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Development of muscle pathology in canine X-linked muscular dystrophy. I. Delayed postnatal maturation of affected and normal muscle as revealed by myosin isoform analysis and utrophin expression

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Abstract Canine X-linked muscular dystrophy (CXMD) is genetically homologous to Duchenne muscular dystrophy and shares the severe myopathy and lethal clinical development of the human disease. We used immunohistochemistry to characterize the time course of postnatal expression of adult fast, adult slow and developmental myosin in the muscle of CXMD dogs, carriers and healthy controls. We also characterized the expression of utrophin and dystrophin. This detailed immunolocalization study confirmed that postnatal muscle maturation is delayed in normal dogs compared to other animals and humans, and is only achieved at around 60 days. In CXMD dogs major derangement of myosin expression became evident from about 15 days; there was a selective loss of fibers expressing fast myosin and persistence of developmental fibers compared to controls. In carriers, the proportion of dystrophin-deficient fibers, which mainly expressed fast myosin, decreased with age. In controls and carriers utrophin was absent from muscle fiber surfaces in 2-day-old animals but present between 15 and 30 days, to mostly disappear by 60 days. In dystrophic animals, sarcolemmal expression of utrophin was more marked and persistent. That immature neonatal muscle from control dogs normally contains sarcolemmal utrophin may have implications for the success of utrophin up-regulation therapy to correct the dystrophic phenotype. The data of this study provide important baseline information for further studies on the development and progression of pathological changes in the muscle of CXMD dogs.

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Introduction

Canine X-linked muscular dystrophy (CXMD) is an inherited degenerative disorder genetically homologous to Duchenne muscular dystrophy (DMD) [5]. DMD and CXMD have progressive clinical signs and severe myopathy in common; in both disorders skeletal muscle shows early fiber necrosis and regeneration together with endomysial and perimysial connective tissue proliferation. Furthermore, histopathological changes in skeletal muscle appear at preclinical stages in both DMD and in CXMD [10, 26, 27]. The other animal models genetically homologous to DMD, the mdx mouse and the cat with hypertrophic feline muscular dystrophy, have mild clinical features and little or no endomysial fibrosis [4, 11]. CXMD is, therefore, the most relevant animal model for DMD particularly in regard to potential therapeutic approaches.

Postnatal skeletal muscle development in the dog is delayed, compared with other mammals (guinea pig, cow and human [8, 9, 28]) as revealed by histochemical fiber typing based on the myosin ATPase reaction [3]. Immunohistochemical characterization of myosin isoform expression in the dog is still lacking.

We have now elucidated the time course of postnatal expression of myosin isoforms in the skeletal muscle of normal dogs, CXMD carriers (in correlation with mosaic expression of dystrophin) and CXMD-affected animals. Developmental, adult fast, and adult slow myosin isoforms were revealed by immunohistochemistry.

We performed this detailed morphological study of early muscle maturation as part of our research on the development of pathological changes in CXMD. Utrophin, a protein closely homologous and structurally similar to dystrophin [16, 18], is normally present at the neuromuscular

junction in adult skeletal muscle [22] and is expressed at the sarcolemma in human fetal and regenerating fibers [13, 17, 20]. It is also expressed – at a low level – at the sarcolemma of DMD patients' muscle [14]. Since skeletal muscle development in dogs is delayed, the mature localization of utrophin exclusively at the neuromuscular junction could also be delayed; we therefore analyzed utrophin expression in CXMD-affected animals, carriers and controls.

Materials and methods

Animals

A colony of CXMD dogs was established from CXMD carriers obtained from Dr. Barry J. Cooper of Cornell University, Ithaca, NY, and maintained in our facilities in accordance with European legislation. Control male and female mix-breed dogs, CXMD carriers and CXMD male dogs of 2, 15, 30, and 60 days, and 2–3 years of age were used (three to six animals in each age group). The 2-day-old dystrophic animals died naturally and were from different litters. Three CXMD individuals that died naturally at 8, 9 and 11 days were also studied. Samples from open biopsies were obtained from the quadriceps femoris vastus lateralis muscle under general anesthesia or immediately after death. In animals from which biopsy samples were obtained from the same muscle at different ages, care was taken to avoid previous biopsy sites. Tissue samples were snap-frozen in isopentane cooled with liquid nitrogen, and stored in liquid nitrogen. In selected cases and in euthanized animals specimens were also taken from the tibialis anterior (cranial tibial).

Plasma CK levels were determined in all animals at birth and in carrier dogs also at later stages. Levels were 140 | 775 ± 20 | 517 U/l (mean ± SD) in dystrophic dogs and 7260 ± 8036 U/l in carriers at birth (normal value: 2370 ± 1286); 470 ± 111 U/l in carriers at 60 days (normal: 141 ± 38) and 35 ± 2.5 in adult carriers (normal: < 50 U/l).

Immunohistochemistry

Serial cryostat sections, 6 μm thick, were collected on gelatin-coated slides for immunohistochemistry. For quantitations, sections from carriers and control dogs were double-stained either for fast myosin plus dystrophin, slow myosin plus dystrophin, or developmental myosin plus dystrophin. In sections from dystrophic animals, dystrophin staining was replaced by staining for the cytoskeletal membrane protein talin, allowing quantitation of fibers in dystrophin-negative muscles. In the youngest dystrophic animals myosins were quantitated on peroxidase-immunostained sections counterstained with eosin, since talin is not expressed.

Monoclonal antibodies to slow, fast and developmental myosin heavy chain isoforms (Novocastra Labs, Newcastle upon Tyne, UK) were diluted, respectively, 1:20, 1:250 and 1:20 in PBS plus appropriate serum. Dystrophin antibodies (diluted 1:500) were rabbit polyclonal antibodies raised in our laboratory against fragments D8 or D11 of the human dystrophin rod domain and C-terminal domain, respectively [21]. Utrophin was localized using two commercial monoclonal antibodies (Novocastra) directed against the N terminus and the C terminus of the protein (the latter corresponding to Mancho-7 [13]) diluted, respectively, 1:10 and 1:5. In selected cases utrophin was also detected by a polyclonal antibody (diluted 1:2000) kindly donated by Drs. Kunkel and Khurana (Children's Hospital and Harvard Medical School, Boston, Mass. [16]). Monoclonal and polyclonal antibodies were detected using a biotin-avidin system.

For co-localization of myosins and dystrophin, cryosections were incubated in a mixture of anti-dystrophin and anti-myosin antibodies, followed by incubation in biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, Calif.), followed by rhodamine avidin D (Vector), and then fluorescein-conjugated goat anti-mouse IgG.

For co-localization of myosins and talin, samples were incubated in anti-talin (monoclonal, from Sigma, St. Louis, Mo.) diluted 1:50, then in biotinylated horse anti-mouse IgG (Vector) and in rhodamine avidin D. Sections were then treated with anti-myosin antibodies and finally with fluorescein-conjugated goat anti-mouse IgG.

As control, in a few assays, secondary antibodies used for double localizations were affinity purified and multiadsorbed (purchased from Jackson ImmunoResearch Labs, West Grove, Pa.). This allowed us to assess the presence of unwanted cross reactivity. Other controls were omission of primary antibodies or, for utrophin detection, substitution with a nonimmune mouse isotype-specific IgG1 (Sigma) on adjacent sections.

For immunoperoxidase staining, sections were incubated in primary anti-myosin monoclonals (diluted as above), then in secondary biotinylated goat anti-mouse IgG followed by peroxidase-conjugated avidin (Vector) and diaminobenzidine development (Sigma). They were washed in PBS and water, immersed for a few seconds in eosin, dehydrated, and mounted.

Immunoblot of utrophin

Muscle cryosections were weighed and solubilized (50 mg/ml) in sample buffer containing 10% SDS, 50 mM dithiothreitol, 10 mM EDTA, 0.1 M TRIS, pH 8.0, and 0.001% bromophenol blue. After electrophoresis of the solubilized tissue in SDS-polyacrylamide gel (3–8%), the gel was blotted onto nitrocellulose paper. The nitrocellulose sheets were incubated with primary anti-N terminus utrophin antibody, then with alkaline phosphatase-conjugated secondary antibody, followed by development with nitro blue tetrazolium.

Quantitations

Fiber counts were performed by two different operators. At least 500 fibers were counted on each section from randomly selected fields; data recorded by the two researchers did not differ by more than 5%. The same fields were chosen on consecutive sections stained for different myosin isoforms. Mean percentages of fiber types were calculated for each age group of affected dogs, carriers and normal dogs. Student's *t*-test was used to compare differences between means from carrier or dystrophic and control muscles.

Both dystrophin-negative fibers and those partially immunostaining for dystrophin were counted as dystrophin-deficient fibers in carriers. Myosin isoform expression was assessed in dystrophin-deficient fibers and the mean percentages calculated for each age group.

Utrophin was quantitated on immunoblot using a densitometer and the BIO-PROFIL software system (Vilber-Lourmat, Tozcy, France). Values were expressed as the ratio of the optical density (OD) of the utrophin band (after subtraction of background) on immunoblot to the OD of the myosin band (used as reference of muscle protein) on the Coomassie blue-stained post-transfer gel.

Results

Evaluation of myosin heavy chain isoforms

The percentages of fibers expressing myosin isoforms according to age in control, carrier and dystrophic dogs are shown in the graphs of Fig. 1.

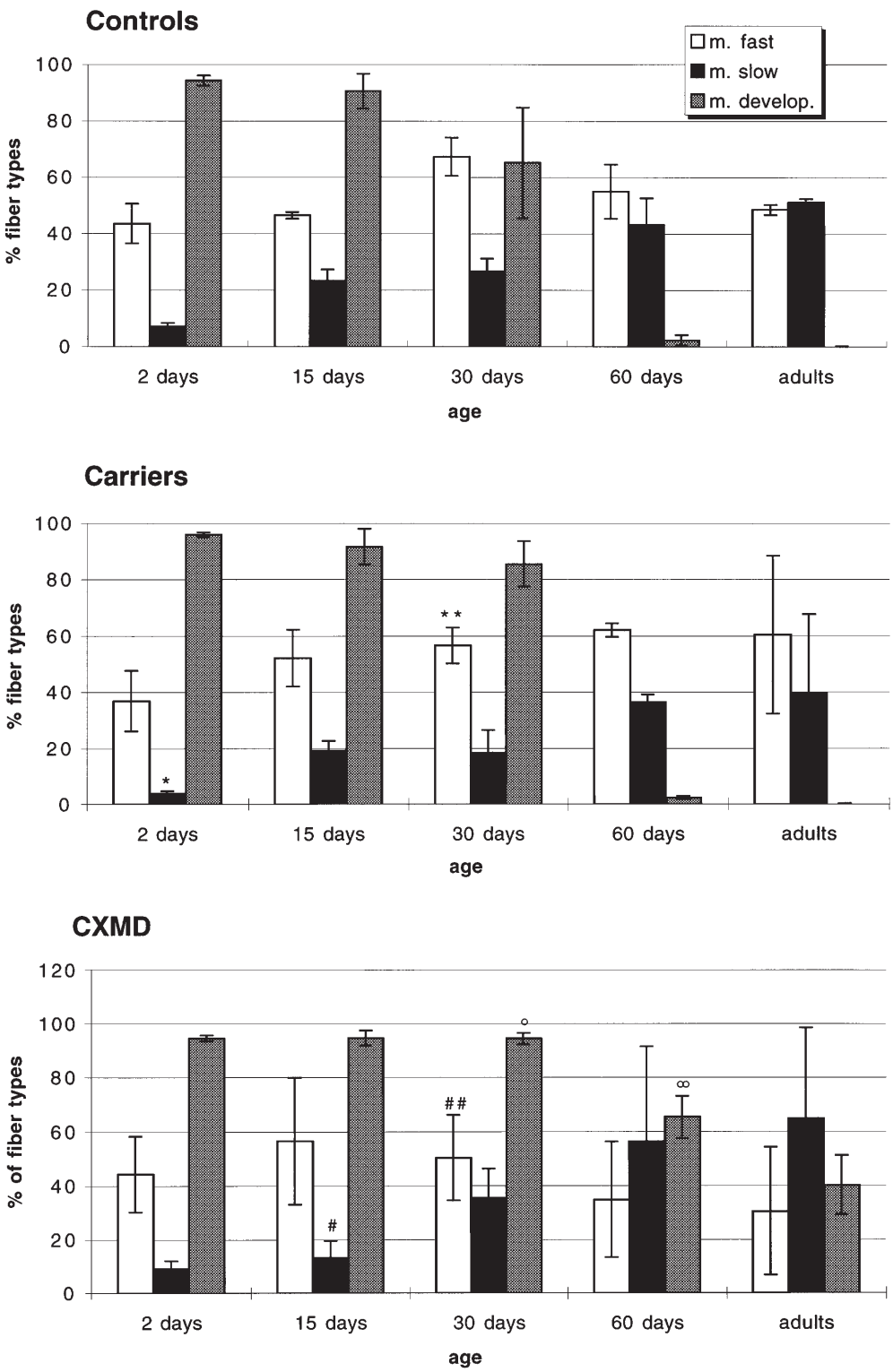

Fig. 1 Graphs showing quantitations of fiber types by myosin expression. Note that sums of percentages in the age groups may be more than 100% because fibers co-express different myosins (*CXMD* canine X-linked muscular dystrophy). * $P = 0.0107$; ** $P = 0.0385$; # $P = 0.0525$; ## $P = 0.0359$; ° $P = 0.0043$; °° $P = 0.0001$

Control dogs

At 2 days most fibers expressed developmental myosin. A proportion of fibers also co-expressed fast myosin. A small percentage of fibers (7.1 ± 1.3%), usually located singly at the center of fascicles, exclusively expressed adult slow myosin and were larger than average (diameter around 8–10 μm, compared to about 6–7 μm) (Fig. 2). Although expressing adult myosin, these larger fibers often had central nuclei.

With increasing age the proportion of fibers expressing slow myosin and fast myosin (some retaining develop-

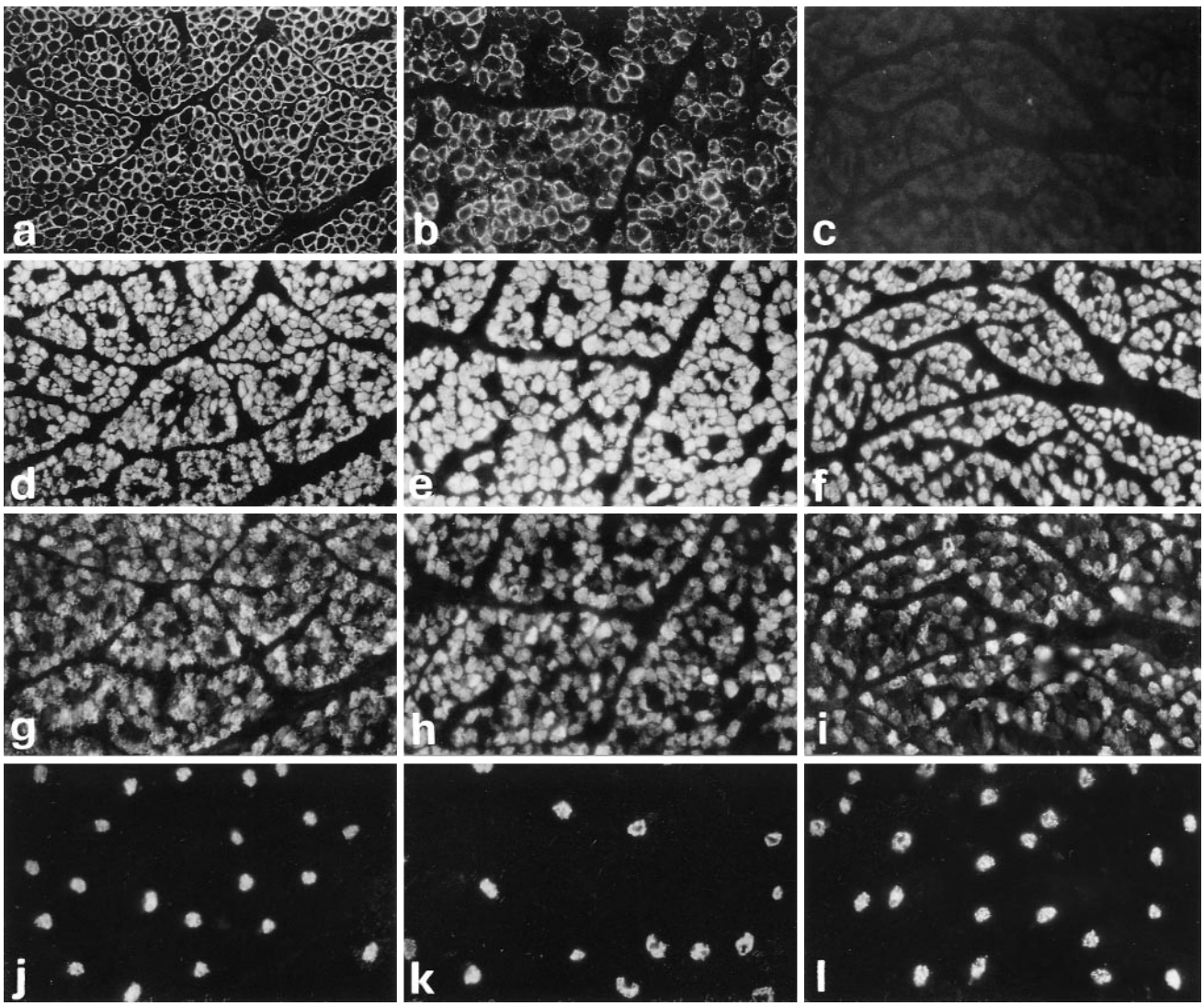

Fig. 2 Dystrophin (**a–c**) and developmental (**d–f**), fast (**g–i**) and slow (**j–l**) myosin expression in 2-day-old control (**a, d, g, j**), carrier (**b, e, h, k**) and dystrophic (**c, f, i, l**) dogs. **a–l** × 400

mental myosin, but some expressing fast myosin only) increased, while the proportion expressing developmental myosin decreased.

Fiber diameters also increased with age: by 15 days fibers expressing fast/developmental myosin were 10–12 μm in diameter and those expressing slow myosin were 18–20 μm (Fig. 3); at 30 days diameters were 12–16 μm and 20–24 μm, respectively, and at 60 days both fast and slow myosin-positive fibers were about 22 μm. With increasing age fiber outline changed from round to polygonal. At all ages there was considerable variation in fiber size within individual muscles; in particular, at 15 and 30 days several puntiform fibers highly and exclusively positive for developmental myosin were observed (Fig. 3). Furthermore, from 15 to 60 days the population of fibers expressing slow myosin consisted of large fibers (very likely those present at birth) and others of smaller and greatly variable size. The size of these smaller fibers increased and became more uniform with age (Figs. 3, 4).

In adult control dogs, muscle fibers were generally homogeneous and about 45 μm in diameter, although occasional isolated small fibers were observed (Fig. 5).

Analysis of the tibialis anterior in a 2-day-old and in two 60-day-old control dogs revealed features similar to those observed in the quadriceps of the same animal.

Carriers

The distribution of myosin expression in the fibers was similar to that in control dogs, although there were less slow myosin-positive fibers (significantly less in some age groups) (Fig. 1), but numbers were highly variable. Fiber diameters and fascicle organization were also similar to controls of the same age (Figs. 2–5).

In the tibialis anterior muscle of a 2-day-old carrier, the proportions of myosins were similar to those in the quadriceps muscle; in one 60-day-old and in two adult

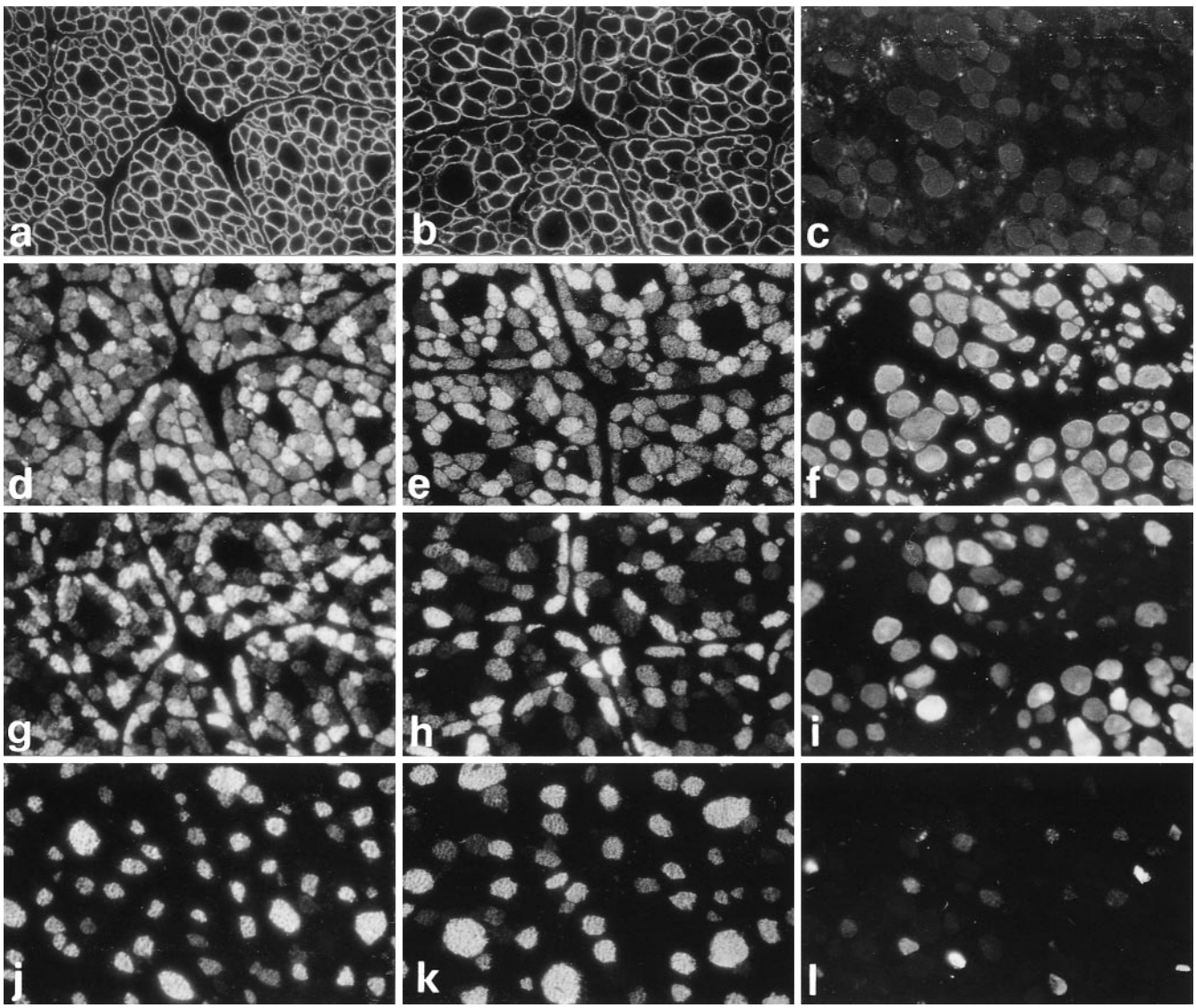

Fig. 3 a–l Dystrophin and myosin expression in 15-day-old dogs. Panel organized as in Fig. 2. **a–l** × 250

carriers the tibialis anterior had more fast and less slow-positive fibers than the quadriceps.

Quantitation of myosin isoforms in dystrophin-deficient fibers showed that these fibers mainly expressed fast myosin, at all ages (Table 1). Almost all dystrophin-deficient fibers expressed developmental myosin at 2 days, but the proportion doing so declined steadily with age.

In the dystrophin-deficient fibers from the tibialis anterior of one 2-day-old, one 60-day-old, and two adult carriers, the proportions of fast, slow and developmental fibers were similar to those found in the dystrophin-deficient fibers of the quadriceps of these animals.

Dystrophic dogs

In 2-day-old puppies, the overall organization of muscle fascicles and proportions of fibers expressing fast, slow and developmental myosin resembled that in normal dogs of the same age (Fig. 2). In the 8-day-old dystrophic puppy, the organization of some fascicles was altered, with increased space between fibers and presence of a few fibers of larger size expressing fast myosin. In the dog of 9 days of age, there was no apparent alteration in fascicle organization. In the 11-day-old dog some fascicles showed altered overall organization and contained several large fibers expressing either fast or developmental myosin or both, but most fascicles appeared normal (data not shown).

At 15 days major derangement of muscle organization was evident in all animals (Fig. 3). Muscle fiber diameter was highly variable (3–50 μm); fascicles consisted of many large round fibers with either slow or fast myosin, and of smaller fibers (some centronucleated) that mainly expressed developmental myosin.

Similarly deranged muscle organization was observed at later stages (Figs. 4, 5). Fiber diameters increased progressively with age, but were highly variable (from 25 μm to more than 200 μm). At 30 and 60 days some fibers co-expressed fast and slow myosin.

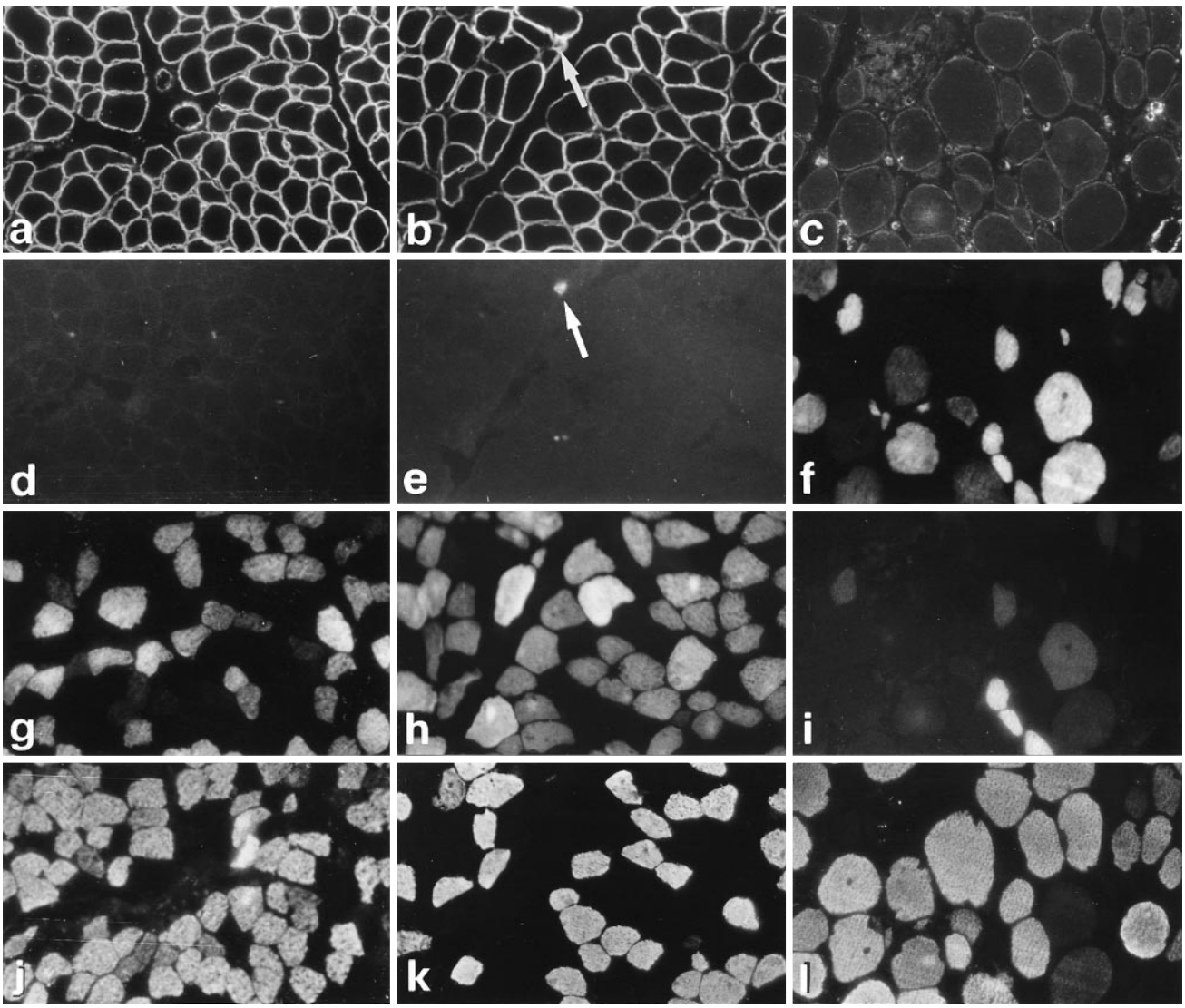

Fig. 4 a–l Dystrophin and myosin expression in 60-day-old dogs. Panel organized as in Fig. 2. Note small developmental myosin-positive fiber *(arrow)* in carrier muscle (**e**). **a–l** × 250

From 15 days on, the proportions of fast myosin-positive and developmental fibers decreased, the latter much slower than in controls, and the proportion of slow myosin-positive fibers increased, more than in controls. In adult animals the number of fast myosin fibers was markedly less than in controls and was inversely related to the number of slow fibers (Fig. 1, graph). Approximately 30% of fibers in these dogs continued to express developmental myosin.

The tibialis anterior of two 2-day-old, two 60-day-old and two adult dystrophic dogs showed higher values of fast fibers at 2 days, similar numbers of fast, slow and developmental fibers at 60 days, and higher percentages of fast fibers at 6 months, compared to the quadriceps in the same animals (data not shown).

Evaluation of utrophin and dystrophin

Immunohistochemistry

Control dogs. Dystrophin immunostaining was patchily distributed on muscle fiber surfaces in 2-day-old dogs. No cytoplasmic dystrophin was observed. From 15 days on, the distribution of dystrophin became progressively more homogeneous at the sarcolemma, although with some variability of intensity in younger animals (Figs. 2–5).

Utrophin was not detected on the muscle fiber surface of 2-day-old control dogs with any of the antibodies used. Large blood vessels, nerves and neuromuscular junctions were intensely positive to the N-terminal monoclonal antibody and to the polyclonal anti-utrophin antibody, while no clear positivity was detected with the C-terminal antibody (Fig. 6 a, b). At this stage the morphology of the neuromuscular junctions was simplified.

At 15 days blood vessels, nerves and neuromuscular junctions were intensely positive to utrophin (N-terminal monoclonal and polyclonal antibodies). Patchy utrophin

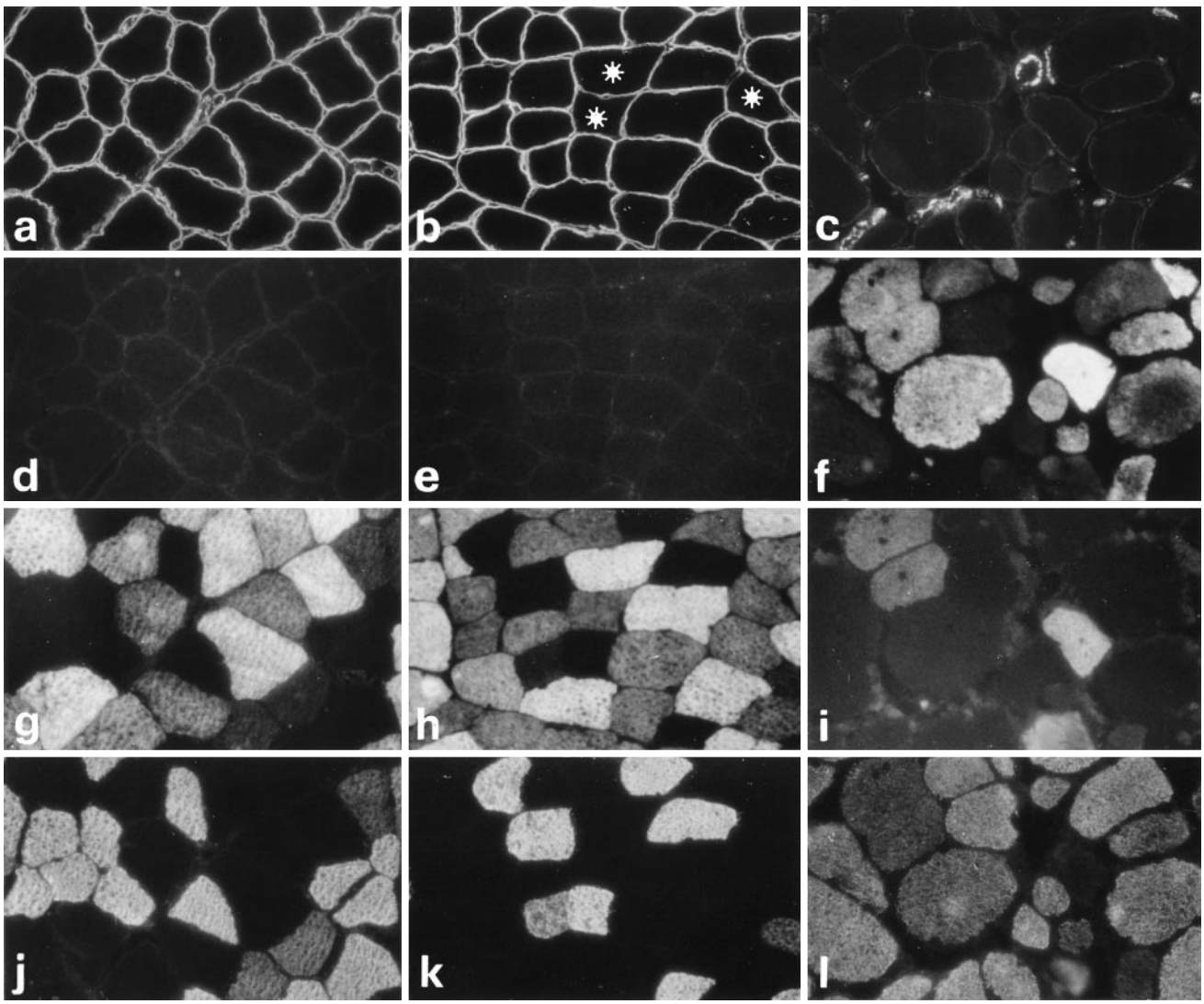

Fig. 5 a–l Dystrophin and myosin expression in adult dogs. Panel organized as in Fig. 2. Dystrophin-deficient fibers in carrier indicated by *asterisks* (**b**). **a–l** × 250

positivity was present on the surface of almost all muscle fibers but intensity varied from fascicle to fascicle; positivity was particularly intense (especially with the N-terminal monoclonal antibody) in fascicles where end plates were present (Fig. 6 d). Neuromuscular junctions appeared still simplified.

At 30 days utrophin distribution was similar to that at 15 days, but immunostaining on fiber surfaces was more intense, especially in fascicles containing neuromuscular junctions (Fig. 6 g, h). By 60 days utrophin positivity on fiber surfaces had become faint or absent except in fascicles with neuromuscular junctions (Fig. 6 i). At this stage serial sectioning showed that utrophin positivity was mainly confined to the area close to neuromuscular junctions. The C-terminal antibody detected faint positivity to utrophin only at neuromuscular junctions from 15 days on (Fig. 6 m).

In tibialis anterior from a 2-day-old puppy, utrophin was absent on the fiber surface and dystrophin was patchy. In tibialis anterior of two 60-day-old dogs dys-

Table 1 Quantitation of myosin expression in the dystrophin-deficient fibers of carriers

Age	Dys-deficient (% of total)	Fast	Slow	Developmental
2 days (n = 4)	30.03 ± 9.09	47.97 ± 18.80	0.89 ± 1.46	99.11 ± 1.46
15 days (n = 3)	30.63 ± 7.18	62.19 ± 27.69	7.49 ± 9.39	84.59 ± 2.02
30 days (n = 4)	30.79 ± 3.60	63.23 ± 8.82	22.71 ± 15.22	78.39 ± 6.65
60 days (n = 3)	33.32 ± 7.60	64.42 ± 5.87	33.45 ± 6.62	3.33 ± 1.41
Adults (n = 6)	5.31 ± 1.43	69.23 ± 15.27	29.88 ± 14.71	0.90 ± 1.45

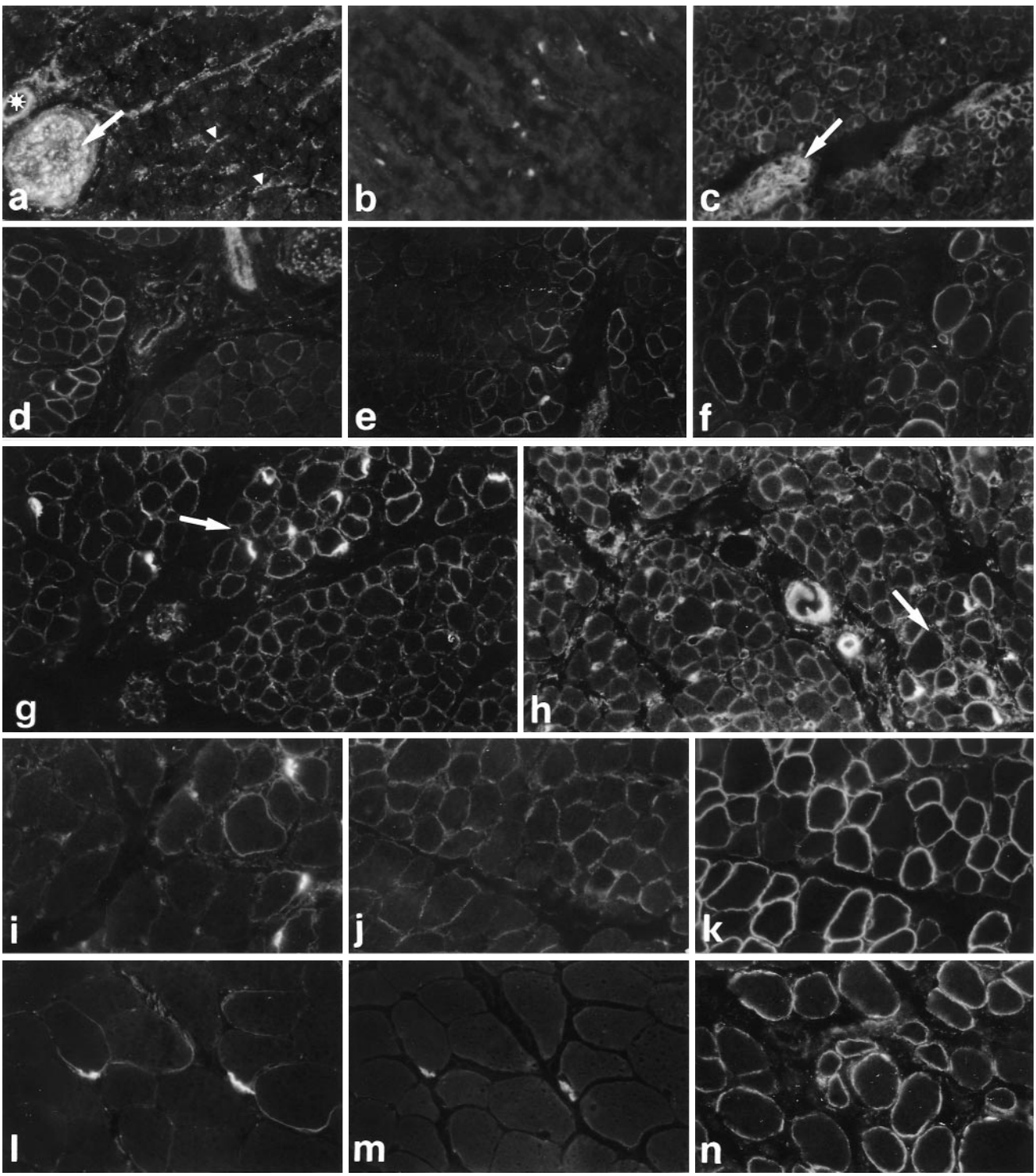

Fig. 6 a–n Utrophin expression at various ages by N-terminal monoclonal (**a–f, h, i, l, n**), polyclonal (**g**) and C-terminal monoclonal (**m**) antibodies. At 2 days (**a–c**), blood vessels (*asterisk*), nerves (*arrows*) and capillaries (*arrowheads*) (**a**) as well as neuromuscular junctions (**b**) are utrophin positive in normal control (**a, b**). **c** At this age utrophin is present on the surface of groups of muscle fibers in dystrophic dog. **d–f** At 15 days utrophin is expressed on the surface of fibers in fascicles containing neuromuscular junctions in control (**d**) and carrier (**e**). **f** In dystrophic animal utrophin is present on most fiber surfaces. **g, h** At 30 days (both controls) utrophin is expressed on most fiber surfaces, more intensely in fascicles containing neuromuscular junctions *(arrows)*; this is most evident with the N-terminal antibody (**h**). **i–k** At 60 days utrophin is expressed on fiber surfaces in fascicles containing neuromuscular junctions in control (**i**). In carriers utrophin is up-regulated on the fibers surface of dystrophin-deficient fibers but also on some dystrophin-positive fibers (**j, k**, serial sections; **k**, dystrophin). **l–n** In adult animals utrophin is present at neuromuscular junctions of controls (**l, m**, serial sections with different antibodies) and is abundant on surfaces of dystrophic fibers (**n**). **a–n** × 400

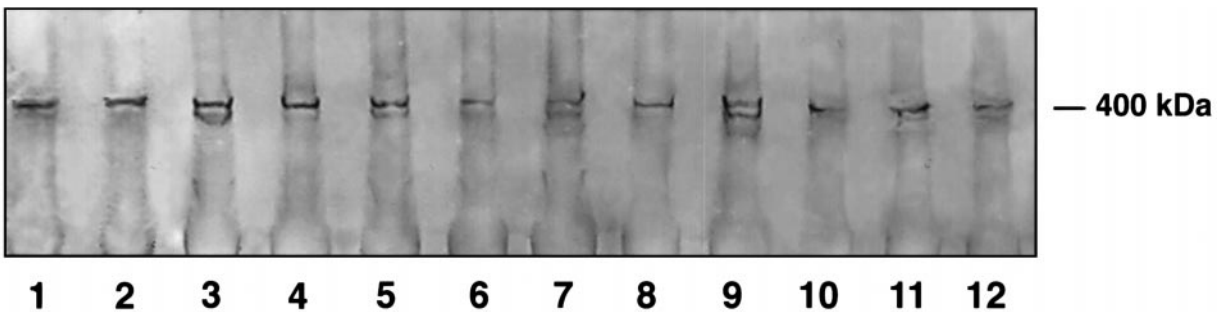

Fig. 7 Immunoblot of utrophin. *Lane 1* Sample from a 2-day-old control dog; *lanes 3, 5, 7, 9* from dystrophic dogs and *lanes 2, 4, 6, 8* from controls, at 15, 30, 60 days and adult, respectively. *Lanes 10–12* Samples from carriers at 15, 30 and 60 days, respectively. Note doublet, more prominent in dystrophic dogs

trophin was uniformly distributed at fiber surfaces and sarcolemmal utrophin was mainly confined to areas close to neuromuscular junctions, as in the quadriceps.

Carriers. Dystrophin was patchily distributed on the sarcolemma at 2 days and its distribution became progressively more uniform with time (Figs. 2–5). Some faint cytoplasmic positivity to dystrophin was observed in the quadriceps of 2-day-old carriers (Fig. 2). The percentage of dystrophin-deficient fibers remained stable at around 30% until age 60 days and then decreased to about 5–6% (Table 1). This was particularly evident in two animals undergoing biopsy on the same muscle at different ages; percentages were 35.05, 36.54 and 6.62 at 30 days, 60 days and 22 months, respectively, in one, and 32.05, 24.64 and 6.65 at the same times in the other. Complete lack of dystrophin was only seen in dystrophin-deficient fibers of younger animals; from 30 days on some faint labeling was usually present on the surface of most dystrophin-deficient fibers.

Utrophin expression in CXMD carriers was similar to that of normal dogs of the same age group (Fig. 6e), including maximum expression on fiber surfaces at 30 days.

In 60-day-old and adult carriers, although dystrophin-deficient fibers tended to express more utrophin on their surface, no clear correlation was found between fibers with increased utrophin and fibers lacking dystrophin – in that dystrophin-positive fibers could also express utrophin (Fig. 6j, k)

In one 2-day-old, one 60-day-old and two adult carriers, the tibialis anterior expressed percentages of dystrophin-deficient fibers similar to those observed in the quadriceps of the same animals. Utrophin was also expressed in this muscle as in the quadriceps and was absent from the sarcolemma at 2 days.

Dystrophic dogs. From 15 days dystrophin immunostaining showed a faint and patchy positivity on most fiber surfaces with both antibodies (Figs. 2–5). In the 15-day-old animals occasional revertant fibers were revealed with both anti-dystrophin antibodies.

Table 2 Densitometric quantitation of utrophin

Age	Controls	Carriers	CXMD
2 days (n = 3)	1.07 ± 0.33	1.02 ± 0.18	1.08 ± 0.21
15 days (n = 3)	0.79 ± 0.04	0.84 ± 0.07	1.23 ± 0.22*
30 days (n = 3)	1.24 ± 0.26	1.24 ± 0.16	1.12 ± 0.10
60 days (n = 3)	0.96 ± 0.1	1.06 ± 0.21	1.09 ± 0.22
Adults (n = 3)	0.65 ± 0.20	0.77 ± 0.09	1.2 ± 0.19**

* P = 0.0132; ** P = 0.0147

As in control and carrier muscles, blood vessels, nerves and neuromuscular junctions were positive to utrophin immunostaining (with the polyclonal antibody and monoclonal antibody to the N terminus). At 2 days muscle fiber surfaces were negative to utrophin immunostaining in two puppies, faintly positive in some fascicles in another (Fig. 6c); and all fibers were variably positive in the fourth puppy. In the 8- and 9-day-old dystrophic animals utrophin was absent on fiber surface; in the 11-day-old puppy all fiber surfaces were utrophin-positive but intensity varied from fascicle to fascicle.

Using the N-terminal monoclonal and the polyclonal antibodies, almost all muscle fiber surfaces were utrophin positive at 15 days, although positivity was patchy and intensity variable (Fig. 6f). Positivity at the sarcolemma was more intense in 30- and 60 day-old and adult dystrophic animals, again with some variability between fibers (Fig. 6n). The antibody against the C terminus showed very faint sarcolemmal positivity in a few fibers from 15 days on; neuromuscular junctions also stained faintly with this antibody.

Utrophin and dystrophin expression in the tibialis anterior was always similar to that in the quadriceps (two 2-day-old puppies, two 60-day-old and two adult dystrophic dogs).

Immunoblot

On immunoblot a band, often a doublet, of about 400 kDa corresponding to utrophin was detected in all animals (Fig. 7). The band was generally of similar intensity in control, carrier and dystrophic dogs until age 30 days; from 60 days on the intensity of utrophin decreased in controls and carriers but less so in dystrophic dogs. Quantitative analysis of utrophin in controls, carriers and dystrophic dogs is reported in Table 2.

Discussion

This study confirms that skeletal muscle development in the postnatal period is delayed in the dog. Complete maturation is achieved in the quadriceps muscle of normal dogs and CXMD carriers at around 60 days, by which time fibers expressing developmental myosin have almost disappeared, and utrophin expression on muscle fiber surfaces has become faint or absent. The tibialis anterior presents a closely similar picture. The presence of utrophin on the muscle fiber surface of 15- and 30-day-old pups is probably a direct consequence of the delayed maturation of canine skeletal muscle, while its absence in 2-day-old controls suggests that, unlike in human muscle [17, 20], utrophin is absent from the sarcolemma during fetal development. It is possible that a fetal utrophin isoform, which we do not detect, continues to be expressed soon after birth; however, we did detect utrophin in blood vessels, nerves and neuromuscular junctions in neonate animals.

Using histochemical fiber typing, Braund and Lincoln [3] reported tissue organization and fiber differentiation findings similar to ours. The type I fibers and undifferentiated type IIC fibers detected by these authors using ATPases seem to correspond, respectively, to fibers expressing slow myosin and those expressing developmental myosin (which we detected by immunohistochemistry). However, the type IIA fibers detected by ATPase do not completely correspond to our fast myosin-positive fibers, at least in the early stages of fiber maturation: Although co-expressing developmental myosin, we found fast myosin-positive fibers at birth, while type IIA fibers appear abruptly in large numbers at around 3 to 4 weeks [3].

Valentine and Cooper [25] reported “moderate lesions” in the quadriceps and cranial tibial at birth in CXMD dogs, but the proportion and pattern of fiber types (by ATPase) were similar to those of control pups up to 4 weeks of age. By contrast, we found deranged skeletal muscle organization, manifest as alterations in fiber typing and organization at around 15 days; differences between individual animals cannot be excluded, however.

We and others [6, 29] found reduced numbers of dystrophin-deficient fibers in adult carriers compared to pups. This was particularly evident from our investigation of the same muscle in the same animal at different ages. Although the percentage of dystrophin-deficient fibers did not decrease greatly until 60 days, CK levels had already declined by that time and were normal in adult carriers with low proportions of dystrophin-deficient fibers. The percentages of fiber types in the tibialis anterior were closely similar to those in the quadriceps of the same animals.

In previous studies by Webster et al. [30] on DMD patients and by us [21] on DMD and BMD carriers, characterization of myosin heavy chain expression in dystrophin-deficient fibers revealed that the proportion of type IIB fast fibers was significantly lower than in healthy controls. We have now found that for CXMD carriers of all ages, fibers lacking dystrophin were preferentially fast fibers; similarly 60-day-old and adult CXMD dogs had fewer fast fibers than controls. This suggests that pathogenetic mechanisms leading to preferred involvement of one fiber type rather than another are similar in DMD patients, DMD/BMD carriers, and CXMD dogs. Furthermore, type IIC and fetal myosin-containing fibers are increased in DMD patients and carriers [21, 30]. We found this also to be the case in CXMD dogs: muscles had significantly higher percentages of developmental myosin-positive fibers than controls at 30 and 60 days and these percentage remained high in adult animals. In carrier dogs percentages of developmental fibers did not differ significantly from those in controls, and were only occasionally detected in adults; likewise in DMD carriers, developmental fibers are not detected in all cases [21].

Faint reactivity to dystrophin antibodies has previously been reported in CXMD dogs [31], and may be due either to the presence of abnormal dystrophin transcripts that produce some protein, or to cross-reactivity with utrophin. The latter explanation seems more likely as we did not observe dystrophin labeling in 2-day-old dystrophic dogs, in whom sarcolemmal utrophin was absent.

We observed up-regulation of utrophin at the sarcolemma of dystrophic pups and the protein remained up-regulated in adults (unlike in controls). This also occurs in DMD muscle [13, 14]. By contrast, Wilson et al. [31] reported sarcolemmal utrophin almost exclusively in regenerating fibers with traces of peripheral utrophin on some large fibers in CXMD dogs. This discrepancy is probably due to differing antibody reactivity. We used three antibodies including the C-terminal antibody (corresponding to the Mancho-7 monoclonal used in [31]) which in our hands shows poor reactivity in human and canine tissues.

Immunoblot revealed a band, sometimes a doublet, of molecular weight corresponding to that of utrophin, at all ages and in all three groups of dogs. This may be attributed to utrophin expression in blood vessels, nerves and neuromuscular junctions. Total estimates of utrophin are influenced by the variable proportion of these components in tissue samples. Furthermore, protein quantitation on immunoblot is influenced by the relative amount of muscle proteins present in the tissue specimen. For this reason our results are given as the ratio of utrophin to myosin bands. Differences in utrophin expression between dystrophic and control animals were significant only at 15 days and in adults.

Utrophin gene therapy, or pharmacological up-regulation [15, 24], seem promising options in DMD as they may avoid immunological rejection problems of dystrophin gene therapy in individuals who have never “seen” the protein [1, 2, 19]. Furthermore, mdx mice made knockout for utrophin, which are severely affected because they lack both dystrophin and utrophin [7, 12], show a marked reduction in dystrophic pathology when they express a transgenic utrophin minigene at high levels [23]. We found utrophin at the sarcolemma in normal dogs in the postnatal period and considerably greater sarcolemmal expression in dystrophic animals. The dys-

trophic process may, therefore, develop irrespective of utrophin up-regulation (as perhaps occurs in DMD). On the other hand without utrophin up-regulation, the rate of muscle fiber necrosis may have been faster and disease progression more severe. Artificially increasing utrophin expression at the sarcolemma is, therefore, likely to be beneficial. In this regard we note that in dystrophic pups, utrophin appears when the first signs of muscle disorganization have become evident; it would, therefore, seem important to begin utrophin gene therapy in CXMD dogs even earlier (i.e., from birth).

Because human DMD patients are usually diagnosed at a much later stage and the pattern of utrophin expression is different in humans [17, 20], utrophin therapy might not have the same effect in humans as in dogs. In any event it would also seem important to begin such therapy at the earliest possible stage, i.e., before pathological changes have become extensive and clinical symptoms have become evident.

In conclusion our detailed study confirms that postnatal muscle maturation is achieved at around 60 days in the dog, considerably later than other animals and humans. In CXMD dogs major derangement of myosin expression becomes evident from about 15 days as a selective loss of fibers expressing fast myosin and excessive persistence of developmental fibers compared to controls. Dystrophin-deficient fibers in carriers decrease with age and mainly express fast myosin. The data of this study provide important baseline information for further studies on the development and progression of pathological changes in the muscle of CXMD dogs.

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