Muscular dystrophy in a family of Labrador Retrievers with no muscle dystrophin and a mild phenotype

Natassia M. Vieira a, Ling T. Guo b, Elicia Estrela a, Louis M. Kunkel a, Mayana Zatz c, G. Diane Shelton b, *

a The Division of Genetics and Genomics, Boston Children’s Hospital, Department of Pediatrics and Genetics, Harvard Medical School, Boston, MA 02115, USA
b Department of Pathology, School of Medicine, University of California San Diego, La Jolla, CA 92093
c Human Genome and Stem Cell Center, Biosciences Institute, University of Sao Paulo, Brazil

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Abstract

Animal models of dystrophin deficient muscular dystrophy, most notably canine X-linked muscular dystrophy, play an important role in developing new therapies for human Duchenne muscular dystrophy. Although the canine disease is a model of the human disease, the variable severity of clinical presentations in the canine may be problematic for pre-clinical trials, but also informative. Here we describe a family of Labrador Retrievers with three generations of male dogs having markedly increased serum creatine kinase activity, absence of membrane dystrophin, but with undetectable clinical signs of muscle weakness. Clinically normal young male Labrador Retriever puppies were evaluated prior to surgical neuter by screening laboratory blood work, including serum creatine kinase activity. Serum creatine kinase activities were markedly increased in the absence of clinical signs of muscle weakness. Evaluation of muscle biopsies confirmed a dystrophic phenotype with both degeneration and regeneration. Further evaluations by immunofluorescence and western blot analysis confirmed the absence of muscle dystrophin. Although dystrophin was not identified in the muscles, we did not find any detectable deletions or duplications in the dystrophin gene. Sequencing is now ongoing to search for point mutations. Our findings in this family of Labrador Retriever dogs lend support to the hypothesis that, in exceptional situations, muscle with no dystrophin may be functional. Unlocking the secrets that protect these dogs from a severe clinical myopathy is a great challenge which may have important implications for future treatment of human muscular dystrophies.

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Keywords: Myopathy; Canine; Animal model

1. Introduction

Duchenne muscular dystrophy (DMD) is a lethal X-linked condition which affects 1 in 3500 to 5000 male births. It is caused by mutations in the DMD gene, which result in the absence of muscle dystrophin [1]. Without any intervention, affected patients carrying a null mutation show a very similar severe clinical course. The onset is usually noticed around 3–5 years of age with loss of ambulation between 9 and 12 years of age. Death occurs in the second or third decade due to respiratory or cardiac failure.

In contrast to the mdx mouse, the murine model of DMD with a mild clinical phenotype despite the lack of muscle dystrophin [2] but a reduced life-span [3], the Golden Retriever Muscular Dystrophy (GRMD) dog represents the best animal model for DMD [2,4]. Affected founder animals carry a frameshift mutation in the dystrophin gene that causes the skipping of exon 7 and a premature stop codon, resulting in the absence of dystrophin in their muscles [5]. GRMD dogs and DMD patients have many phenotypic and biochemical similarities including early progressive muscle degeneration and atrophy, fibrosis, contractures and markedly elevated serum creatine kinase (CK) activities. However, unlike humans, GRMD dogs may have dysphagia and esophageal dysfunction, but loss of ambulation is uncommon. Early death may occur within the first weeks of life but is most frequently around 1–2 years of age as a result of respiratory failure or cardiomyopathy.

A striking feature of GRMD is the degree of phenotypic variability that occurs as a result of dystrophin deficiency. We have previously reported on a Brazilian GRMD dog colony (Gene Dog) with an exceptional dog, Ringo, who showed a very mild course despite the complete absence of muscle dystrophin [6]. Ringo was born in 2003 and survived with good motor
function until 2014. Ringo had 49 offspring born from natural intercourse with 4 different females. Among them, Sulflair, born in 2006, also shows a milder phenotype although more severe than Ringo.

Dystrophin deficient muscular dystrophy in Labrador Retriever dogs (LRMD) is another canine model of DMD. In the first clinical report of LRMD in a male puppy, severe clinical signs were described including dysphagia, weakness and inspiratory stridor [7]. Clinical signs began in the immediate postnatal period. Over the years 2002 to 2010, muscle biopsies were submitted to the Comparative Neuronal Laboratory, University of California San Diego (GDS) for pathological and immunohistochemical diagnosis on 12 additional young, male Labrador Retrievers. In all of these 12 cases, a dystrophic phenotype was identified and clinical information described moderate to severe generalized myopathic signs (Shelton, unpublished). Generalized weakness and gait abnormalities were present progressing to variable atrophy and hypertrophy of different muscle groups including the diaphragm, esophagus and pharyngeal muscles. Cardiomyopathy and respiratory insufficiency resulted in early death or euthanasia. The serum CK activities were markedly increased which helped differentiate this form of muscular dystrophy from other congenital myopathies affecting young Labrador Retriever puppies such as centronuclear myopathy [8], X-linked myotubular myopathy [9] and collagen VI deficiency [10]. A mutation in the dystrophin gene has been described in severely affected LRMD dogs with an insertion between exon 19 and 20 resulting in a premature stop codon [4].

In 2010 we evaluated a muscle biopsy from a young male Labrador Retriever puppy for which markedly and persistently increased CK activities were noted in the absence of clinical signs of myopathy. Over the subsequent 4 years, we followed this dog and the large family of related Labrador Retrievers from the northeastern United States. All affected males had markedly elevated serum CK activities but showed no obvious clinical signs of myopathy. Dystrophin deficient muscular dystrophy was confirmed in 12 male dogs from this family evaluated by histopathology, immunohistochemistry and western blot.

Here we describe the pathological, immunohistochemical and western blot results on this family of clinically asymptomatic Labrador Retrievers with dystrophin deficiency. The elucidation of the mechanism that is protecting these exceptions from the deleterious effect of the dystrophin gene mutation is still unknown, but it could open new avenues for human DMD future therapies. Our findings add additional support to the observations that it is possible to have a functional muscle in a large animal despite the absence of muscle dystrophin, as previously reported in GRMD [6] and recently described in humans [11].

2. Methods

2.1. Animals and archived tissues

All affected Labrador Retriever puppies evaluated in this study were presented to general veterinarians for pre-surgical screening prior to neuter. Physical examinations were performed on all dogs. Routine blood evaluations including a complete blood count (CBC) and serum biochemical profiles with measurement of CK activity were performed in all dogs. Unless indicated, muscle biopsies were performed at the time of neuter for histopathologic diagnosis and immunostaining. Archived frozen muscles from previously diagnosed severely affected Labrador Retriever puppies and normal young dogs were used for comparison in immunohistochemical studies. Muscle from GRMD and normal Golden Retrievers from the GRMD Genedog colony at the University of Sao Paulo, Brazil were also used for comparison.

2.2. Histopathology and immunohistochemistry

Biopsies from the vastus lateralis or biceps femoris muscles of affected Labrador Retrievers were collected under general inhalational anesthesia and shipped by an overnight express service under refrigeration to the Comparative Neuromuscular Laboratory, University of California San Diego. Immediately upon receipt, the biopsies were flash frozen in isopentane pre-cooled in liquid nitrogen and stored at −80 °C until further processed. All control muscles were similarly processed for a direct comparison to the affected Labrador muscles. A standard panel of histochemical stains and reactions was performed on 8 μm muscle cryosections [12]. Additional cryosections were used for immunohistochemical stainings using antibodies against the rod (1:100, NCL-DYS1, Novocastra Laboratory) and carboxy terminus (1:100, NCL-DYS2 Novocastra Laboratories) of dystrophin, α-sarcoglycan (1:200, gift from Eva Engvall), β-sarcoglycan (1:100, NCL-βSG, Novocastra Laboratories) and γ-sarcoglycan (1:100, NCL-γSG, Novocastra Laboratories), laminin α2 (1:200, gift from Eva Engvall), utrophin (1:20, NCL-DRP2, Novocastra Laboratories), developmental myosin heavy chain (1:20, NCL-MHCd, Novocastra Laboratories), dysferlin (1:50, NCL-hamlet, Novocastra Laboratories) and spectrin (1:10, NCL-SPEC2, Novocastra Laboratories). Stainings were visualized using immunofluorescent techniques as previously published [13].

2.3. Western blot

Muscle sample proteins were extracted using RIPA buffer. Samples were centrifuged at 13,000 g for 10 minutes to remove insoluble debris. Soluble proteins were resolved by 6% SDS-PAGE, and transferred to nitrocellulose membranes (Hybond; Amersham Biosciences). All membranes were stained with Ponceau (Sigma-Aldrich) to evaluate the amount of loaded proteins. Membranes were blocked for 1 hour in Tris-buffered saline Tween (TBST) containing 5% powdered skim milk and incubated overnight with the following primary antibodies: anti-dystrophin NCL-DYS1 and NCL-DYS2 (1:1000, both from Novocastra Laboratories); 6–10 (1:1000) [14] and AB15277 antidiastrophin antibody (1:1000, Abcam). Horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000, Santa Cruz Biotec2) was used to detect bound antibodies with enhanced chemiluminescence (ECL) plus kit (GE Healthcare).
2.4. PCR genotyping for the GRMD dog mutation

DNA from four of the affected Labrador Retriever dogs, and affected and carrier dogs from the GRMD colony in Brazil, were tested for the GRMD mutation as previously described [15]. The cDNA region flanking the dystrophin exon 7 was amplified using the following primers: Forward-GATTGCAACAAACCAACAGTG and Reverse-AACTTCTTTCAGTTGCTGATTCT and a 57 °C annealing temperature.

2.5. PCR genotyping for the LRMD dog mutation

DNA from two of the mildly affected Labrador Retriever dogs, three severely affected dogs, a dog with GRMD and a control dog from the GRMD colony in Brazil, were tested for the LRMD mutation. The region flanking the 6363 bp insertion in intron 19 of dystrophin gene was amplified using the following primers: Forward-TGAAAGTAAGAGCTGAGTCATGG and Reverse-TCGCCAAAAGTGAATTGAAA and a 60 °C annealing temperature.

3. Results

3.1. Affected male Labrador Retrievers had markedly elevated CK activities in the absence of clinical muscle weakness

A 7 month old male Labrador Retriever puppy without obvious clinical signs of muscular weakness, atrophy or other medical problems was presented for pre-neuter screening (Fig. 1A). No abnormalities were found on general physical and neurological examination. Laboratory evaluations including

Fig. 1. (A) A seven month old male Labrador retriever puppy (dog 1 in panel B) with a markedly elevated CK activity, histopathologic phenotype consistent with a muscular dystrophy, and the absence of muscle dystrophin. At the time of neuter there was no clinical evidence of a myopathy. (B) Pedigree showing relationships of 3 generations of affected Labrador retriever dogs and obligate carriers.
a CBC and serum biochemical analysis with CK activity were performed. The CK activity was markedly increased (>40,000 IU/L, reference <200 IU/L), and upon recheck one week later, remained elevated (>30,000 IU/L). Because of the markedly increased CK activities, biopsies were collected from the vastus lateralis muscle at the time of neuter. Over the following 2 years, muscle biopsies from 10 additional young male Labrador Retriever puppies were submitted by general veterinarians due to markedly elevated CK activities identified prior to neuter or after identification of relatedness of all 11 dogs (Fig. 1B, pedigree). Muscle weakness, atrophy or dysphagia was not noted in any of the additional dogs. A further 3 asymptomatic, related, male Labrador Retriever puppies with markedly elevated CK activities and no clinical signs of myopathy were also identified, but muscle biopsies were not collected. All muscle biopsies, including that from the original case were submitted by veterinarians in the Northeastern United States.

3.2. Histopathology and immunohistochemistry showed a dystrophic phenotype, absence of or greatly diminished muscle dystrophin, and utrophin in regenerating fibers

Hematoxylin and eosin stained cryosections from the first affected puppy (Puppy 1, Fig. 1B) confirmed degenerative and regenerative changes consistent with a dystrophic phenotype (Fig. 2). Degenerating fibers undergoing necrosis and phagocytosis (Fig. 2Aa), groups of basophilic regenerating fibers (Fig. 2Ab) and calcific deposits (Fig. 2Ac) were noted. Immunohistochemical staining using an antibody against developmental myosin heavy chain confirmed the presence of

![Fig. 2. Representative cryosections from dog 1 in Fig. 1B stained with hematoxylin and eosin show the degenerative (a) and regenerative (b) changes typical of the dystrophic phenotype (A). Calcific deposits were occasionally noted (c). Immunostains from additional affected dogs (dogs 6–10 in Fig. 1B) showed clusters of regenerating fibers using an antibody against developmental myosin heavy chain. Bar in panel A(c) = 50 μm for all figures.](image-url)
clusters of regenerating fibers in all dogs (Fig. 2B). Based on histopathology from all 11 related dogs, a dystrophic myopathy was diagnosed.

To further investigate a specific type of muscular dystrophy, immunostaining of muscle cryosections was performed using antibodies against dystrophin associated proteins as described in methods. In general, staining was markedly diminished or absent with antibodies against the rod domain and C-terminus of dystrophin (Fig. 3). Rare presumptive revertant fibers (2–3 per 500 fibers) were observed. Stainings for all other dystrophin associated proteins were similar to controls.

To further investigate the pattern of utrophin expression, serial cryosections from mild and severely affected Labrador Retrievers, and control muscle, were incubated with antibodies against utrophin and developmental myosin heavy chain (Fig. 4). Myofibers expressing utrophin were limited to regenerating fibers in both the mild and severely affected Labrador Retrievers. Neither sarcolemmal labeling of utrophin nor staining of regenerating fibers were found in the control dog muscle.

3.3. Dystrophin protein was absent using Western blot analysis

To confirm the absence of dystrophin expression in the muscles of LRMD dogs, we performed a Western blot analysis (Fig. 5). Total muscle protein from LRMD dogs 8 and 15 were compared with two GRMD dogs, and normal human samples. The dystrophin band of 427 kDa was completely absent in samples from dogs 8 and 15 as in the GRMD dogs, but was present in normal amount in the control samples. No small dystrophin bands with molecular weights below approximately 400 kDa were identified. Four different dystrophin antibodies were used to confirm the findings: two against the rod-domain
3.4. Mildly affected Labrador Retrievers with dystrophin deficiency do not carry the GRMD mutation

To verify whether these dogs carried the same dystrophin mutation as Golden Retrievers with GRMD [6], genotyping was performed using DNA from 2 of the mildly affected Labrador Retriever puppies (dogs 5 and 8 in Fig. 1B), 2 affected Golden Retrievers, a Golden Retriever female carrier and a wild type Golden Retriever. The affected Labradors did not carry the same mutation found in GRMD dogs (Fig. 6A). The cDNA region flanking the deleted exon of dystrophin in the GRMD dog model was amplified from a LRMD dog and a normal control. Bands of expected size were observed in both samples, which showed no deletion (Fig. 6B).

3.5. Neither mildly or severely affected Labrador Retriever dogs with dystrophin deficiency carried the LRMD Mutation

To verify whether these dogs carried the same dystrophin mutation as previously reported in Labrador Retrievers with LRMD [4], genotyping was performed using DNA from 2 of the mildly affected Labrador Retriever puppies (dogs 8 and 15 in Fig. 1B), 3 severely affected LRMD dogs from the tissue archives of the Comparative Neuromuscular Laboratory, a GRMD dog from the Brazil colony and a normal dog (Fig. 6C).

Fig. 4. Representative fluorescence immunostaining of serial cryosections from a mildly affected Labrador Retriever (dog 8 in Fig. 1B), a previously diagnosed severely affected Labrador Retriever and a control dog using the DRP2 antibody against utrophin and dMHC antibody to detect regenerating fibers. The sarcolemma of regenerating fibers was positively stained while the sarcolemma of non-degenerating fibers was unstained in both the mild and severe cases. Sarcolemmal staining of control muscle was not detected. Nuclei were highlighted with DAPI. Bar = 50 μm for all images.

Fig. 5. Dystrophin expression in the muscles of LRMD dogs (dogs 8 and 15 in Fig. 1B), an affected Golden retriever (GR) and control dog and human muscle was assessed by western blot. The dystrophin band of 427 kDa was completely absent in the LRMD and GR dogs, but was present in the dog and human control samples. Four different dystrophin antibodies were used to confirm the findings. Beta-actin was used as a loading control.

Fig. 6. LRMD dogs do not carry the same mutation as the GRMD dogs. (A) PCR genotyping using DNA from four of the affected Labrador retriever dogs (dogs 1, 5, 7 and 8 from Fig. 1B), and affected and carrier dogs from the GRMD colony in Brazil confirmed the LRMD dogs did not carry the GRMD mutation. (B) Amplification of dystrophin cDNA region flanking exon 7 shows normal size bands (994 bp) in both a mildly affected LRMD dog (8) and normal Labrador (Dog) including a negative water control (C). (C) Neither mildly affected nor severely affected LRMD dogs carry the previously reported dystrophin mutation since bands of expected size (460 bp) were observed. The absence of the 6363 bp insertion in the intron 19 of dystrophin gene was confirmed in samples from mildly affected LRMD dogs (8, 15) and severely affected LRMD dogs (S) as compared to a GRMD dog (GR) and normal dog (N).
Bands of expected size were observed in all samples, confirming the absence of the 6363 bp insertion.

4. Discussion

The identification of the dystrophin gene and its protein product provided an explanation for the difference in severity between DMD and its allelic form, Becker muscular dystrophy [16]. While DMD is caused by the absence of muscle dystrophin, the same protein is present but reduced in size or quantity in BMD. This led to the conclusion that there was a correlation between the amount of muscle dystrophin and the severity of the phenotype. Based on this conclusion, most therapeutic trials for DMD, such as cell or gene therapy [17], exon skipping [18,19] or the read through approach [PTC therapeutics [20]] aimed to increase dystrophin expression in an attempt to ameliorate the course of the disease. However, the quantity of dystrophin required to rescue the DMD phenotype and establish a functional muscle has been an unresolved question.

As in humans, dystrophin deficient muscular dystrophy is the most common form of muscular dystrophy in dogs [21] and mutations in the dystrophin gene have been identified for several breeds. In addition to the Golden Retriever [5,6], dystrophin mutations have been identified for the Rottweiler [22], German Short-haired Pointer [23], Cavalier King Charles Spaniel [24], Welsh Corgi [25] and Labrador Retriever [4]. The GRMD mutation was also passed to the Beagle breed by mating with GRMD dogs [26]. In contrast to mdx mice, which are almost asymptomatic despite the absence of muscle dystrophin, most dystrophin deficient dogs show a severe phenotype that is comparable to human DMD. Where a large enough number of dystrophic dogs have been identified of certain breeds, such as the Golden Retriever and Labrador Retriever, a spectrum of clinical severity has been identified with most dogs showing a severe myopathic phenotype, but exceptions can be noted with milder phenotypes [6,27].

One exceptional dog, Ringo, was identified in the colony of Brazilian dystrophic dogs. Ringo and one of his descendants, Sulfair, had a mild clinical course despite no muscle dystrophin. This observation led us to an investigation of a protective mechanism that could explain this mild phenotype and suggest a new therapeutic approach for DMD.

Here we report that LRMD dogs, without muscle dystrophin, may also be asymptomatic. Evidence that a muscle disease was present was only indicated on pre-surgical screening of bloodwork that showed a markedly and persistently elevated CK activity. Clinical examinations at this early time-point did not indicate a myopathic phenotype. The diagnosis of a dystrophic pathologic phenotype was made by biopsy at the time of neuter. In the following 4 years after the original diagnosis, clinical signs of mild muscle weakness did develop and included reluctance or inability to jump into a car or stand on the pelvic limbs. In this family of dogs with LRMD, the mild clinical phenotype and absence of dystrophin has been transmitted in at least 3 generations.

An interesting finding in both the mild and severely affected Labrador Retrievers in our study was the absence of the previously reported insertion between exons 19 and 20 [4] in the dystrophin gene in LRMD (Fig. 6C). As in humans with dystrophin deficiency, this finding suggests that more than one mutation in dystrophin affects the Labrador Retriever breed.

A striking finding was robust regeneration in the muscles of all dystrophin deficient asymptomatic Labradors at the time of muscle biopsy (Fig. 2B). A direct comparison of regeneration in muscle biopsy specimens of minimally affected Labrador Retrievers with that of severely affected LRMD dogs with dystrophin deficiency was not possible as different muscles were evaluated and at different time points. Expression of utrophin in regenerating fibers in CXLMD in Golden Retrievers has been previously described [28]. Here, utrophin expression in both the mild and severely affected Labrador Retrievers was limited to regenerating fibers and not expressed on control dog muscle (Fig. 4). A comparable finding was observed in the Brazilian dogs, where utrophin expression in the mildly affected Ringo and Sulfair did not differ from severely affected GRMD dogs [6].

Our observations in this Labrador Retriever family, the exceptional dogs from the Brazil GRMD colony [6] and rare DMD patients [11] confirm a paradigm-shift that large muscles can be partially functional without dystrophin. Interestingly, although immunostaining and western blot analysis confirmed the absence of muscle dystrophin in the LRMD dogs, we did not find any detectable deletion, duplication or small rearrangement in the dystrophin gene. Even though a specific mutation has not yet been identified, our findings lend further support to the hypothesis that, in exceptional situations, muscle with no dystrophin may be functional independently of the specific mutation or haplotype. Sequence analysis of the entire dystrophin cDNA and promotor is currently underway to search for point mutations or variants in the promoter region.

In other recent investigations from our group [29], Pelatti et al. 2014 unpublished] we found that human mesenchymal stem cells injected into GRMD dog muscles improved their clinical phenotype and resulted in an increased lifespan, despite the fact that in most experiments no dystrophin expression was found in the injected muscles. This finding also demonstrates that other factors may have a therapeutic effect protecting muscles without dystrophin. In short, the observation that a large animal such as the Golden Retriever or Labrador Retriever dogs without muscle dystrophin may be almost asymptomatic opens new avenues for research. Finding the secrets that protect these dogs is a great challenge which may have important implications for future treatment.

4.1. Conclusions

Our observations in this Labrador Retriever family, the exceptional dogs from the Brazil GRMD colony [6] and rare DMD patients [11] confirm a paradigm-shift that large muscles can be partially functional without dystrophin. Searching for modifying factors that protect these dogs and patients from the deleterious effects of dystrophin deficiency opens new avenues for research and may have important implications for treating this devastating condition.

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Authors’ contributions

NMV performed all molecular studies, western blotting and evaluated affected dogs. LTG conducted the pathological and immunohistochemical studies and GDS interpreted the results and determined the clinicopathological diagnosis. EE performed the pedigree analysis. GDS, LMK, MZ, LMK and GDS interpreted the data and drafted the manuscript. All authors read and approved the final manuscript.

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Abbreviations

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<td>CK</td>
<td>creatine kinase</td>
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<td>GRMD</td>
<td>Golden retriever muscular dystrophy</td>
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<td>LRMD</td>
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