Acta Neuropathol (2001) 101 : 469–478
DOI 10.1007/s004010000308

REGULAR PAPER

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Development of muscle pathology in canine X-linked muscular dystrophy. II. Quantitative characterization of histopathological progression during postnatal skeletal muscle development

Received: 21 June 2000 / Revised: 30 August 2000 / Accepted: 11 September 2000 / Published online: 17 March 2001

Abstract We have characterized the time course of muscle pathology development during the postnatal maturation of quadriceps and tibialis anterior muscle in dystrophic golden retriever dogs. We determined the percentages of degenerating, regenerating, calcium-positive, hypercontracted, albumin-positive, and C3 complement fraction-positive muscle fibers and the extent of connective tissue proliferation in animals from neonate to adult. Necrotic fibers increased from days 2 to 30, decreased at 60 days (to 0.8%) and increased in older animals to a stable level of around 2%. Hypercontracted fibers peaked at 15 days (19.1%) and declined to 3.7% in adults. Regenerating fibers were numerous at 15 and 30 days (10%), declined at 60 days to 4.7% and declined further in adults. Calcium- and albumin-positive fibers peaked at 30 days (6.5% and 13.8%, respectively) and then declined to around 3% and 5%, respectively, in older dogs. In dystrophic dogs, the extent of fibrosis was significantly greater on 15 days than in controls, but did not then increase with age. In carriers, calcium- and albumin-positive fibers always expressed dystrophin abnormally. Muscle damage occurs before completion of muscle maturation in dystrophic dogs. While necrosis and hypercontraction remain stable in adults, fiber regeneration declines to very low levels. In contrast to Duchenne muscular dystrophy, muscle fibrosis in the muscle studied does not increase with age.

Keywords Canine X-linked muscular dystrophy · Duchenne muscular dystrophy · Dystrophin · Muscle fiber necrosis

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Introduction

Canine X-linked muscular dystrophy (CXMD) is the animal model most similar to Duchenne muscular dystrophy (DMD). CXMD is genetically homologous to DMD and shares its severe myopathy and lethal clinical development. In both diseases, fiber necrosis and regeneration begin early in skeletal muscle and are associated with connective tissue proliferation [6, 24, 25]. CXMD has been found in several breeds; the best characterized at the molecular, clinical and histopathological levels is that in golden retrievers [6, 18, 21, 24, 25, 26].

We have previously characterized the time course of the postnatal expression of adult fast, adult slow and developmental myosin, as well as the expression of utrophin and dystrophin in this breed [15]. We confirmed delayed postnatal muscle maturation in normal dogs compared to other animals and humans, which is only achieved at around 60 days. We have also shown that major derangement of myosin expression in dystrophic CXMD dogs becomes evident from about 15 days, while sarcolemmal expression of utrophin is marked and persistent, in contrast to control and carrier animals where utrophin is mainly expressed on fiber surfaces between days 15 and 30.

In the present study we have characterized the time course of muscle pathology development during postnatal skeletal muscle maturation in dystrophic golden retriever dogs at the same ages as those of our previous study. On consecutive muscle sections we determined the percentages of degenerating, regenerating, calcium-positive and hypercontracted fibers by conventional histopathological staining, and the percentages of prenecrotic and necrotic

fibers by immunohistochemical detection of albumin and C3 complement fraction. Albumin is normally present only in the extracellular fluid but penetrates into muscle cells following sarcolemmal damage [7, 19]. The complement system contributes to the removal of necrotic muscle, irrespective of the underlying pathological process [9, 16]. Albumin and complement fractions within muscle fibers can, therefore, be taken as markers of prenecrosis and necrosis, respectively.

The aim of the study was to elucidate the mechanisms of fiber degeneration and fibrosis due to dystrophin deficiency and to provide baseline information for evaluating the effect of therapies in this X-linked muscular dystrophy model.

Materials and methods

Animals

Control, carrier and dystrophic dogs of 2, 15, 30 and 60 days, and adult age were the same as studied previously [15]. Four additional 6-month-old dystrophic dogs were also included. The quadriceps muscle was examined in all animals and the tibialis anterior muscle was analyzed in selected cases.

Histological and immunochemical staining

Serial cryostat sections, 6 μm thick, were collected on gelatin-coated slides. Unfixed sections were stained as follows: with hematoxylin-eosin, modified Gomori's trichrome, acridine orange, and alizarin red S, and for complement fraction C3 and albumin. Some selected muscle samples from carriers were also immunostained for dystrophin.

Canine complement fraction C3 was localized using a polyclonal antibody raised in goat (Nordic Immunological Labs, Tilburg, The Netherlands) diluted 1:2,000 in PBS plus appropriate serum.

Albumin was detected with rabbit polyclonal antibody (diluted 1:10,000) raised in our laboratory against canine albumin purchased from Sigma (St. Louis, Mo.). This polyclonal antibody detected a band of about 66 kDa on immunoblots.

For albumin localization, cryosections, fixed in 4% paraformaldehyde, were incubated in avidin and biotin for 15 min each (avidin blocking kit; Vector Labs, Burlingame, Calif.), and subsequently in PBS plus 10% appropriate serum, followed by the primary antiserum. C3 and albumin were detected with biotinylated donkey anti-goat IgG or biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, Pa.), respectively, followed by Cy2-labeled streptavidin (Jackson ImmunoResearch). They were then mounted in glycerol-based fluorescence mounting medium.

As a control for albumin, either the primary antibody was omitted, or a preimmune serum or serum preadsorbed with albumin was applied on consecutive sections. As control for C3, primary antibody was omitted or a nonimmune goat serum was applied on adjacent sections.

For co-localization of dystrophin and C3 in carrier muscle, cryosections were incubated in a mixture of anti-dystrophin (rabbit polyclonal antibodies raised in our laboratory [15]) and anti-C3, followed by biotinylated donkey anti-goat IgG, by Cy2-labeled streptavidin, and then rhodamine-labeled goat anti-rabbit IgG (Jackson ImmunoResearch).

Mononuclear cell infiltrates were characterized on consecutive sections of selected muscles using Ber-MAC3 (Dako, Denmark), and anti-canine CD4 and CD8 (Serotec, Oxford, UK) monoclonal antibodies recognizing macrophages, and CD4- and CD8-positive T lymphocytes, respectively, followed by revelation with the avidin-biotin system.

Washings (several between each incubation) were in PBS. All steps were performed at room temperature in a humid chamber. Specimens were examined under a Zeiss Axiophot fluorescence microscope.

Quantitation

Fiber counts in muscle biopsy specimens were performed by two operators. From each animal, 500–1,000 fibers were counted from three or four 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 with trichrome, acridine orange, alizarin red, anti-C3 and anti-albumin. Mean percentages for abnormal fibers were calculated for each age group of dystrophic dogs.

The extent of connective tissue was measured on photographs of trichrome-stained sections at ×20 magnification using the NIH

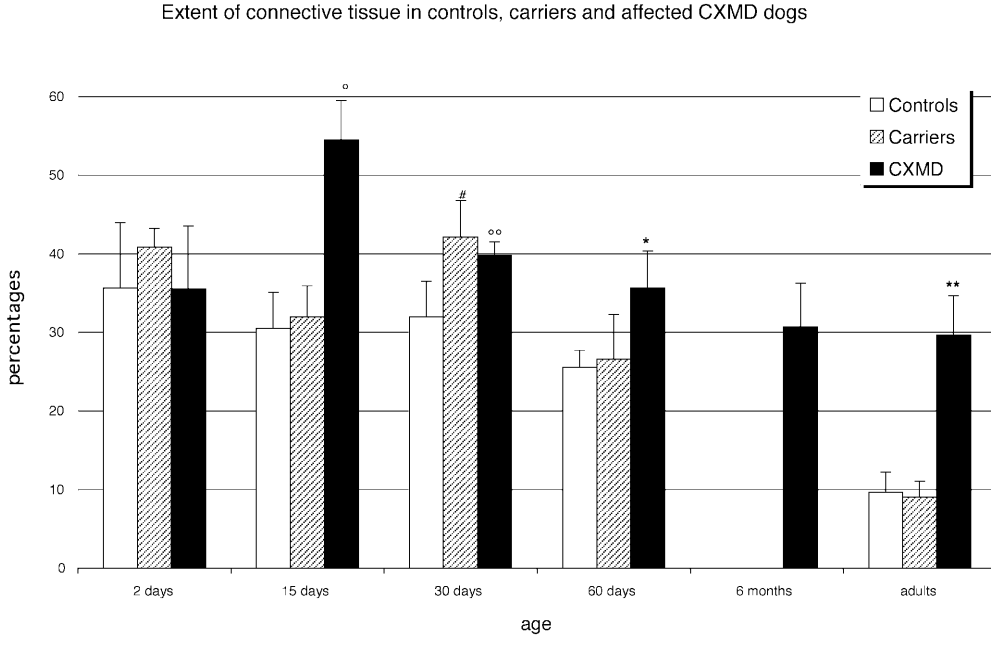


Fig. 1 Extent of connective tissue in controls, carriers and dystrophic CXMD dogs (*CXMD* canine X-linked muscular dystrophy)

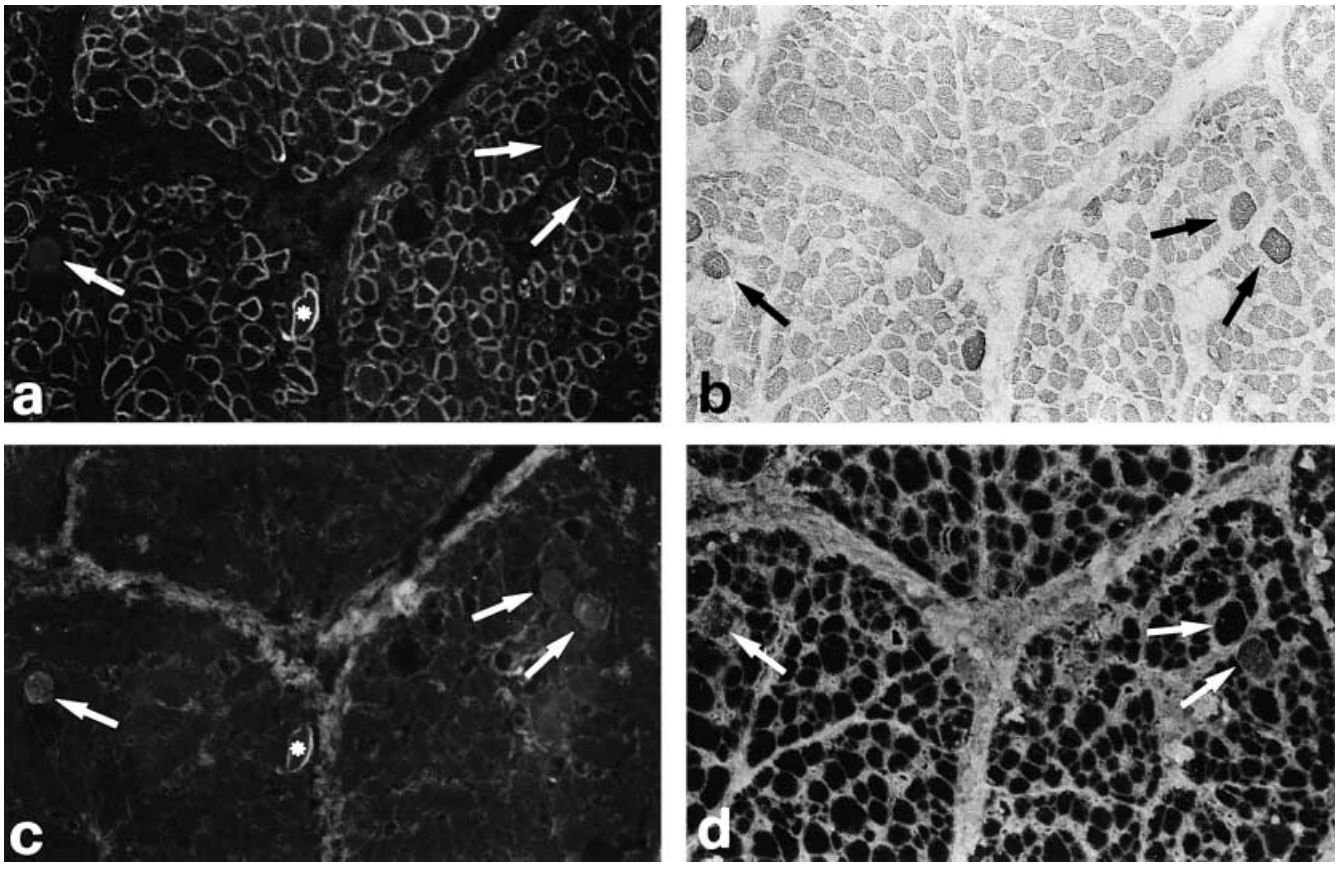


Fig. 2 Co-localization of dystrophin (**a**) and complement (**c**) on a skeletal muscle section from a 60-day-old carrier, and, on consecutive sections, alizarin red staining (**b**) and albumin immunostaining (**d**). *Arrows* indicate dystrophin-deficient fibers containing calcium, albumin or complement. Note also a dystrophin-positive fiber (*asterisk*) expressing C3 on the surface. **a**–**d** ×250

Fig. 3 Quantitations of necrotic, hypercontracted and regenerating fibers in dystrophic dogs at various ages

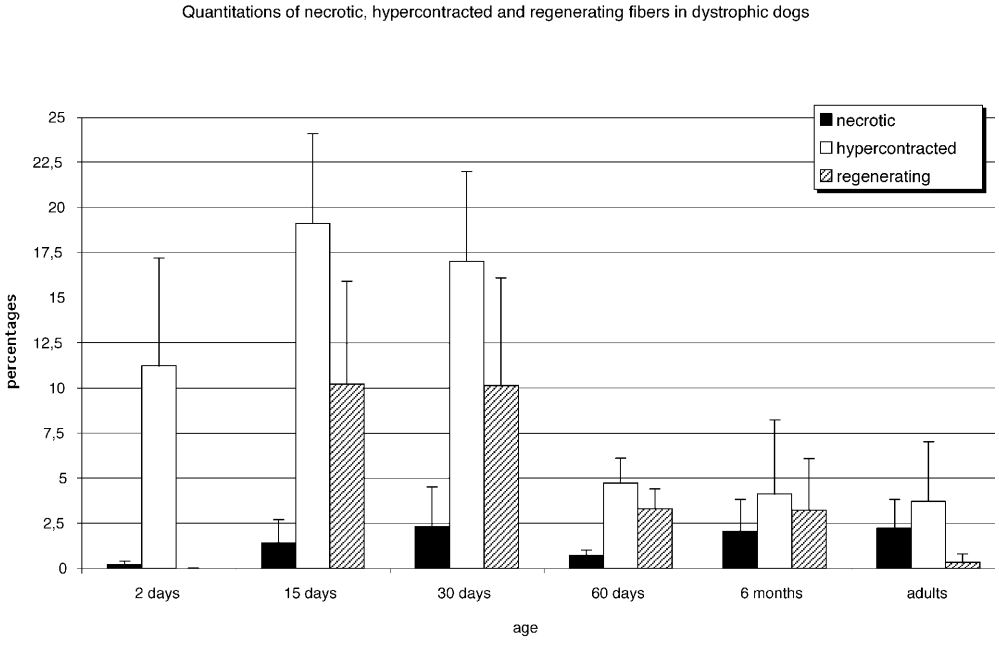


Image software. At least three fields from each animal were analyzed and the mean area calculated for each age group. We used the unpaired Student *t*-test to test the significance of differences in extent of connective tissue proliferation between dystrophic, carrier and control animals.

Results

Control dogs

No necrotic fibers were observed in control dogs at any age on trichrome-stained sections. Rare hypercontracted fibers were observed in 2- and 15-day-old animals (0.2% and 1.5%, respectively), but not in 30- or 60-day-old puppies or adults. Hypercontracted fibers were more frequent in fascicles containing a nerve.

Occasional calcium-positive, albumin-positive and C3-positive fibers were detected in normal 2-day-old puppies (0.8%, 0%, and 0.1% of total fibers, respectively) and in 15 day-old animals (1.4%, 1.5% and 0.1%), and usually corresponded to the hypercontracted fibers seen on trichrome-stained sections. No calcium-positive, albumin-positive or C3-positive fibers were detected in older animals.

Connective tissue was present in animals at all ages mainly perimysially; it was more abundant in young animals (Fig. 1). Mononuclear cells were not observed.

Carriers

A few necrotic fibers were observed in 15-day-old animals (mean 1.1%); they were rare at 60 days (0.2%) and none were found in adults. Occasional hypercontracted fibers were detected on trichrome-stained sections in 15-, 30- and 60-day-old animals (1.3%, 0.5% and 1.2%, respectively). Acridine orange-positive regenerating fibers were found extremely rarely in 30- and 60-day-old animals, and were absent in younger and older animals.

A few calcium-positive, albumin-positive and C3-positive fibers were observed mainly at 15 days (1.2%, 1.1% and 0.4%, respectively); percentages in 30, and 60, day-old animals were 0.3%, 0% and 0%, and 1.1%, 0.7% and 0.9%, respectively. These abnormal fibers almost always showed either reduced, patchily distributed or absent dystrophin on their surfaces (Fig. 2). No calcium-positive, albumin-positive or C3-positive fibers were observed in adult carriers. In 30-day-old carriers fibrotic tissue was significantly more extensive than in normal controls; at all other ages it was in the normal range (Fig. 1). Mononuclear cells were observed mainly in 30- and 60-day-old carriers; they were almost exclusively macrophages and were singly dispersed within the perimysium and endomysium.

Dystrophic dogs

The percentages of necrotic, hypercontracted and regenerating fibers in dystrophic dog quadriceps are shown in Fig. 3 according to age. The percentages of calcium- albumin- and C3-positive fibers in the same dystrophic muscles are shown in Fig. 4. The extent of fibrosis is shown in Fig. 2. Quantitations in tibialis anterior muscle (data not shown) were similar to those in the quadriceps.

Trichrome staining revealed necrotic, hypercontracted and regenerating fibers at all ages. Necrotic fibers were usually present in small numbers; they tended to increase from day 2 to day 30, declined at day 60, and increased again in older animals where they remained stable at around 2%. The level of hypercontracted fibers was 11.2% in neonates, peaked at 15 days (19.1%) and then decreased to about 4% in adult animals. Regenerating

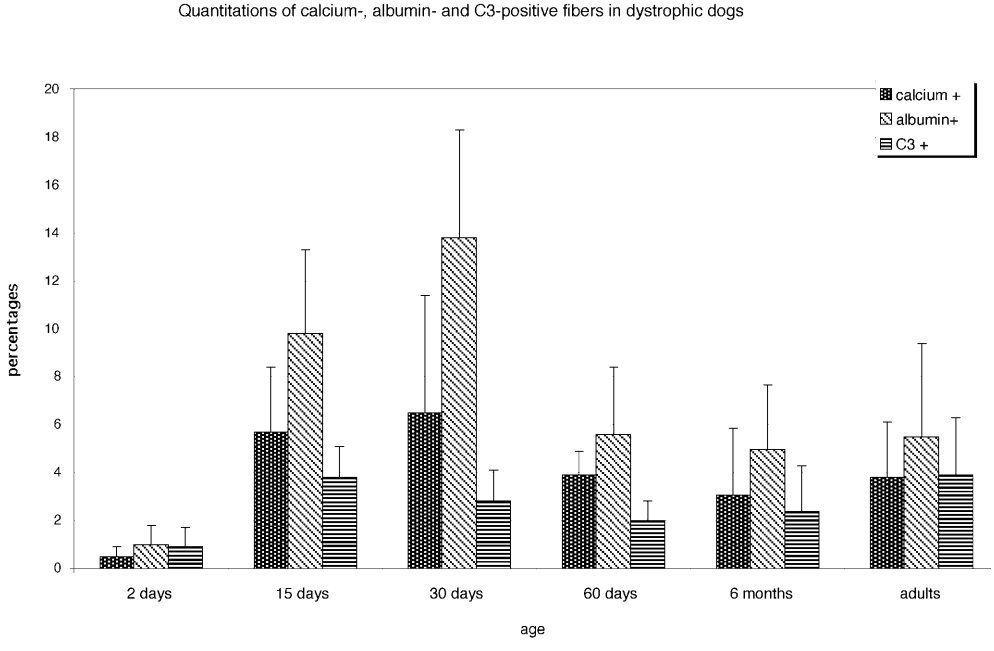


Fig. 4 Quantitations of calcium-, albumin- and C3-positive fibers in dystrophic dogs at various ages

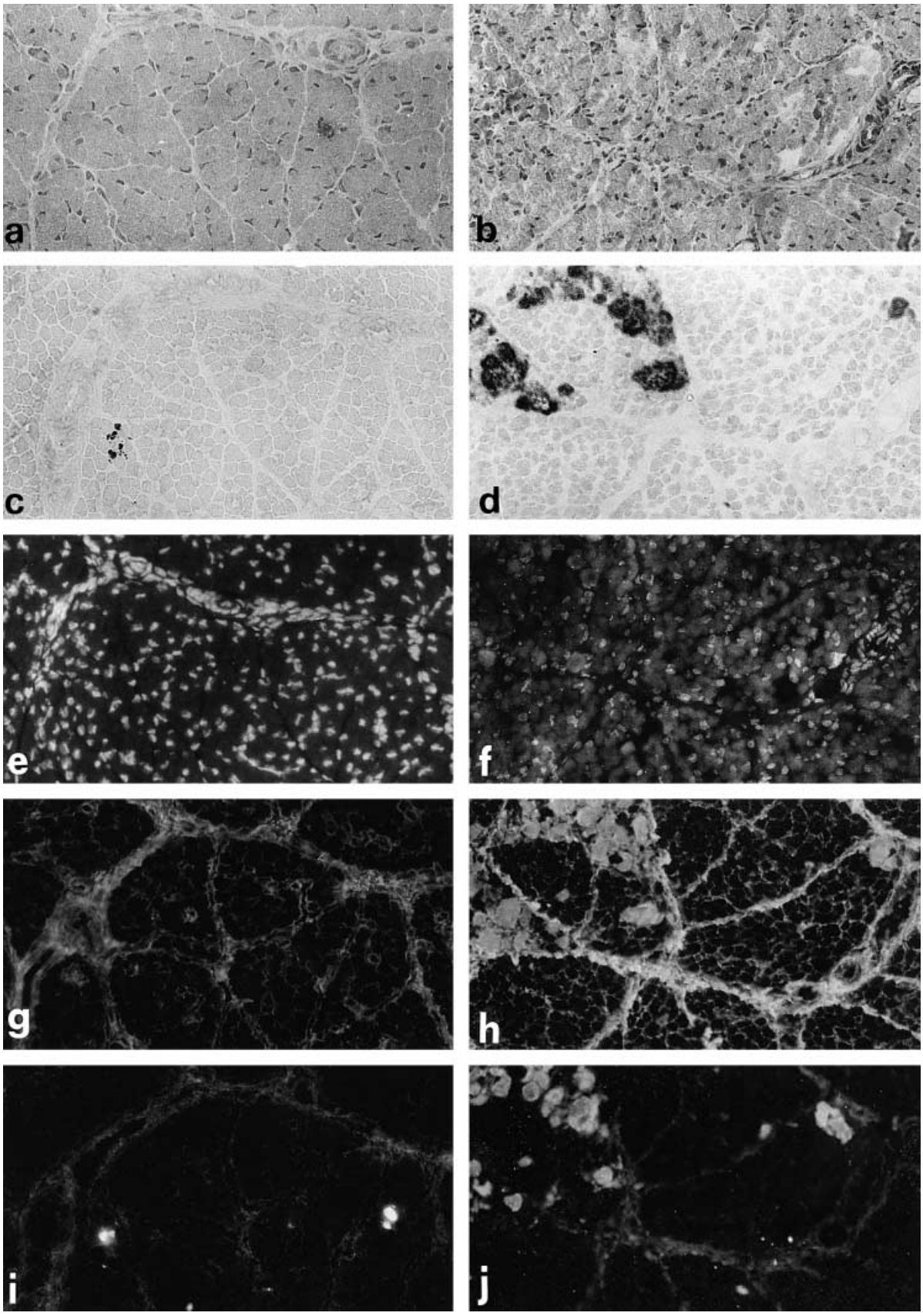
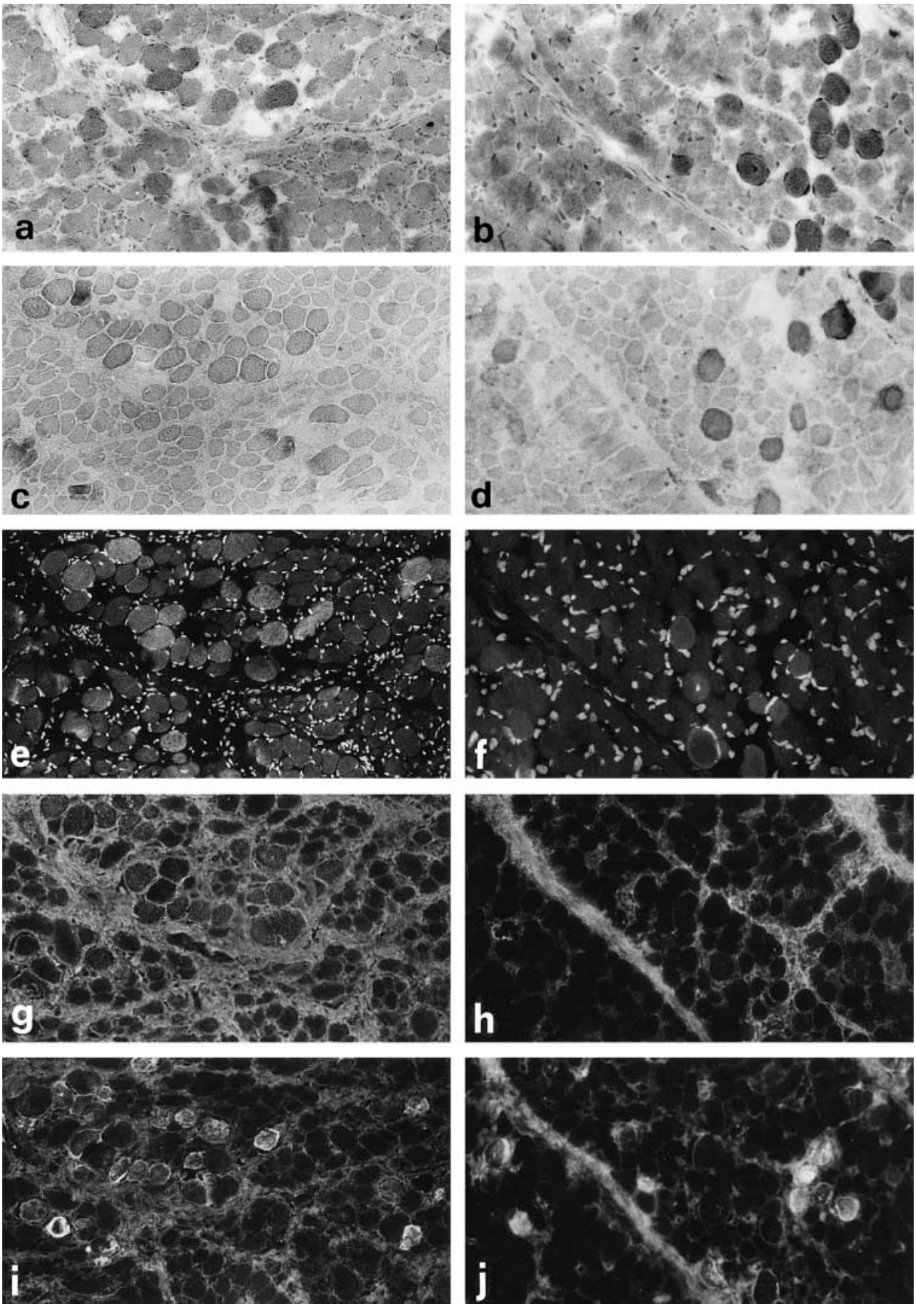


Fig. 5 Trichrome (**a,b**), alizarin red (**c,d**), acridine orange (**e,f**), albumin immunostaining (**g,h**), and C3 immunostaining (**i,j**) on serial sections of skeletal muscle from a 2-day-old dystrophic dog. The *left panel* shows almost normal morphology; the *right panel* shows that several fibers within a fascicle are pathological. Note that albumin and C3 positivity is present in endomysium and perimysium as well as fibers. **a**–**j** ×450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fibers positive to acridine orange were often observed in small groups. They were not detected in 2-day-old animals. At 15 and 30 days small acridine orange-positive fibers were common, they decreased in number at 60 days to 4.7% and which was further reduced in adult animals (Figs. 5, 6, 7).

Calcium-positive fibers were observed at all ages; percentages were higher at 15 and 30 days (Figs. 5, 6, 7). Sometimes fiber calcification was also observed. Calcium positivity was present mostly in hypercontracted fibers, but also in some necrotic and normal fibers.

C3-positive fibers were observed at all ages. C3 positivity was bright and located intracellularly in necrotic fibers or appeared as a peripheral rim on the fiber surface in non-necrotic (most often hypercontracted) fibers (Figs. 5, 6, 7). When the latter fibers were observed in sections a few micrometers away they usually appeared necrotic.

A few albumin-positive fibers were present in 2-day-old animals and in greater proportions in older dogs. The greatest proportions were observed at 15 and 30 days. Like calcium positivity, albumin positivity was mainly in hypercontracted fibers, but also in necrotic and normal fibers (Figs. 5, 6, 7).

The proportions of abnormal fibers varied from animal to animal, and in the same animal from fascicle to fascicle. This was particularly evident in neonates where most of the muscle appeared normal with no evident necrosis, but entire fascicles full of calcium-positive, C3-positive and albumin-positive fibers (Fig. 5, right panel) were observed.

In 30- and 60-day-old puppies central nuclei were observed in very small regenerating fibers, while in regenerating fibers of larger size the nuclei were mostly peripheral. Central nuclei were present in 12% of fibers in 6-month-old dogs. In adults usually less than 1% of fibers had central nuclei, although in one animal the figure was 10%.

Endomysial and perimysial fibrosis was significantly increased, compared to controls of the same age, in all dystrophic animals from 15 days on (Fig. 1), with some variability from animal to animal. Findings in 6-month-old animals were similar to those in adults.

Mononuclear cells in dystrophic dogs were almost exclusively macrophages before 30 days. In 30-day-old dogs rare CD4- and a few CD8-positive cells were seen. Infiltrates were present in most muscle biopsies mainly from 60 days on, and were found most often in necrotic fibers but also around non-necrotic fibers. In the selected muscles studied these infiltrates were composed mainly of macrophages (64.74±7.12%), with CD4-positive (14.67± 9.57%) and CD8-positive (15.69±13.00%) T lymphocytes. Macrophages (13–56/field at ×20) and rare CD8- and CD4-positive T lymphocytes were singly dispersed in the perimysium and endomysium at 60 days and in older dogs.

◀ **Fig. 6** Trichrome (**a,b**), alizarin red (**c,d**), acridine orange (**e,f**), albumin immunostaining (**g,h**), and C3 immunostaining (**i,j**) on serial sections of skeletal muscle from 15-day-old (*left panel*, ×250) and 30-day-old dystrophic dog (*right panel*, ×300). Many fibers are faintly positive to albumin (**g**); C3 positivity is both inside fibers and on the surface as a peripheral rim of several fibers (**i,j**); note also that albumin and C3 positivity is present in the endomysium and perimysium

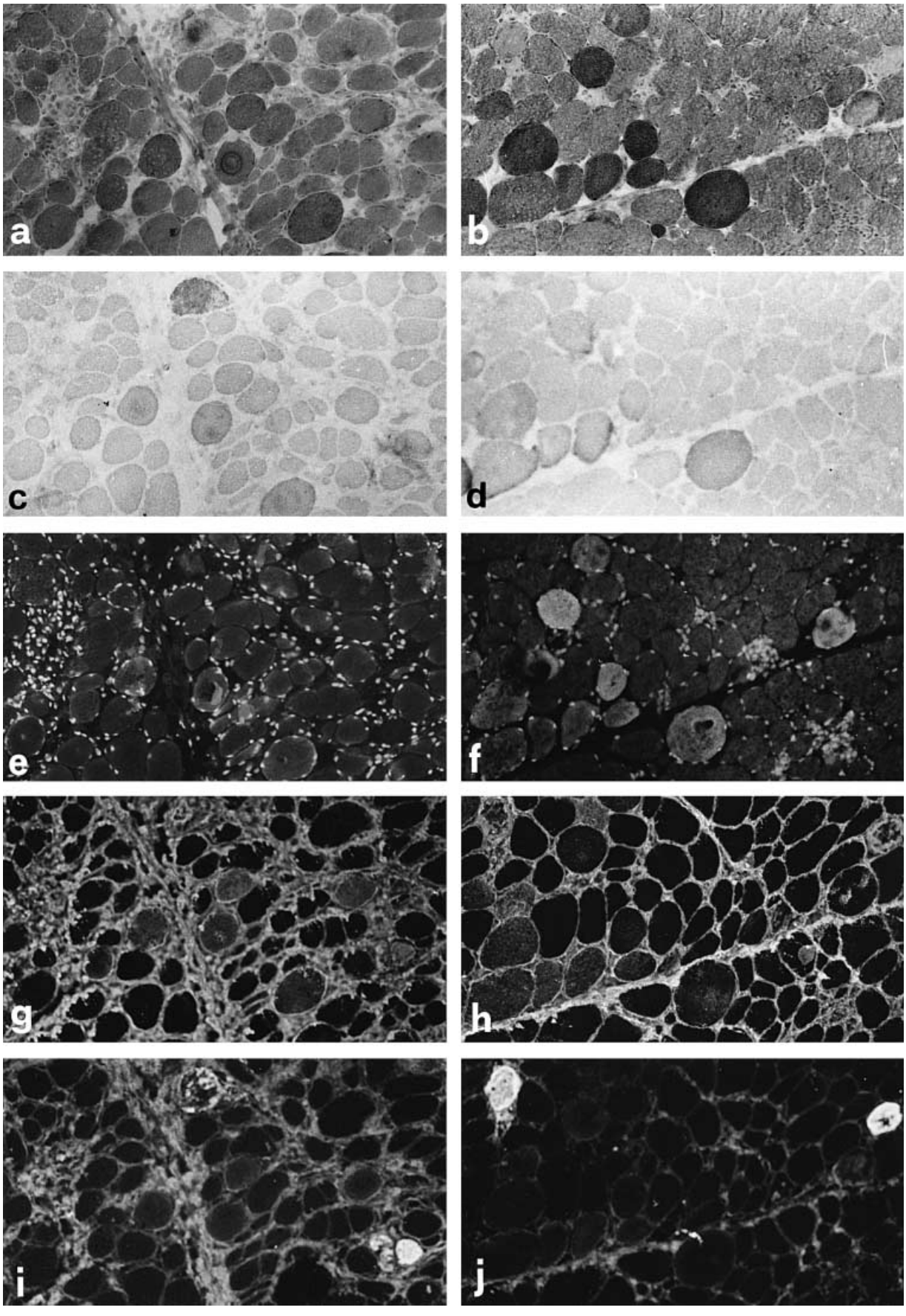
Discussion

The observation of focal defects in the plasma membrane of non-necrotic fibers in DMD (so-called delta lesions) [17] led to the structural hypothesis which clearly identified DMD as a membrane disease prior to the discovery of dystrophin. According to this hypothesis, membrane stresses associated with muscle contraction and tension induce plasma membrane ruptures, resulting in loss of muscle enzymes and influx of extracellular fluids [3, 7, 10]. This leads to intracellular calcium overload, which is likely to trigger segmental hypercontraction and may also activate endogenous proteases so that segmental degeneration follows [8, 15]. Calcium may also accumulate in mitochondria causing impairment of mitochondrial function, leading to severe energy deficit within the fiber and eventually fiber death. The complement cascade is also implicated in the final stages of necrosis [7, 9, 16]. Satellite cells may be able to effect fiber repair at least for some time. Studies, after dystrophin had been identified, confirmed the role of dystrophin in protecting the sarcolemma from membrane stresses developed during muscle contraction [20]. It has also been suggested that dystrophin is involved in the control of sarcolemmal ion permeability [12, 13].

Hypercontracted fibers were the most significant pathological finding in neonate dystrophic dogs, whose muscle was otherwise almost normal. Starting from 11% of the total, hypercontracted fibers peaked at 15 days (about 20%), and then declined. Calcium- and albumin-positive fibers were also present at birth but in small numbers and peaked later at 30 days. The peak of hypercontracted fibers corresponds to the stage at which puppies become ambulatory and muscle contraction becomes significant. After this stage, the percentage of hypercontracted fibers decreased probably because an equilibrium between hypercontraction, subsequent fiber necrosis, and fiber repair is established for some time, and because of reduced movement in older animals. Although much less abundant, hypercontracted fibers were also observed in 2- and 15-day-old normal dogs, suggesting that hypercontraction may be physiological in young puppies.

We found several C3-positive fibers in some fascicles of 2-day-old animals, but few visibly necrotic fibers in trichrome-stained sections, probably because they are too small to be visible at this age unless marked by a specific stain; C3 complement immunostaining was, therefore, useful for detecting such fibers. The detection of albumin inside some apparently normal fibers indicated the utility of this protein as a prenecrotic marker, particularly since in carriers albumin-positive fibers largely coincided with dystrophin-deficient fibers.

These findings indicate that the dystrophic process in CXMD dogs begins before birth – as also occurs in DMD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[4] – although the extent varies from animal to animal. Moreover, connective tissue proliferation is already significant in 15-day-old puppies, again indicating previous loss of muscle fibers. Note also that the muscles we studied are those in which pathological changes are reported to be less precocious than in diaphragm or tongue [23], and whose early deterioration would account for the frequent death of neonate dystrophic puppies (a feature of our colony).

The dystrophic process, including fibrotic substitution of muscle, was clearly evident from 15 days on, well before completion of muscle maturation. Therefore, muscle maturation seems not to be a critical factor in the initiation of fiber necrosis in the CXMD dog, in contrast to what has been recently suggested for DMD [14].

The percentages of necrotic and hypercontracted fibers declined to moderate and stable levels in older dystrophic animals, as did the percentages of calcium-, albumin- and C3-positive fibers. By contrast, regeneration, which was prevalent during the initial stages of muscle damage, declined to very low levels in adult animals. Valentine et al. [18] also reported fewer regenerating fibers in 8-month-old and older dogs than young animals. This decline may be due to exhaustion of the regenerative potential following repeated cycles of degeneration-regeneration. Clearly regeneration is less significant in this animal model than in the mdx mouse. Furthermore, central nuclei are not a primary feature of adult dystrophic dog muscle as is the case in the mdx mouse. In larger regenerating fibers the nuclei were mostly peripheral, although in one CXMD dog 10% of fibers were centrally nucleated.

We found that the extent of connective tissue proliferation in dystrophic dogs was significantly greater than in normal controls from 15 days on. Mantegazza et al. (personal communication) have similar findings. However, unlike in DMD [2], the extent of fibrosis tended to plateau in adult life. The decrease from 15 to 60 days was probably due to a decrease in perimysium following the reorganization of muscle tissue that occurs in normal dogs as part of the maturation process.

The failure of connective tissue to increase in adult animals may be due to exceptionally the severe disease in two of the 6-month-old dogs (that had to be killed), while the older dogs were clinically less affected. Extent of connective tissue proliferation is variable also in DMD patients, and, although fibrosis generally increases with disease progression, it may not be simply age related [27].

The lower connective tissue proliferation than in DMD may also arise because the fibrotic process differs between the human and canine diseases. For example, macrophages are more abundant in CXMD than DMD muscle [1], and this might result in more efficient removal of substances that could contribute to focal release of fibrogenic factors. In humans, however, macrophages may stimulate the fibrotic process by producing fibrogenic factors such as transforming growth factor-β1 [2].

Our findings in CXMD carriers paralleled those we described in DMD carriers [7]. In both cases only a small percentage of dystrophin-deficient fibers showed pathological features, and fibers containing calcium, albumin and complement were almost exclusively those that lacked dystrophin or expressed it abnormally. The absence of pathological fibers in the muscle of adult canine carriers, taken together with our previous finding that dystrophin-deficient fibers decline from about 30% (in young animals) to about 5% in adults [15] indicate positive selection in favor of dystrophin-positive fibers in adult life.

In conclusion our study shows that the dystrophic process in CXMD dogs is already underway at birth, well before the completion of muscle maturation. In adults, fiber regeneration decreases to very low levels, while necrosis and hypercontraction remain stable; however, the muscle does not atrophy since the diameter of the surviving fibers increases. Unlike in DMD, extent of fibrosis does not increase continuously with age. Additional research is required to understand the mechanisms of fibrosis in CXMD and how they differ from those in DMD, while ultrastructural and other investigations of the earliest (prenatal) stages of the disease will shed further light on the pathogenesis of the dystrophic process.

◀**Fig. 7** Trichrome (**a,b**), alizarin red (**c,d**), acridine orange (**e,f**), albumin immunostaining (**g,h**), and C3 immunostaining (**i,j**) on serial sections of skeletal muscle from 60-day-old (*left panel*) and adult dystrophic dog (*right panel*). Albumin and C3 positivity is present in endomysium and perimysium as well as fibers. **a**–**j** ×200

Acknowledgements The authors thank Drs. Renato Mantegazza and Pia Bernasconi for invaluable discussion and Don Ward for help with the English.

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