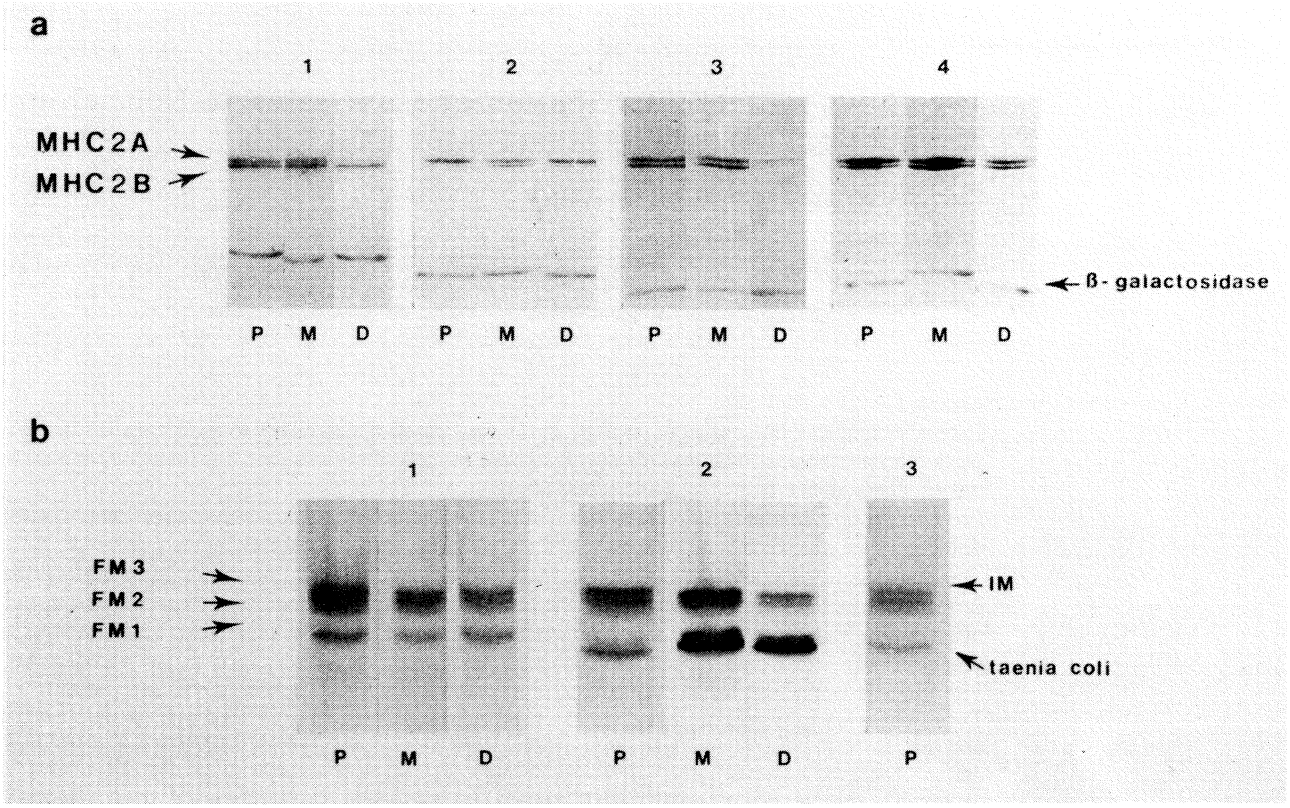

Figure 1. Gel electrophoresis of native myosin and myosin heavy chains. P, M, D refer to proximal, median and distal levels of the muscles. a. SDS-PAGE; 1, Control EDL muscle; 2, 30 days regenerating muscle; 3, 60 days regenerating muscle; 4, 120 days regenerating muscle. β-galactosidase is used as an internal standard.
b ,PPi-PAA gel electrophoresis; 1. Control EDL muscle; 2, Regenerated EDL muscle at 120 days post surgery; 3, Control EDL muscle with intermediate myosin (IM). An extract of guinea-pig taenia coli is used as an internal standard to calculate the isomyosins mobilities.

Materials and Methods

Surgery

Twenty-four male Wistar rats 60 days of age were anaesthetized with Thalamonal (0.2 ml/100 g body weight) injected subcutaneously. The right hind limb was shaved and the skin was cut on the anterior side from the foot to the knee. The tibialis anterior muscle was carefully separated from the peroneus longus. The extensor digitorum longus was excised by cutting the proximal and distal tendons as close as possible to the ends of the muscle fibres. Great care was taken to keep the adjacent muscles undamaged. The EDL was minced with scissors in a Petri dish at room temperature and the mince was grafted orthotopically. Tibialis anterior, peroneus longus and the skin were sutured with Ethicon thread (5/0).

Regenerates and contra-lateral EDL were dissected 30, 60 or 120 days post-surgery. The longest period of regeneration was set at 120 days post-surgery because regeneration is stabilized at that time [29]. Muscles were frozen in isopentane cooled with liquid nitrogen and they were stored at -80°C until use.

Histochemistry

The muscles were cut perpendicularly to their long axis in three parts (here after called proximal, median and distal). Median sections were obtained from the middle of the median part. Sections (10 μm thick) were obtained at each level and stained for type I fibres by the histochemical ATPase reaction at pH 4.25 [7]. The proportion of type I fibre was evaluated by comparison of their areas.

Electrophoresis

The remaining parts of the muscles were weighed and extracted in 3 vol. Guba-Straub buffer (mM: NaCl 300; NaH_2PO_4 100; $MgCl_2$ 1; $Na_4P_2O_7$ 10; Na_2HPO_4 50; Na azide 0.1%, pH 6.5). After centrifugation for 10 min at 20,000 rpm, the supernatant was mixed with 1 vol. glycerol.

(i) Polyacrylamide gel electrophoresis in non dissociating conditions (PPi-PPA) was performed as described by Hoh *et al.* [18] and d'Albis *et al.* [13]. An extract of taenia coli from adult guinea-pig was added as an internal standard for mobility (Rf) measurement [24]. The supernatants with glycerol were diluted in buffer (mM: $Na_4P_2O_7$ 200; glycerol 50%; β-mercaptoethanol 0.1%, pH 8.6) at 1/30 for the control and 1/15 for the regenerated muscles; 10 and 20 μl of the diluted extracts were used for electrophoresis. Gels were stained (20 min at 65° C) with a Coomassie brilliant blue solution (Serva R250 25 mg; methanol 20 ml; acetic acid 70 ml in 1 l H_2O) and destained in 7% acetic acid.

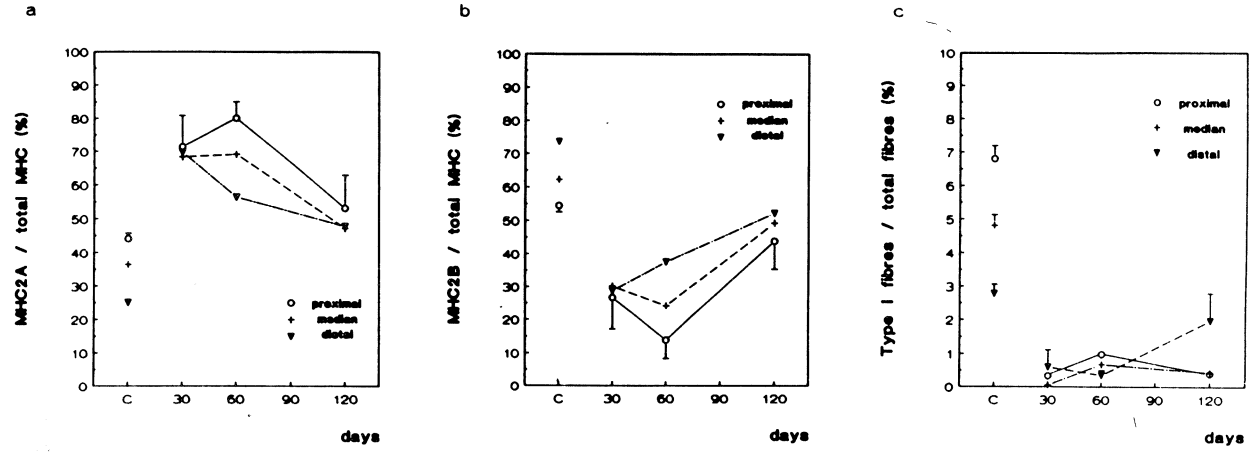

Figure 2. Relative proportions of myosin heavy chains and type I fibres in control and regenerating EDL muscles. **a.** *Proportion of myosin heavy chain 2A (MHC2A); b, Proportion of myosin heavy chain 2B (MHC2B); c, Proportion of type I fibres. Each point is an average value at proximal, median and distal levels for control (C) and regenerating muscles at 30, 60 and 120 days post-surgery. The vertical bar indicates the SEM. The SEM is approximately the same for the two other levels.*

(ii) SDS polyacrylamide-gel electrophoresis (SDS-PAGE) was performed as described by Danieli-Betto *et al.* [14], including 33% (weight/vol) glycerol in both separating and stacking gels [6]. The supernatants with glycerol were diluted in a buffer (mM: Tris 62.5; SDS 2.3%; glycerol 10%; β-mercaptoethanol 5%, pH 6.8) at 1/900 for the control and 1/60 for the regenerate. The electrophoretic run (10 mA) was performed with 4 or 8 μl of the diluted extracts and 5 μl of β-galactosidase diluted in the above buffer (dilution of 1/20) added as a reference for myosin heavy chains mobility.

Staining was done overnight in a Coomassie blue solution (Serva G250 40 mg; perchloric acid 70% 3 ml in 100 H_2O) and gels were destained in 7% acetic acid.

Termin *et al.* [39] reported another electrophoretic method to separate myosin heavy chains with which they could detect a new isoform (MHC2d) present in rat EDL. Using their method, we could not observe MHC2d in our EDL samples. This may be due to some variations between strains. This interpretation is supported by the fact that analyses of rat soleus also gave different results: while Termin *et al.* [39] observed only one type of myosin heavy chains (MHC1) we observed two types (MHC1 and MHC2A) and even three types in some samples (MHC1, MHC2A and MHC2B). In the method used in present experiments , MHC2d, if present, comigrates with MHC2b. We made no further attempts to study separately the distributions of MHC2b and MHC2d.

Gels were analyzed by densitometry. Densitograms display a peak for each isomyosin. The Rf values of the peaks of native myosins were expressed relatively to the Rf of taenia coli myosin arbitrarily set at 147: the Rf of SM_2 is then equal to 100 [25]. For myosin heavy chains, the Rf values of the peaks were expressed relatively to the Rf of β-galactosidase (set at 100). If the temperature in PPi-PAA gels electrophoresis falls under 1° C the taenia coli myosin separates into two zones (3). In this case, the mobility of the slowest migrating component was taken for reference. The proportions of each isomyosin were evaluated by the areas of each peak of the densitograms expressed as fractions of the total area of skeletal myosins.

Results

CONTROL MUSCLES

Myosin heavy chains

In the control heterolateral muscles, two fast-twitch myosin heavy chains are detected, MHC2A and MHC2B (Fig. 1a). The proportions of the myosin heavy chains are shown in Fig. 2 (a, b, control data). As no significant differences could be detected between the three age matched control groups (90, 120, 180 days), all the data were pooled. The relative proportions of MHC2A and MHC2B vary inversely along the length of the muscle. The proportion of MHC2A decreases from the proximal end to the distal end while that of MHC2B shows the opposite change. Slow-twitch myosin heavy chain MHC1 was observed in only two muscles out of twenty-three, but in a low proportion, less than 10% of the total heavy chains. In both cases, the proportions of MHC1 were higher in the proximal end and decreased to the distal end.

Since it is conceivable that the heterogeneity observed in control heterolateral muscles could result from some reaction to the surgery, we checked that EDL muscles from unoperated animals present a similar longitudinal distribution (data not shown).

Myosin isozymes

The three native isomyosins typical for fast-twitch muscles (FM_3, FM_2 and FM_1, Fig. 1b) are present in all control muscles. The relative proportions of native isomyosins are shown in Fig. 3 (a, b, c, control data). We observed approximately 40% of FM_3, 36% of FM_2 and 24% of FM_1, in agreement with previous reports [24, 25, 33, 40]. The fastest migrating component (FM_1) is the least abundant and its proportion is constant along the muscle length. The proportion of FM_3 is significantly larger at the proximal end than at the distal end of the muscle. Inversely, the proportion of FM_2 is significantly larger at the distal end. The middle portion of the muscle is similar to the proximal end.

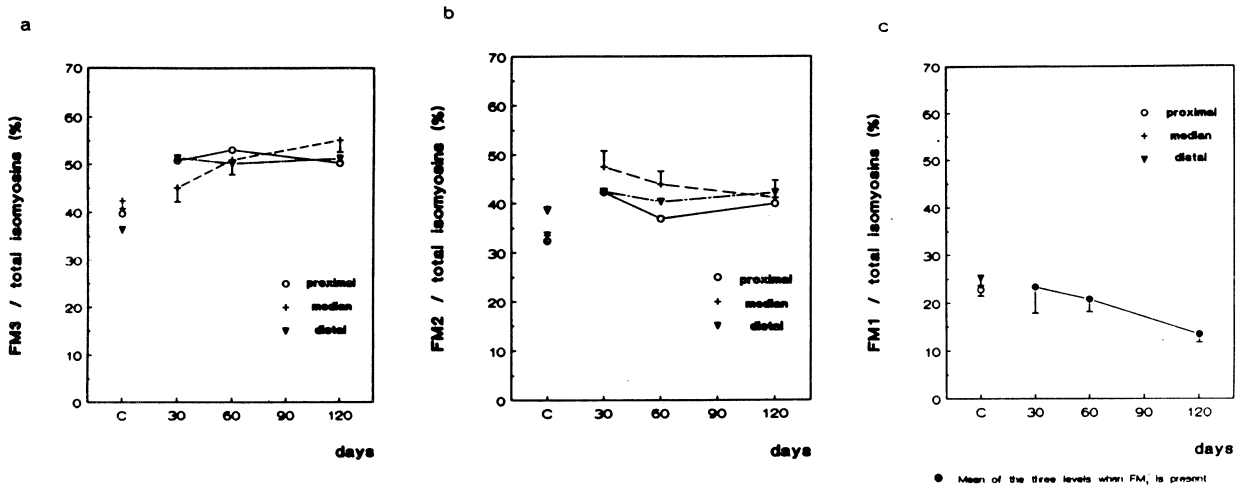

Figure 3. Relative proportions of native myosin isoforms in control and regenerating EDL muscles. a, Native myosin FM_3; b, Native myosin FM_2; c, Native myosin FM_1. Each point is an average value at proximal, median and distal levels for control (C) and regenerating muscles at 30, 60 and 120 days post-surgery. The vertical bar indicates the SEM.The SEM is approximately the same for the two other levels.

Seven out of 23 control muscles (Tab. 1) contain the intermediate myosin (IM) identified by a mobility of 113.5 ± 0.6 [25]. The isomyosin IM is localized at the proximal end with an average proportion of 23%. Only one out of seven muscles had IM in its middle portion, and none contained IM at its distal end.

Type I fibres

The proportion of MHC1 in rat EDL muscle is small [25] and probably under the detection level of the electrophoretic method in most of the muscles studied in the present work. We have thus complemented the electrophoretic analysis with an histochemical staining for the ATPase reaction of type I fibres to evaluate the distribution of MHC1.

In confirmation of earlier work of Balice-Gordon and Thompson [1], we observed that type I fibres are mainly localized in the anterior part of the muscle (Fig. 4 A, B, C). We further observed that type I are preferentially localized at the proximal end of the muscle. The proportion of type I fibres decreases significantly from 6.8 ± 0.4% at the proximal end to 4.8 ± 0.3% at the median section and 2.8 ± 0,3% at the distal end. Simultaneously, the area rich in type I fibres shrinks (Fig. 2c and 4 A, B, C).

Regenerating Muscles

Myosin heavy chains

EDL muscle regenerating from a mince contains the same myosin heavy chains that are present in control muscle but their relative proportions are reversed. At 30 days post-surgery, there is approximately 70% of MHC2A and 30% of MHC2B (Fig. 1a, 2; Fig. 2 a, b). During regeneration the proportions of the myosin heavy chains tend to return to control values but the recovery is not yet completed 120 days post-surgery. No differences in proportions of myosin heavy chains are observed along the length of the muscles.

Myosin isozymes

At 30, 60, 120 days post-surgery two isomyosins are present with mobilities equal to those of FM_3 and FM_2 of control muscles. Their relative proportions are shown in Fig. 3 (a, b). Muscles regenerating for 30 days have significantly more FM_3 and FM_2 than their controls: FM_1 is observed in only 2 cases out of seven. Later in the regeneration, isomyosins FM_3 and FM_2 do not appear to change, but the relative abundance of FM_1 tends to decrease (Fig. 3c).

The intermediate form (IM) was seen in only two experiments: one at 30 days at the proximal end and the median level, and one at 120 days at the proximal end.

Type I fibres

In regenerating muscle, we observed very few type I fibres (0.7 ± 0.2%), with no differences in their proportions between the three cross-section levels (Fig. 2, c). They are randomly distributed throughout the muscle either isolated or in small groups of three or four fibres (Fig. 4 D, E, F). There is no significant evolution during the course of muscle regeneration.

Discussion

Isomyosins and fibre types in control EDL

Rat EDL is considered a typical fast-twitch muscle, but its fibres are of various types. According to Okada *et al.* [28], most of its fibres are either of type IIB (71%) or IIA (23%), with a small fraction of type I (6%). The histochemical ATPase activity reflects the myosin heavy chain composition [35, 36]. Thus the proportions of the myosin heavy chains must reflect those reported for the fibres types. We have indeed observed proportions of heavy chains coherent with fibre types: 73.5% of MHC2B and 26.5% of MHC2A (Fig. 2). The slow myosin heavy chain, MHC1, is observed only in two muscles: it is probable that the proportion of this heavy chain is too small to be efficiently detected by the electrophoretic analysis. As a substitute, we have thus examined the distribution of type I

Table 1. Frequency of myosin isoforms detection in control and regenerating EDL muscles.

	IM	FM_3	FM_2	FM_1
Mobility	113.5 ±0.6	118.1 ±0.2	122.2 ±0.2	127.0 ±0.2
Frequency in control muscles (n = 23)	7/23	23/23	23/23	23/23
30 days (n = 7) post surgery	1/7	7/7	7/7	2/7
60 days (n = 8) post surgery	0/8	8/8	8/8	5/8
120 days (n = 8) post surgery	1/8	8/8	8/8	4/8

IM = intermediate myosin; FM = fast twitch myosin; n = number of muscles. The data show the frequency with which the isomyosin are detected. The mobility is the electrophoretic mobility (mean ± SEM) of the isomyosin (see methods).

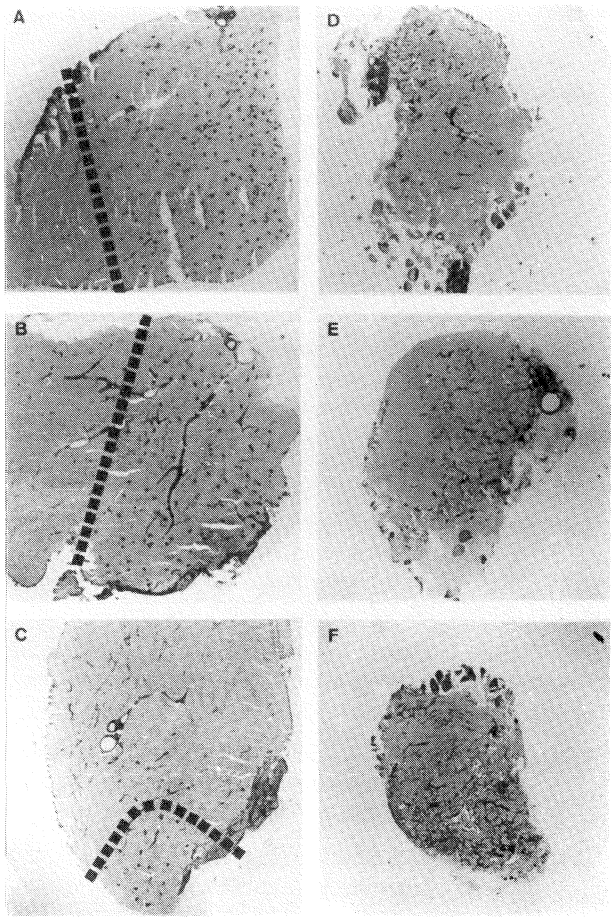

Figure 4. Micrographs of cross-sections of control and regenerated muscles (X14). A, B, C, control muscle; type I fibres are mainly localized on the right side of the dotted lines; D, E, F, regenerate muscles at 120 days post-surgery; A, D, proximal end; B, E, median section, C,F, distal end.

fibres by histochemistry, and found a frequency of 4.8 ± 0.3%, in fair agreement with Okada *et al.* [28].

In earlier works, the distribution of fibres in EDL muscles has been examined in cross-sections obtained from the middle part of the muscle [31, 28, 1]. We have extended the observations to cross-sections obtained from the proximal and distal ends of the muscle, and we have found that there also exists a longitudinal distribution of myosin heavy chains (Fig. 2 a, b) and, by inference, of fibre types. Proximal sections contain relatively more MHC2A and are presumably richer in type IIA fibres, whilst the reverse is true for distal sections, which contain relatively more MHC2B, and are thus presumably richer in type IIB fibres. The proximo-distal gradient IIA/IIB can be estimated from the ratio MHC2A/MHC2B: 0.81 proximally, 0.50 medially and 0.34 distally. This ratio decreases nearly linearly along the leg axis. Since type IIB fibres which contain MHC2B contract faster [32] and give more mechanical power [4] than Type IIA fibres, EDL muscle appears to contract faster distally than proximally.

The native isomyosins FM_3, FM_2 also show a proximo-distal distribution (Fig. 3), the proportions of FM_3 being highest proximally, and those of FM_2 being highest distally. The proportions of FM_1 are constant at all levels. It is known that FM_1 is an homodimer of LC_{3f}, FM_2 an heterodimer of LC_{1f}, LC_{3f} and FM_3 an homodimer of LC_{1f} [22]. One may thus infer from our observations that the distal end is proportionally richer in LC_{3f} than in LC_{1f} when compared to the proximal end. The physiological significance of the light chains repartition between LC_{1f} and LC_{3f} is still uncertain; if we accept the frequent suggestion that the velocity of contraction increases in proportion with LC_{3f}, we again come to the conclusion that the distal end of EDL contracts faster than its proximal end. Eventually, we note that intermediate isomyosin, IM, which contracts more slowly than the fast myosin [27] is localized proximally in the few EDL muscles where it is present; this observation further supports the conclusion that the velocity of contraction in EDL muscle increases from its proximal end to its distal end.

Besides the longitudinal distribution of isomyosin, EDL muscle shows also a transversal organization that is known

since a long time and which we confirm in this work. Type I fibres are segregated in the anterior part of the muscle [31, 1]. This segregation is preserved at all levels, but the relative area of distribution shrinks proximo-distally (Fig. 4 A, B, C). This fact is again in line with the idea that EDL muscle contracts more slowly at its proximal end than at its distal end.

Isomyosins and fibre types in regenerating EDL

The distribution of fast myosin heavy chains undergoes interesting changes during regeneration. In early phase of regeneration (30 days), the proportion of MHC2B is less than 30%, approximately half of that observed in control muscles (Fig. 2b). Later, this proportion rises to 40-50% (120 days), still less than in control muscle. Obviously, there is a trend in regenerating muscle to normalize the proportions of myosin heavy chains. It is probable that this change is mediated by the activity pattern of motoneurons which determines the expression of fast or slow isomyosins [8, 17, 20]. This trend may also account for the increase in shortening velocity observed as regeneration proceeds [10, 11, 15, 16, 26], since the shortening velocity of fibres containing only MHC2B exceeds that of those containing only MHC2A [32]. Contrasting with the changes in myosin heavy chains during regeneration, the proportions of native isomyosins do not change. They are, however, not identical to control and do not show any evolution towards control values (Fig. 3). Regenerating muscles contain especially low amounts of FM_1. This might indicate by inference that regenerating muscles have less fast myosin light-chains 3 (LC_{3f}) than controls. Such a deficit in LC_{3f} has already been reported in rat regenerating muscle by Kelly and Rubinstein [19], and by Strohman and Matsuda [37] in the case of regenerating chicken pectoralis major.

The proportion of type I fibre is smaller than in controls (Fig. 2c), and their distribution is random, in contrast with the spatial arrangement observed in control muscle. The latter fact could be explained by the mechanism of innervation which appears different during regeneration and embryogenesis. According to Balice-Gordon & Thompson [1], the typical fibre distribution of adult EDL muscles depends on the embryonic division of the nerve into two distinct branches and their subsequent divisions. In our experimental procedure, the nerve is cut proximally to its bifurcation. If axons growing into the regenerates innervate the regenerating fibres at random, the typical architecture of EDL muscle cannot be reconstructed. The smaller proportion of type I fibre might be explained by the competition between the numerous axons of fast motoneurons and the few axons of slow motoneurons: once innervated, myotubes become refractory to further innervation [2, 33]. Such an explanation could also account for the observation that type I fibres are often present in groups of two or three fibres, probably innervated by the same axon of a slow motoneuron.

Spatial organization in regenerating EDL

Regenerating muscles completely lack the remarkable spatial organization of control muscles in which myosin heavy chains as well as fibre types are arranged not only along an antero-posterior axis but also along the proximo-distal axis of the muscle. The mincing procedure destroys this architecture, and the regenerative processes do not restore it, even after 120 days. The reasons for this failure might be found in the hypothesis of random innervation already proposed to account for loss of the spatial organization of type I fibres.

Acknowledgments

This study was supported by Fonds National de la Recherche Scientifique of Belgium (contract n 3451385) and the Association Nationale d'Assistance aux Handicapés (Rotary Clubs de Belgique).

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