Myofiber Injury and Regeneration in a Canine Homologue of Duchenne Muscular Dystrophy

ABSTRACT


Objective: To test the hypothesis that differential skeletal muscle involvement, previously observed in dogs with a homologue of Duchenne muscular dystrophy, correlates with the histochemical markers of myofiber injury and regeneration.

Design: Evidence of injury (cellular penetration by Evans blue dye, immunoglobulin G expression, hematoxylin and eosin staining of necrotic figures), myofiber regeneration (fetal myosin heavy chain isoform expression), and morphologic indices in the cranial sartorius (CS), long digital extensor, and vastus lateralis muscles were examined in five dogs with dystrophy and five normal dogs.

Results: Only the CS muscle, at 1 mo, demonstrated significant differences in injury when compared with age-matched controls. By 6 mo, the long digital extensor and vastus lateralis also suffered greater than normal injury. Only the dystrophic CS tissue expressed a notable increase in mean myofiber diameter when compared with other muscles at 6 mo. Normal CS muscles revealed a distinct population of small myofibers at this age.

Conclusion: The CS seems unique in its selective pathologic involvement. These differences may contribute to the marked regenerative response of this muscle in the dystrophic state. An improved understanding of mechanisms by which some dystrophin-deficient canine muscles remain spared from injury may provide clues to investigate and prevent the degenerative processes in humans.

Key Words: Duchenne Muscular Dystrophy, Animal Muscular Dystrophy, Skeletal Muscle, Muscle Fibers
Duchenne muscular dystrophy (DMD) is a devastating inherited muscle disease that affects 1/3500 live male births worldwide. Affected boys become progressively weak because of muscle wasting and contractions, frequently leading to wheelchair use by the age of 12 yr. Dystrophin, the deficient protein in DMD, is normally located on the internal surface of the myofiber membrane. Two important models, the mdx mouse and dogs with golden retriever muscular dystrophy (GRMD), also lack dystrophin. Unlike the mdx mouse, which remains relatively normal clinically, dogs with GRMD suffer a progressive, ultimately fatal disease similar to DMD. Myofiber injury without complete repair in boys with dystrophy and dogs results in fibrosis, contractures, and weakness. Although the genetic defect in DMD is known, the sequence of molecular events that lead to cell injury has not been elucidated. Investigators have noted that dystrophin deficiency leads to a differential pathologic involvement of the affected muscle. Selective involvement of the iliotibial tract has been proposed to cause premature loss of ambulation in patients with DMD. An improved understanding of the processes by which some muscles remain relatively spared from injury might provide clues to further investigate and prevent the degenerative processes in human patients.

Serial in vivo force studies in GRMD pelvic limb muscles have shown that dogs with GRMD are weaker than normal dogs at all ages. However, the differences between normal and GRMD force measures were more pronounced for flexion at 3 mo of age, whereas the extension force values were more reduced at later ages, suggesting a preferential involvement of selected muscles over time. Other studies have similarly reported a preferential involvement of muscle groups in humans with DMD. Moreover, morphometric studies in dogs with GRMD yielded additional data illustrating selective muscle involvement. Therefore, this muscle may serve as a model to study the mechanisms that contribute to injury in dystrophin-deficient muscles, and even more importantly, the factors that lead to regeneration.

This study was undertaken to address the following questions in dogs with GRMD: (1) Does selective muscle involvement previously observed in the pelvic limb correlate with the histopathologic markers of myofiber injury and regeneration over time (at 1 and 6 mo of age)? (2) Are the markers of muscle regeneration in the cranial sartorius (CS) muscle selectively expressed at 1 mo, then relatively decreased by 6 mo of age? In contrast, are the markers of regeneration in the long digital extensor (LDE) muscle minimally expressed at 1 mo but markedly increased by 6 mo of age, as suggested in earlier studies of force measures?

METHODS

Animal Care. Dogs used in this project were obtained from the colony with GRMD at the University of Missouri-Columbia and were cared for according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Dogs with dystrophy were identified at birth by their increased creatine kinase levels. Normal dogs from the same litter were used as controls.

Evans Blue Dye. Evans blue dye, also called T-1824, contains a membrane-impermeant molecule (a tetrasodium diazzo salt) that penetrates injured or “leaky” cells, but does not permeate intact normal or dystrophic myofibers. The dye can also be detected by a fluorescence microscope before or coincident with myofiber necrosis. Using this in vivo tracer technique, we counted fluorescent-positive (presumably injured or necrotic) myofibers (Fig. 1) to estimate muscle membrane damage.

Muscle Biopsies. Samples were obtained from the Cs, LDE, and vastus lateralis (VL) muscles at 1 mo (right limb) and 6 mo (left limb) of age. Dogs were anesthetized and muscle biopsies were performed according to established protocols. Longitudinal sections of muscle (approximately 1 × 0.5 × 0.5 cm) were removed sharply.

Muscle Cryosection. Preparation. Muscle samples were snap frozen in liquid nitrogen and sectioned at 10 μm. Cryosections from each muscle sample, including one antibody-neg-
ative control, were incubated in ice-cold acetone at −20°C for 10 min, washed 3 × 10 min with phosphate buffered saline, mounted with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA), stored at −80°C, and thawed to room temperature just before imaging.

**Microscopy.** Data were recorded for each muscle specimen in five dogs with GRMD and five normal dogs. After randomization and coding, serial cryosections were imaged and observed under a microscope by one investigator (MKC) without knowledge of the identity of the muscle sections. Slides were previewed under low magnification to identify areas devoid of staining artifacts. For each staining method, 1000 myofibers were digitally analyzed (NIH Image, Scion Corp., Frederick, MD) by examining 100 myofibers in ten adjacent fields-of-view, under 200× magnification. To provide a quantitative estimate of damage, the number of myofibers positively stained were counted and recorded.

**Determination of Myofiber Injury and Necrosis.** Degenerating muscle fibers emit bright red fluorescence after intravenous Evans blue dye and green-light activation (590-nm wavelength). Accordingly, cryosections were prepared and observed using an IMT-2 inverted epifluorescence microscope (Olympus Optical Company, Ltd., Tokyo, Japan) equipped with a green-activation filter (Omega Optical, Brattleboro, VT) permitting visualization of injured myofibers permeated by Evans blue dye (Fig. 1).

Assessment of myofiber necrosis was also achieved by examining serial transverse cryosections stained with hematoxylin and eosin using light microscopy. Evidence of myofiber necrosis consisted of identifying “hyaline” or hypercontracted fibers, fibers undergoing myophagocytosis (fibers surrounded by phagocytic cells), or empty sarcolemmal tubes (Fig. 2).

**Figure 2:** Digital light micrograph (200×) of a 10-μm cryosection from the cranial sartorius muscle of a 1-mo-old dog with golden retriever muscular dystrophy stained with hematoxylin and eosin. “Hyaline” or hypercontracted fibers (asterisks), fibers undergoing myophagocytosis (left arrow) or empty sarcolemmal tubes (right arrow) were used to count necrotic figures.

Activation of the complement cascade occurs early during muscle damage; thus, immunoglobulin G (IgG) binding of C9 is one method of demonstrating recent (within 24 hr) injury to myofibers. Consequently, muscle cryosections were treated with monoclonal antibodies to canine IgG. Primary IgG antibodies were localized with a cyanine dye (Cy-2) conjugated with goat antimouse IgG antibody (Jackson ImmunoResearch Laboratories) and visualized using epifluorescence microscopy methods (Fig. 3).

**Figure 3:** Digital fluorescence micrograph (200×) of a 10-μm cryosection from the cranial sartorius muscle of a 1-mo-old dog with golden retriever muscular dystrophy. Endogenous IgG was seen (arrows) both intracellularly and peripherally. Asterisks identify the same myofibers seen in the other methods described in Figures 1 and 2.

**Morphometric Assessment.** To determine the mean-lesser myofiber diameter for each muscle, at least 2000 fibers were measured from each muscle by a technician without knowledge of the identity of the slide. Transverse muscle cryosections stained with hematoxylin and eosin were digitally imaged at 200× magnification and imported to software with image measurement capabilities (NIH Image). To ensure that the di-
ameters were not overestimated, the smallest measurement was obtained by trial-and-error fit of a straight line connecting the outer myofiber membrane through the center (hence, the term “lesser diameter”).

Statistical Analysis. Student’s t test (paired) was used to compare withingroup data (1 vs. 6 mo of age data), whereas Student’s t test (unpaired) was used to evaluate GRMD vs. control data. All analyses were performed using Sigmasstat software (SPSS Inc., Chicago, IL).

RESULTS

Histochemical data are summarized in Table 1. In general, positive findings varied markedly between the five normal and five dogs with dystrophy, among the three individual dystrophic muscles studied, and between the two age groups.

Dystrophic CS muscles, at 1 mo of age, expressed significantly (P < 0.05) more IgG when compared with age-matched normal controls (32 ± 23 vs. 0.5 ± 1.0 positive fibers counted per 1000). Other markers of myofiber injury (hematoxylin and eosin, IgG, and Evans blue dye stains) were increased in dystrophic VL and LDE muscles, but these differences were not significant. In contrast, all markers of myofiber injury were increased (P < 0.05) in each dystrophic muscle compared with normal controls at 6 mo of age.

The CS muscle had greater than normal FMHC expression at 1 mo of age (184 ± 191 vs. 0.75 ± 1.5 positive fibers per 1000), but these differences were not significant. In contrast, dystrophic LDE and VL muscles expressed little FMHC at this age (54 ± 25 and 4.8 ± 6.9 positive fibers per 1000, respectively). By 6 mo, however, all dystrophic muscles expressed significantly (P < 0.05) greater than normal FMHC. Notably, dystrophic LDE muscles had greater FMHC (111 ± 41 positive fibers per 1000) when compared with dystro-

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### TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>GRMD (1 mo)</th>
<th>Normal (1 mo)</th>
<th>GRMD (6 mo)</th>
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<tr>
<td>Cranial sartorius</td>
<td></td>
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<tr>
<td>H&amp;E</td>
<td>47 ± 38</td>
<td>2.5 ± 4.4</td>
<td>24 ± 11&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>IgG</td>
<td>32 ± 23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5 ± 1.0</td>
<td>17 ± 10&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Evans blue dye</td>
<td>47 ± 36</td>
<td>0.25 ± 0.5</td>
<td>21 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>FMHC</td>
<td>184 ± 191&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.75 ± 1.50</td>
<td>30 ± 23&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Long digital extensor</td>
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<tr>
<td>H&amp;E</td>
<td>30 ± 39</td>
<td>0.2 ± 0.4</td>
<td>36 ± 21&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>IgG</td>
<td>23 ± 26</td>
<td>6 ± 13</td>
<td>24 ± 7&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>Evans blue dye</td>
<td>43 ± 59</td>
<td>3.0 ± 6.7</td>
<td>22.5 ± 3.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>FMHC</td>
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<td>28 ± 63</td>
<td>111 ± 41&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td>Vastus lateralis</td>
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<tr>
<td>H&amp;E</td>
<td>11 ± 16</td>
<td>0.8 ± 1.7</td>
<td>21 ± 16&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td>IgG</td>
<td>2.0 ± 4.5</td>
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<td>4.4 ± 4.8</td>
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<td>Evans blue dye</td>
<td>8.2 ± 16</td>
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<td>13.0 ± 7.9&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>FMHC</td>
<td>4.8 ± 6.9</td>
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Data reported as mean ± SD of number of positive findings observed per 1000 myofibers; number of dogs studied = 10.

H&E, hematoxylin and eosin; GRMD, golden retriever muscular dystrophy; FMHC, fetal myosin heavy chain.

<sup>a</sup> Significantly different (p < 0.05) from age-matched controls.

<sup>b</sup> Significantly different (p < 0.05) between values for cranial sartorius and vastus lateralis.

<sup>c</sup> Significantly different (p < 0.05) between values for cranial sartorius and vastus lateralis.

<sup>d</sup> Significantly different (p < 0.05) between values for long digital extensor and vastus lateralis.

<sup>e</sup> Significantly different (p < 0.05) between 1 and 6 months within the same disease group (GRMD or normal).
phic CS and VL muscles (30 ± 23 and 4.6 ± 7.8 positive fibers per 1000, respectively).

Results of the morphometric analysis of mean-lesser myofiber diameters are summarized in Table 2 and Figure 5. The mean ± SD lesser-myofiber diameter for the dystrophic CS was greater than normal at 1 mo of age, whereas the dystrophic and normal values were similar for the VL and LDE muscles. By 6 mo of age, dystrophic CS muscles showed a striking increase in mean-fiber diameter (37.9 ± 16.0 μm) when compared with both normal control CS (21.5 ± 13.5 μm) and dystrophic VL (30.9 ± 11.1 μm) and LDE muscles (30.9 ± 12.9 μm).

DISCUSSION

Severity of muscle injury and regeneration, as indicated by the indices studied, varied markedly between normal and dystrophic dogs, among the three individual dystrophic muscles studied, and between the two age groups. The CS and LDE muscles were likely injured earlier and subsequently underwent more marked regeneration than the VL muscle. Only the CS muscle demonstrated statistically greater injury, as reflected by the IgG values. The greater expression of FMHC in the CS vs. the VL was the only significant (P < 0.05) difference among the dystrophic muscles at 1 mo of age. Evidence of

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<tbody>
<tr>
<td>Cranial sartorius</td>
<td>12.3 ± 6.5</td>
<td>10.8 ± 4.8</td>
<td>37.9 ± 16</td>
<td>21.5 ± 13.5</td>
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<tr>
<td>Long digital extensor</td>
<td>12.3 ± 4.3</td>
<td>11.8 ± 3.1</td>
<td>30.9 ± 12.9</td>
<td>31.2 ± 11.5</td>
</tr>
<tr>
<td>Vastus lateralis</td>
<td>9.1 ± 3.0</td>
<td>10.6 ± 3.6</td>
<td>30.9 ± 11.1</td>
<td>32.4 ± 10.7</td>
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Data reported as mean ± SD; number of dogs studied = 10.
GRMD, golden retriever muscular dystrophy.

Figure 5: Frequency distribution (per 5 μm) of the mean-lesser myofiber diameters observed in the pelvic limb muscles from dogs with golden retriever muscular dystrophy (GRMD) (A, C) and normal (B, D) dogs 1 (A, B) and 6 (C, D) mo of age. Note the bimodal distribution in the normal CS (A) CS, cranial sartorius muscle; VL, vastus lateralis muscle; LDE, long digital extensor muscle.

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early injury and regeneration in the CS is in keeping with studies by Valentine and Cooper\textsuperscript{12} and our own unpublished pathologic data.

These data suggest that the age of the dog contributes to the phenotypic variation, with some muscles selectively involved early in life and others later. We hypothesize that injury occurs early in the CS muscle, perhaps in response to active crawling, but as the pups mature, damage is lessened as the dogs begin to walk upright. In contrast, VL and LDE muscles are less affected in early neonatal life, but then they incur damage and regeneration between 1 and 6 mo of age, the LDE more so than the VL muscle.

We were surprised to identify a bimodal distribution of normal CS myofiber diameter at 6 mo of age (Fig. 5D). Because it is generally believed that small-diameter myofibers reflect recent myotube formation,\textsuperscript{26} these data suggest that a substantial subgroup of CS fibers may develop later during early growth, and thus escape early muscle damage. One explanation attributes preferential early pathologic changes in neonatal GRMD flexor muscles to their predominant role in neonatal crawling, thus incurring increasing contractile forces.\textsuperscript{12}

Experimental findings in the \textit{mdx} mouse further support the idea of contraction-associated injury, as greater than normal penetration of membrane-impermeable dye occurred immediately after eccentric (lengthening) contractions.\textsuperscript{27} An increased rate of necrosis was also observed in \textit{mdx} vs. normal mouse muscles after lengthening contractions.\textsuperscript{23} Moreover, increased intracellular calcium (putatively damaging to the cell) has been detected in contracting DMD myotubes.\textsuperscript{28} Furthermore, a protective effect from stretch-induced injury in \textit{mdx} mouse muscles was reported after injection of a minidystrophin gene.\textsuperscript{29}

**CONCLUSIONS**

We tested the hypothesis that differential involvement previously observed among muscles in dogs with a homologue of DMD correlates with the histochemical markers of myofiber injury and regeneration. Among the pelvic muscles studied, only the dystrophic CS muscle, at 1 mo, demonstrated significant differences in injury when compared with age-matched controls. By 6 mo, however, the dystrophic LDE and VL muscles also suffered greater than normal injury. Thus, the CS muscle seems unique in its selective pathologic involvement. These differences may contribute to the marked regenerative response of this muscle in the dystrophic state.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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