Morphological and biochemical analysis of regeneration after cardiotoxin injury of *Xenopus laevis* fast muscles

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ABSTRACT The anterior brachial muscle of *Xenopus laevis* forelimb was characterized as a fast-type muscle composed of type II fibers exclusively. Larval and adult muscles showed three distinct isomyosins composed by two different heavy chains, HCl and HCl, respectively, associated with the same fast light chains. Muscle regeneration was examined after degeneration of the myofibers by injection of cardiotoxin, a snake toxin. 24 h after the injury no myofibers and no myosin were detected. New myosins of larval and adult fast types started to be synthesized two weeks after the injury, during a stage of proliferation of mononucleated cells. 1 month after the injury, the regenerated muscles which showed structural differences with the normal muscle contained only fast isomyosins. The precocious larval to fast heavy chain transition observed in regenerating muscles of the adult *X. laevis* without any thyroid hormone influence shows that the myogenic program in adult muscle regeneration is regulated by factors that are different from those regulating normal development.

KEY WORDS: myosin isoforms, fast muscle, regeneration, *Xenopus*

Introduction

Degeneration and then regeneration of the muscle fibers take place following muscle injury (Allbrook, 1981; Plaghki, 1985). The mononucleated satellite cells, which in normal muscle are located between the plasma membrane and the basal lamina, proliferate and fuse to form new fibers (Mauro, 1961; Snow, 1977; Campion, 1984). In mammals, the same myosin isoforms, i.e., embryonic, neonatal and adult, are synthesized during regeneration as observed during normal development, therefore suggesting that regeneration recapitulates the developmental process (Carraro et al., 1983; Marechal et al., 1984; Plaghki, 1985; d'Albis et al., 1989; Whalen et al., 1990). Nevertheless, d'Albis et al. (1987b, 1988) have shown that the appearance of adult isomyosins was more precocious in regenerating as compared to developing muscles in adult rat and mouse.

It is known that thyroid hormone regulates the neonatal to adult myosin isoform transition during normal development (d'Albis et al., 1987a; d'Albis and Butler-Browne, 1993). The acceleration of synthesis of fast myosin in regenerating muscles of adult mammals has been attributed to differences in thyroid hormonal status between newborn and adult animals (d'Albis et al., 1987b).

In amphibians as observed in mammals, there is a myosin isoform transition during the course of muscle development. This transition is characterized by the progressive replacement of larval isomyosins by the adult isoforms during the time of anatomical metamorphosis, is correlated with transitory increase of thyroid hormone levels (Chanoine et al., 1987, 1990a). An essential difference between thyroidal status of amphibians and that of mammals is that thyroid hormone is not detected in serum of adult amphibians (Leloup and Buscaglia, 1977; Chanoine et al., 1987). So, the amphibian model makes it possible to study the regeneration process without any thyroid hormone influence.

In this work, we study the regeneration of fast type muscles following cardiotoxin injury in the adult *Xenopus laevis*. We carry out our analysis at the histological and biochemical levels. The myosin isoforms and their heavy chain subunits synthesized during the new myogenesis were characterized in relation to the histological stage of the regenerating muscle.

Results

Normal muscle features

Analysis of the myosin ATPase profile of adult muscle revealed a uniform strong staining of the myofibers after preincubation at pH 9.4 (Fig. 1A). In contrast, we showed a complete reversion of the staining following acidic preincubation (pH 4.35); all myofibers displayed only a weak staining reaction (Fig. 1B) and were classified as type II fibers, which are characteristic of the fast-type muscles.

Larval and adult fast muscles were characterized by three myosin isoforms, the three adult isoforms migrating slightly slower than the three larval ones (Fig. 2). Comigration of myosin extracted from larval and adult muscles showed that the two faster migrating
adult isoforms comigrated with the two larval isoforms migrating slower.

Analysis of the myosin heavy chains using SDS-PAGE in presence of 37.5% glycerol showed that larval and adult fast myosins were composed of two different heavy chains, namely HCl and HC!, respectively (Fig. 3).

Two-dimensional gel analysis revealed the same three fast light chains, LC1f, LC2f and LC3f in larval and adult muscles (Fig. 4).

Regenerating muscles

Histological analysis

By 24 h post-injection of cardiotoxin, all the myofibers were degenerated (Fig. 5B). The degenerated muscle retained the appearance of continuous tubes due to the persistence of the basal lamina sheaths of the myofibers (Couteaux et al., 1988). The fifteenth day post-injection was characterized by an important proliferation of mononucleated cells (Fig. 5C). New myotubes with central nuclei were observed as early as the nineteenth day post-injection (Fig. 5D-G) and the progressive transformation of myotubes into myofibers took place, characterized by the migration of the central nuclei to the periphery. One and 2 months following cardiotoxin injection, the regenerated muscle presented morphologic features distinguishing it from normal muscle (Fig. 5H-J). Myofibers were less closely juxtaposed than in normal muscle, which was clearly observed by immunohistochemistry using antibodies against laminin (Fig. 5K-M). A large scatter of the diameter of the new myofibers was also visible. Moreover, many myonuclei are abnormally located in the center of the fibers.

Analysis of myosin isoforms

No myosin isoforms were detected before the fifteenth day post-injection where four isoforms were observed in pyrophosphate gel electrophoresis (Fig. 6a). As early as 1 month post-injection, only the three isomyosins with the slower electrophoretic mobility were detected (Fig. 6c). These observations suggested that larval and adult fast isomyosins were expressed as early as the beginning of the regeneration process. That was confirmed by the analysis of myosin heavy chains in SDS-PAGE in the presence of 37.5% of glycerol.
Fig. 5. Hematoxylin-eosin histological stains of degenerated (B), regenerating (C-I) and control adult (A,J) muscles. The degenerated and the regenerating muscles were taken at 24 h (B), 15 days (C), 19 days (D), 21 days (E,F,G), 1 month (H) and 2 months (I) after cardiotoxin treatment. Immunohistochemical staining with a laminin antibody of control (K) and regenerating muscles taken at 21 days (L) and 2 months (M). All the sections of muscle were transverse sections except for (F) and (G) which were longitudinal sections. Bars: A-E and K-M, 20 μm, F-G and I-J, 10 μm.

glycerol (Fig. 7). Larval and fast heavy chains were expressed during the first stage of regeneration, at 15 days post-injection, when the mononucleated cells proliferated. As early as 1 month post-injection, when the new fibers were formed, only the fast heavy chain was detected. It should be noted that the fast myosin heavy chain was mostly expressed as early as the beginning of muscle regeneration.

Discussion

Only a few reports have studied the biochemical features of muscles from anuran amphibians (Pliszka et al., 1981; Lannergren, 1987). In this work, we show that the anterior brachial muscle of *X. laevis* is a fast-type muscle composed exclusively of type II fibers. Kordylewski *et al.* (1989) demonstrated that larval and fast muscles...
of *X. laevis* are composed of three distinct isomyosins. We have shown that the three larval and the three fast myosin isoforms are characterized by two different heavy chains, HCl and HCl, respectively, associated with the same three fast light chains. These isomyosins probably represent different combinations of the three light chains either with HCl or with HCl as previously observed in mammals (d'Albis *et al.*, 1985), urodelan amphibians (Chanoine and Galien, 1989; Chanoine *et al.*, 1991) or fishes (Chanoine *et al.*, 1990b; Martinez *et al.*, 1990).

Cardiotoxin purified from the venom of *Naja mossambica* offered the advantage of selective action. It induced degeneration of the myofibers but did not affect the satellite cells, blood vessels and muscle innervation (Couteaux and Mira, 1988). New myofibers were formed during the following days and the progressive transformation into myofibers took place.

The structural differences observed between the architecture of the regenerates and normal muscle could be attributed to different explanations. On the one hand, in avian muscles, it has been shown that the connective tissue plays an organizing role in muscle development (Chevallier and Kieny, 1982). Couteaux *et al.* (1988) suggested that the presence of an altered connective tissue at the beginning of muscle regeneration might explain in part some of the deviations from normal myogenesis observed during this process. On the other hand, the structural features of the regenerates muscle could be imputed to properties of satellite cells which might be different from that of embryonic myoblasts.

Our analysis of muscle regeneration following cardiotoxin injury showed a myosin isofom expression pattern which clearly differs from that observed during normal development. Fast myosin heavy chain was expressed as early as the first stage of muscle regeneration. This contrasted with what happened during normal muscle development where fast heavy chain began to appear at the time of anatomical metamorphosis during the terminal differentiation of the developing muscle (Kordylewski *et al.*, 1969; Radice and Malacinski, 1989).

It has been clearly demonstrated that the increase of thyroid hormone levels during anatomical metamorphosis regulates the larval to fast myosin isofom transition, T3 playing an essential role in initiating the disappearance of the larval isoforms in amphibian muscles (Chanoine *et al.*, 1987, 1989). In the adult rat, the precocious expression of fast myosin heavy chain following cardiotoxin injury has been attributed to the high, constant plasma concentration of thyroid hormone in comparison to the low concentration observed in the newborn, hyperthyroidism promoting the synthesis of fast isomyosins and hypothyroidism promoting that of neonatal isomyosins (d'Albis *et al.*, 1987b). The precocious larval to fast heavy chain transition observed in regenerating muscles of the adult *X. laevis* without any thyroid hormone influence shows that the myogenic program in adult muscle regeneration is regulated by other factors than those regulating normal development. This suggests that satellite cells and embryonic myoblasts might be endowed with specific developmental programs and would agree with the proposal of Chevallier *et al.* (1997) that satellite cells and embryonic myoblasts represent non-equivalent myogenic cells.

The precocious larval to fast heavy chain transition during muscle regeneration without any thyroid hormone influence seems to be a common feature of amphibians since similar results had been previously observed during limb regeneration of urodelan amphibians following amputation (Saadi *et al.*, 1993).

**Materials and Methods**

**Muscle injury and sampling**

Injury was performed on adult *Xenopus laevis*. Animals were anesthetized with tricaine methane sulfonate (MS222) and pure cardiotoxin from *Naja mossambica nigricollis* venom (Latoxan, France) (10^3 M in 0.9% NaCl) was injected into the right anterior brachial muscle of the forelimb. We checked that intramuscular injection of the solvent did not induce any deterioration of the muscles. Samples for histological, histochemical and immunohistochemical studies were plunged in isopenate and frozen in liquid nitrogen. For biochemical studies, samples were placed in cryotubes and immediately frozen in liquid nitrogen.

**Biochemistry**

**Myosin extraction**

Preparation of crude myosin was performed in ice according to Pliszka *et al.* (1981). The muscles were cut in small pieces and washed with 4 vol. of 600 mM KCl, 40 mM NaHCO₃, 10 mM Na₂CO₃, 1 mM MgCl₂, 10 mM Na₃P₂O₅, pH 8.8 (buffer A). Myosin was then extracted with 4 vols. of buffer A; after 90 min of gentle agitation, the mixtures were centrifuged at 10000 x g.

![Fig. 6. Electrophoretic analysis under non-dissociating conditions of native myosin extracted from regenerating muscles. 15 days (a). 21 days (b) and 1 month (c) after cardiotoxin treatment. Adult (e) and larval (f) myosin isoforms used as controls. (d) Coagulation of (c)+(f).](image)

![Fig. 7. SDS-PAGE in presence of 37.5% glycerol of myosin heavy chains extracted from regenerating muscles. 15 days (b), 19 days (c), 21 days (d) and 1 month (e) after cardiotoxin treatment. Larval (a,g) and adult (f) heavy chains used as controls. (f) Larval heavy chain.](image)
and the myosin containing supernatants were diluted twice with glycerol for conservation and study of native myosin.

Electrophoretic analysis of native myosin
Non-dissociating conditions were used (d’Albis et al., 1985) but for different modifications (Chanoine et al., 1990b). Running buffer was 20 mM Na2HPO4, pH 8.5, 10% glycerol, 0.01% 2-mercaptoethanol, 2 mM MgCl2 and 2 mM ATP. Cylindrical gels (8x0.5 cm) were 4% in polyacrylamide; about 1 μg myosin per band was loaded. Electrophoresis was carried out at 80 V for 16h30 at -2°C/3°C.

Two-dimensional electrophoretic analysis of myosin light chains
Crude myosin extracts were dissolved in 9 M urea, 2% nonidet P-40, 2.2% ampholines (pH 4-6.5) and 5% 2-mercaptoethanol. Analysis of myosin light chains was performed by the technique of O’Farrell (1975) using 2.2% ampholines in the first dimension and 14% polyacrylamide; 0.1% SDS in the second dimension (Laemmli, 1970).

Electrophoretic analysis of myosin heavy chains in the presence of SDS
This was performed according to Carraro and Catani (1983) in 5% polyacrylamide slab gels, in the presence of 0.1% sodium dodecyl sulfate (SDS) and 37.5% glycerol. The extracts were diluted in 20% glycerol, 5% SDS, 5% 2-mercaptoethanol, 1 mM EDTA and boiled for 2 min. Electrophoresis was carried out at 55 V for 18 h. Gels were stained with silver nitrate according to Ansorge (1983).


Acknowledgments
This work was supported by grants from the Centre National de la Recherche Scientifique and from the Association Francaise contre les Myopathies.

References


Accepted for publication: May 1994