

Fig. 3. Origin, proliferative activity, and Berp1 expression of subpopulations of muscle SP cells. (A,B) C57BL/6 mice were transplanted with whole BM from GFP Tg mice, and 3 months later, SP cells from untreated muscle (A) or regenerating muscle (3 days after CTX injection) (B) were further analyzed for CD31, CD45, and GFP expression. Note that CD45⁺ SP cells (middle and lower panels) are almost all negative for GFP, indicating that they do not originate from BM. In contrast, more than 90% of muSP-45 cells were GFP⁺ (upper panels). (C–H) Ki67 expression (green) and nuclei stained with DAPI (blue) on muSP-45 (C,D), muSP-DN (E,F), and muSP-31 (G,H) cells. The percentages of Ki67-positive cells were expressed as means \pm SD of three independent experiments. muSP-45 (I), muSP-DN (J), and muSP-31 (K) were sorted from regenerating muscle and stained for Berp1 (green) and nuclei (blue). Only muSP-31 cells were stained positive for Berp1 (K). Bar: 50 μ m.

myoblasts derived from satellite cells. muSP-DN cells from untreated muscle rapidly proliferated in vitro as observed in regenerating muscle (Fig. 2C). On the contrary, muSP-31 cells hardly expanded. After 2–3 weeks co-culture, both muSP-DN cells and muSP-31 cells differentiated not only into multinucleated myotubes co-expressing GFP and sarcomeric- α -actinin (Figs. 5E–G, only muSP-DN culture is shown) but also mononucleated myocytes (shown in insets). The frequency of mononucleated

myocytes was too low to quantify, but existence of these cells suggests that myogenic differentiation of SP cells could occur without fusion. Strikingly, the myotube-forming activity (the frequency of GFP⁺ myotubes, see Materials and methods for details) of muSP-DN cells was approximately 10-fold that of muSP-31 cells (Fig. 5H, lane for cont. 0.026 ± 0.007 vs 0.002 ± 0.001). In the experiments using SP cells from regenerating muscle at 3 days after CTX injection, muSP-DN cells showed the highest

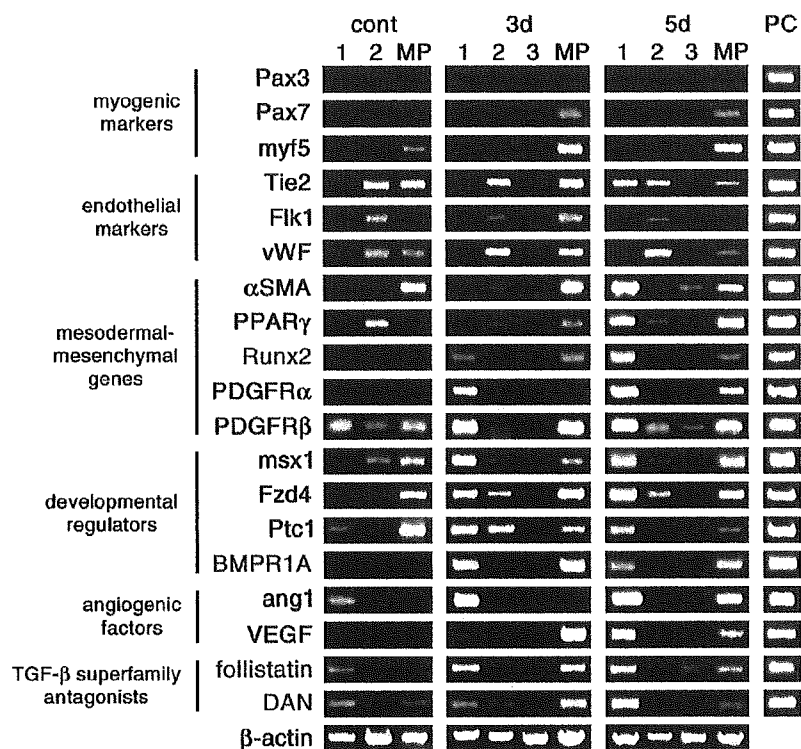


Fig. 4. Gene expression profiles of muscle SP cells during muscle regeneration. muSP-DN (lane 1), muSP-31 (lane 2), muSP-45 (lane 3), or MP cells were collected from untreated (cont) and regenerating muscle at 3 days (3d) or 5 days (5d) after CTX injection, and RT-PCR was performed against the indicated genes. Total embryo extract (E13) was used as a positive control (PC). β -actin was amplified to confirm that the quantities of mRNA were equal.

myotube-forming activity, although each SP subpopulation did form myotubes co-expressing GFP and sarcomeric- α -actinin (Fig. 5H, lane for CTX3d). This clearly demonstrates that muSP-DN cells have the highest myogenic potential among SP sub-fractions *in vitro*. For comparison, we quantified the myotube-forming activity of satellite cell-derived myoblasts. The value was 0.09 ± 0.01 , indicating that myogenic activity of myoblasts is much higher than that of muSP-DN cells.

Myogenic potential of muscle SP cells *in vivo*

To evaluate the myogenic potential of muscle SP cells *in vivo*, we performed transplantation experiments. muSP-DN or muSP-31 cells from untreated muscle of GFP Tg mice were directly transplanted into CTX-treated TA muscles of immunodeficient NOD/*scid* mice. Three weeks after transplantation, muSP-DN cells had generated myofibers more efficiently than muSP-31 cells (Figs. 6A and B, and Table 1), indicating that muSP-DN cells had relatively higher myogenic potential *in vivo* as well as *in vitro*. Contrary to our expectation, muSP-DN cells formed no GFP-positive adipocytes after transplantation.

Discussion

Muscle SP cells have been suggested to be multipotent and can contribute to skeletal muscle regeneration

[4,9,10,23]. However, most of these studies dealt with whole muscle SP cells as one functional unit. We subdivided, for the first time, muscle SP cells using CD31 and CD45 markers and revealed functional heterogeneity of muscle SP cells. CD31⁺CD45⁻ SP cells (muSP-31 cells) are a main subpopulation in non-regenerating muscle, but CD31⁻CD45⁻ SP cells (muSP-DN cells) which represent a minor subpopulation in non-regenerating muscle have the greatest differentiation potentials and become predominant subpopulation of SP cells upon muscle injury.

Differentiation potential of muscle SP cells

Phenotypic and immunohistochemical analysis suggested that muSP-31 cells are a subset of endothelial cells of capillaries and veins. They poorly proliferate after injury or *in vitro* culture, and their differentiation potentials are limited both *in vitro* and *in vivo*.

CD45⁺ muscle SP cells (muSP-45 cells) were shown to have both hematopoietic and myogenic potentials, and hematopoietic potential of muscle-derived cells was exclusively found in this fraction [8,9]. We previously reported the contribution of muSP-45 cells to muscle regeneration [14]. In this study, we identified novel subpopulation that possesses much higher myogenic potential than muSP-45, muSP-DN.

muSP-DN cells showed the highest differentiation potential of all the mesenchymal lineages tested among

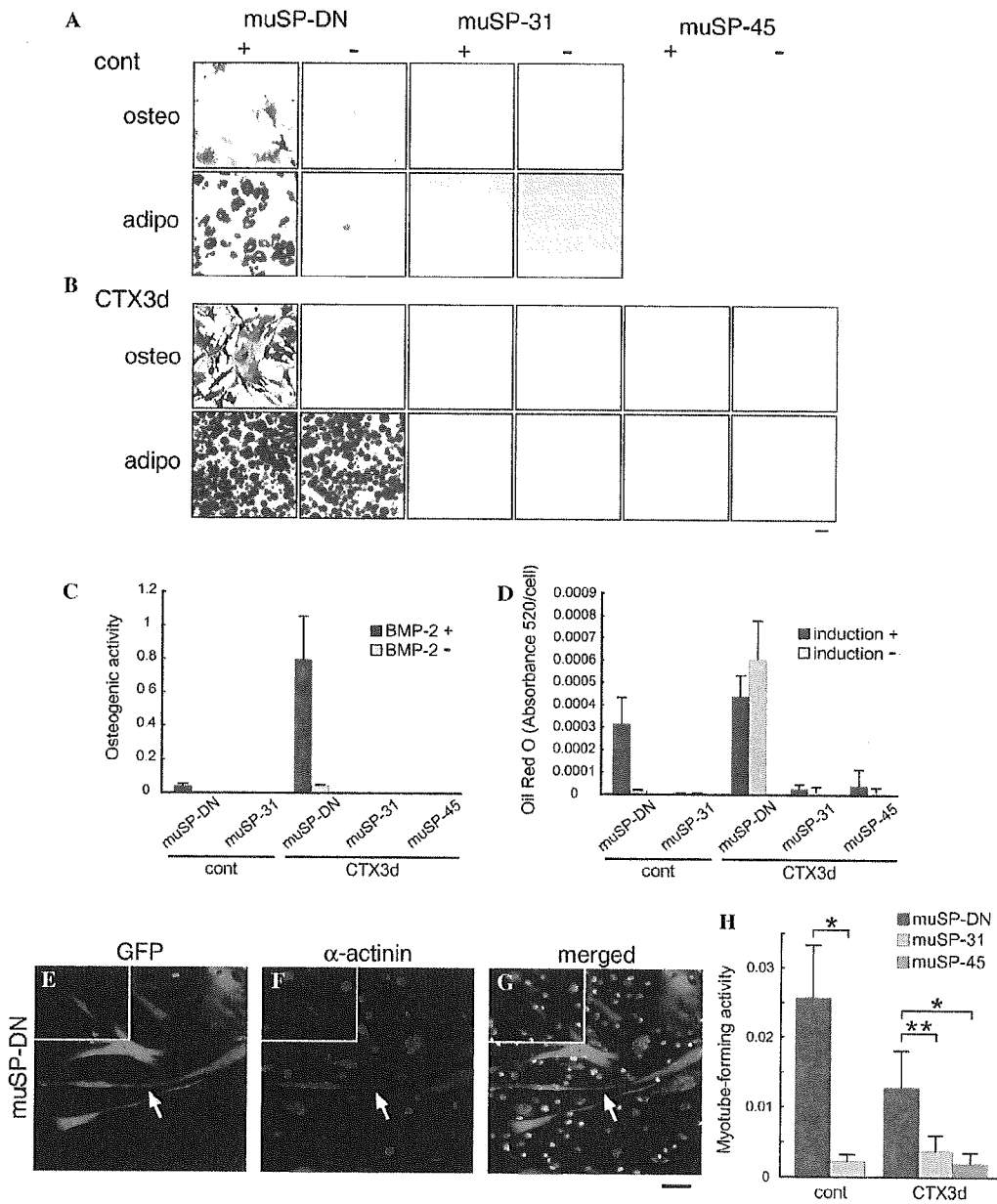


Fig. 5. muSP-DN cells differentiate into osteogenic cells, adipocytes, and skeletal muscle cells. (A,B) Three subpopulations of SP cells prepared from untreated (A) or regenerating (B) muscle were induced to differentiate into osteogenic or adipogenic cells. Uninduced cells (–) and induced cells (+) were then examined for alkaline phosphatase expression (osteo) or oil deposits (adipo). Bar: 50 μ m. (C,D) Osteogenic (C) and adipogenic (D) activities of subsets of SP cells prepared from control (cont) or regenerating muscle at 3 days after CTX injection (CTX3d) were quantified. Values are the average of three independent experiments. Error bars represent SD. (E–G) Co-culture of muscle SP cells with myoblasts. muSP-DN cells from GFP Tg mice were sorted and co-cultured with WT primary myoblasts in differentiation medium. Cells were stained with anti-GFP (green) and anti-sarcomeric α -actinin (red) antibodies. Nuclear staining with DAPI (blue) is shown in merged images (G). Insets show GFP⁺ mononucleated myocyte. Bar: 50 μ m. (H) Myotube-forming activities of muSP-DN cells (red bars), muSP-31 cells (blue bars), and muSP-45 cells (green bar) are shown. Each subpopulation was prepared from untreated (cont) or CTX-treated regenerating muscle (CTX3d). Values are the average of three independent experiments. Error bars represent SD. * $P < 0.01$, ** $P < 0.05$.

SP subpopulations. They were negative for lineage-specific markers under the non-regenerating condition, but after muscle injury or in *in vitro* expansion, they actively proliferated and were readily induced to express several mesenchymal genes. Their differentiation potential seems to be restricted to mesenchymal lineages because we did not

detect hematopoietic colonies derived from muSP-DN cells *in vitro* and muSP-DN cells failed to rescue the lethally irradiated mice (data not shown). These observations indicate that muSP-DN cells are enriched for primitive mesenchymal cells. This notion is further supported by gene expression pattern of muSP-DN cells. muSP-DN cells

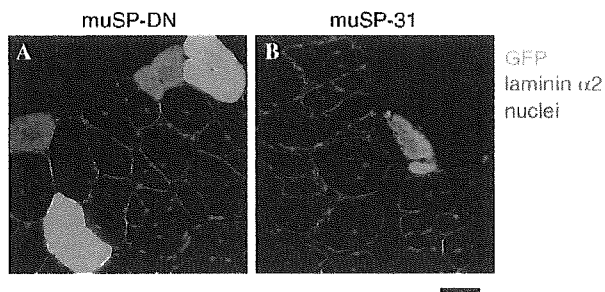


Fig. 6. muSP-DN cells participate in myofiber formation during muscle regeneration. (A,B) muSP-DN (A), muSP-31 (B) were transplanted into CTX-injected NOD/*scid* muscles. Each subpopulation was prepared from untreated muscle of GFP Tg mice. Muscle sections were stained with anti-GFP (green) and anti-laminin $\alpha 2$ (red) antibodies 3 weeks after transplantation. More GFP-positive myofibers were detected in muSP-DN-transplanted muscles (A) than in muscles transplanted with muSP-31 cells (B). Bar: 40 μ m.

specifically expressed *ang1* under the non-regenerating condition and during the early phase of regeneration (Fig. 4, lane 1, cont or 3d). Perivascular cells, such as pericytes, express *ang1* [24,25], and several groups suggest that multipotent mesenchymal stem cells may be derived from pericytes [26–28]. A recent report demonstrated that vascular mural precursor cells are negative for endothelial markers but positive for Tie2 and smooth muscle cell markers [29]. Likewise, muSP-DN cells were negative for *Flk1* and *vWF* throughout the regeneration process (Fig. 4, lane 1), but began to express *Tie2* and *α SMA* during late phases of regeneration (Fig. 4, lane 1, 5d). Given the similarity between muSP-DN cells and those reported perivascular primitive cells, muSP-DN cells would represent perivascular primitive mesenchymal cells in skeletal muscle.

Roles of muscle SP cells in muscle regeneration

muSP-DN cells actively proliferated and significantly increased in number upon muscle injury. The precise fate of muSP-DN cells has remained to be determined, since the number of muSP-DN cells returned to normal level at late stage of muscle regeneration.

We noted that angiogenic factors and TGF- β superfamily antagonists were strongly expressed in muSP-DN cells during muscle regeneration. Previous reports showed that *Ptc1*⁺ interstitial mesenchymal cells in muscle produce angiogenic factors, including *ang1*, and promote muscle regeneration after ischemia [30,31]. Some members of the TGF- β superfamily, such as myostatin and TGF- β 1, are known to act as negative regulators of myogenesis [32,33]. Inversely, one of the TGF- β superfamily antagonists, follistatin, has been reported to promote myoblast recruitment and fusion [34]. Therefore, muSP-DN cells might promote muscle regeneration by producing regeneration-regulating factors.

muSP-DN cells preferentially differentiate into myogenic cells after intramuscular transplantation, implying that normal muscle environment facilitates myogenic differenti-

ation of muSP-DN cells. However, we revealed that muSP-DN cells have a high tendency to differentiate into osteogenic or adipogenic cells in vitro. Therefore, it is possible that muSP-DN cells differentiate into osteogenic or adipogenic cells in some pathological conditions such as Duchenne muscular dystrophy [35,36]. Recent finding that microvascular pericytes can differentiate into adipocytes [37] further supports the notion that muSP-DN cells might be implicated in pathological changes.

In conclusion, we identified novel subpopulation of muscle SP cells, CD31⁻CD45⁻ SP cells, which possesses capacity of mesenchymal differentiation in vitro and reveals myogenic differentiation potential in vivo. Our findings might provide new insights that may well be useful in understanding adult skeletal muscle regeneration and in designing therapeutic strategies of muscular dystrophy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.01.037.

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ORIGINAL ARTICLES

Intracellular Localization of Dysferlin and its Association with the Dihydropyridine Receptor

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Mutations in the *dysferlin* gene underlie two phenotypically distinct muscular dystrophies: Miyoshi myopathy and limb-girdle muscular dystrophy 2B. Dysferlin was proposed to have a putative functional role in mediating the fusion of intracellular vesicles to the sarcolemma during injury-induced membrane repair; but dysferlin has been found not only at the sarcolemma but also within the cytoplasm of skeletal muscle fibers by immunohistochemistry. In this study, we examined the subcellular localization of dysferlin in skeletal muscle by immunohistochemical and biochemical analyses to elucidate other functional roles of dysferlin. Immunohistochemistry confirmed granular cytoplasmic expression pattern of dysferlin in muscle fibers. Subcellular membrane fractionation revealed that a portion of dysferlin associated with a T-tubule-enriched intracellular membrane fraction as well as a sarcolemmal fraction. This indication was consistent with subsequent results that dysferlin coprecipitates by immunoprecipitation with the dihydropyridine receptor (DHPR), a protein complex localized in T-tubules. Moreover, both proteins were observed to partially colocalize by double immunofluorescent labeling in skeletal muscle fibers. We also found that caveolin-3, previously shown to interact with dysferlin, coprecipitates with DHPR. These results demonstrated that dysferlin may be involved in the formation of an oligomeric complex with DHPR and caveolin-3. Caveolin-3 has been also reported to participate in an insulin-regulated transport mechanism in muscle, and caveolin-3-containing vesicles might traffic between intracellular sites and target sites on the sarcolemma and T-tubules. Therefore, it is very intriguing to assume that dysferlin might be involved in the fusion of caveolin-3-containing vesicles with T-tubules.

Key Words: dysferlin, T-tubules, caveolin-3

Introduction

Two phenotypically distinct forms of muscular dystrophy with autosomal recessive inheritance, Miyoshi myopathy and limb girdle muscular dystrophy 2B (LGMD 2B) are reported to be caused

by genetic mutations in the *dysferlin* gene (*DYSF*). Distal or proximal muscles are known to be predominantly involved in Miyoshi myopathy or in LGMD 2B, respectively [1,2]. Both diseases are clinically characterized by a late onset and slow progression of symptoms, as well as elevated serum creatine kinase levels [3]. *DYSF* was identified through positional cloning and mapped to the 2p13 gene locus [4,5]. It consists of over 55 exons transcribed into an 8.5 kb major transcript predominantly expressed in skeletal muscle [4,5].

The functional role of dysferlin in muscle physiology and pathology has been the subject of extensive research over the years. Studies of its cDNA sequence predict a protein of approximately 230 kDa, typically a type II transmembrane protein. Dysferlin contains sequences of calcium-binding C2 domains. These are motifs found in a number of proteins involved in signal transduction and membrane trafficking [6] such as the calcium-sensing synaptotagmins [7] and protein kinase C [8]. In addition, dysferlin has recently been identified as a member of a unique family of proteins: the ferlins. These proteins, which include myoferlin [9] and otoferlin [10], have as an important functional characteristic a high sequence homology to the *C. elegans* fer-1 protein, reported to play a critical role in the fusion of intracellular vesicles with the plasma membrane in sperm cells [11]. This has suggested a putative role for dysferlin in mediating the fusion of intracellular vesicles with the sarcolemma of skeletal muscle fibers in a calcium-dependent manner. Indeed, dysferlin knockout mice developed a slowly progressive muscular dystrophy due to an inability of intracellular vesicles

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to fuse with the sarcolemma during injury-induced membrane repair [12]. The fusion process has also been suggested to involve an interaction between dysferlin and the annexins; calcium and phospholipid-binding proteins implicated in membrane repair [13]. The presence of dysferlin has been shown not only at the sarcolemma [12-16] but also within the cytoplasm [12,16] of skeletal muscle fibers by immunohistochemistry and it has been proposed that dysferlin present on the intracellular vesicles is involved in the injury-induced fusion process [17].

In the present study, we systematically examined the localization of dysferlin in the muscle fiber by immunohistochemical and biochemical analyses in order to characterize the intracellular localization of dysferlin. Our immunohistochemical results confirmed the presence of dysferlin at the sarcolemma and within the cytoplasm of muscle fibers. In membrane fractionation experiments, dysferlin was shown to be associated with a T-tubule-enriched intracellular membrane fraction in addition to a sarcolemmal fraction. This association with a T-tubule-enriched membrane fraction was consistent with subsequent results shown by immunoprecipitation experiments that dysferlin coprecipitates with the dihydropyridine receptor (DHPR), a protein complex localized in T-tubules. We also found that caveolin-3, previously shown to interact with dysferlin, coprecipitates with DHPR by immunoprecipitation.

Materials and methods

Animals

Wistar rats and mice (Balb/c and SJL) were purchased from Clea Japan Inc. (Tokyo, Japan) and Charles River Japan Inc. (Tokyo, Japan) respectively. The SJL mouse is a naturally occurring animal model of dysferlin deficiency caused by an inframe deletion mutation in the *dysferlin* gene. It has been shown that expression of truncated dysferlin is greatly reduced [18,19] in skeletal muscles of this animal model. All animal handling procedures were performed in accordance with protocols approved by the National Institute of Neuroscience, NCNP, Kodaira, Japan.

Antibodies

For immunoprecipitation studies, a polyclonal antibody against dysferlin was raised by immunizing rabbits with a polypeptide corresponding to the

amino acid sequence of human dysferlin 1999 to 2016 conjugated with keyhole limpet hemocyanin, and emulsified with the adjuvant TiterMax Gold (CytRx Corp., Atlanta, GA). The antiserum was affinity-purified by absorption onto a polypeptide coupled to thiopropyl-Sepharose 6B (Amersham Biosciences Corp., Piscataway, NJ) followed by elution with 4 M MgCl₂. In immunoblot analysis, the antibody specifically reacted with dysferlin in microsomes of mouse skeletal muscle (data not shown). Monoclonal antibodies against dysferlin (NCL-Hamlet) and dystrophin (NCL-DYS2) were purchased from Novocastra Laboratories Ltd Newcastle, UK. Polyclonal antibody against α -actinin was obtained as described [20]. Antibodies against triadin, DHPR (α 2 subunit), ryanodine receptor and sarco/endoplasmic reticulum calcium ATPase1 (SERCA1) were obtained from Affinity Bioreagents, Inc. (Golden, CO). Antibodies against NaK-ATPase α 1 and DHPR subunit α 1 were obtained from Upstate Biotech (Lake Placid, NY). Monoclonal antibodies against annexin A2 and caveolin-3 were from BD Transduction Labs. (San Jose, CA). Anti-NSF antibody was from ICN Biomedicals, Inc. (Aurora, OH). Anti-desmin antibody was from Progen Biotechnik GMBH (Heidelberg, Germany). Goat polyclonal antibodies against DHPR α 1, SERCA1 and caveolin-3 were from Santa Cruz Biotech., Inc. (Santa Cruz, CA). The Alexa-conjugated secondary antibodies were purchased from Molecular Probes Inc. (Eugene, OR).

Immunohistochemical analysis

Animals were killed by CO₂ inhalation followed by cervical dislocation. Tibialis anterior (TA) muscles were rapidly dissected out and snap-frozen in liquid nitrogen-cooled isopentane. 6 μ m thick cryostat sections were fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, at 4°C for 2 min, dehydrated in 70% ethanol at 4°C for 10 min and then permeated with 0.1% triton X-100 in PBS at room temperature for 10 min. Sections were washed and incubated for 25 s with proteinase K solution (550 U/ml, Wako Pure Chemical Industries Ltd, Osaka, Japan) at a dilution of 1:300 in TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. After washing, sections were incubated in primary antibody diluted in 2% casein in PBS at 4°C overnight. For double immunostaining, sections were incubated overnight in a mixture of the relevant primary antibodies diluted in 2% casein in PBS. After primary

antibody incubation, sections were washed three times for 5 min with PBS and then incubated for 1 h with the relevant secondary antibodies. After washing, sections were mounted in VectaShield® mounting medium (Vector Labs, Burlingame, CA) and coverslipped. Fluorescent images were captured by confocal-laser scanning microscope (Leica TCS SP, Leica, Heidelberg, Germany)

Subcellular membrane fractionations

Two different subcellular membrane fractionation protocols were used in the present study. The first protocol used (Fig 2A) was a slight modification of that previously reported by Dombrowski *et al* [21]. Briefly, muscles (~1 g) were excised from the hindlimbs of mice, cut into small pieces with scissors and frozen in liquid nitrogen. The frozen muscles were pulverized by the use of Cryo-Press chilled with liquid nitrogen and homogenized at 4°C with Polytron PT3000 equipped with a DA 3012/2S probe (Kinematica, AG, Littau Lucerne, Switzerland) at 16,000 rpm for 10 s. The original homogenizing buffer was changed to buffer A containing 20 mM sodium pyrophosphate, 20 mM sodium phosphate, 0.25 M sucrose, 0.5 mM O, O'-bis(2-aminoethyl)ethyleneglycol-N, N, N', N'-tetraacetic acid and 1 mM MgCl₂ (pH 7.1) in order to decrease non-specific aggregation. Discontinuous sucrose density gradient centrifugation experiments were omitted in this study.

In the second protocol (Fig 3A), sarcolemma was purified by the method of Munoz *et al* [22]. Homogenization steps were slightly modified as follows. Freshly dissected mouse hindlimb muscles (~6 g) were homogenized twice with a Polytron homogenizer (PT3000) at 4°C, 13,000 rpm for 20 s and centrifuged. The pellet was resuspended and again homogenized twice with the Polytron homogenizer at 17,000 rpm for 30 s. After centrifugation, the two supernatants were pooled and the remaining procedures were carried out in accordance with Munoz *et al*. Protease inhibitor cocktail, complete EDTA free (Roche Diagnostics GmbH, Mannheim, Germany) was included in all buffers used. All other reagents were purchased from Wako Pure Chemical.

Fractionation of microsomal proteins

Fractionation of digitonin-solubilized microsomes from rabbit skeletal muscle by wheat-germ agglutinin (WGA) gel chromatography was performed as previously described [23].

Immunoprecipitation

Besides anti-dysferlin antibody prepared in this study, anti-DHPR α 1 (Upstate Biotech), anti-DHPR α 2 and anti-caveolin-3 (Santa Cruz) antibodies were used for immunoprecipitation. Each IgG was coupled with the gel by use of Affi-Gel Hz immunoaffinity kit (Bio-Rad Labs, Hercules, CA). Prepared gels were washed once before use with the elution buffer: 0.15 M glycine-HCl, pH 2.5 and 0.1% digitonin before use. The WGA-bound microsomal proteins were mixed with approximately 50 μ l of the gel equilibrated with the binding buffer: 0.15 M NaCl, 20 mM Hepes-NaOH, 0.1% digitonin, protease inhibitor cocktail Complete EDTA-free (Roche Diagnostics GmbH) in a MicroSpin column (Amersham Biosciences) and incubated overnight at 4°C with gentle agitation. The gel in the column was then washed many times with the binding buffer and eluted with the elution buffer. Washing and eluting procedures were performed by gravity drip from the column. The eluted proteins were neutralized with 2 M Tris-HCl, pH 9.0 and ultrafiltrated close to dehydration with Microcon 30 (Millipore Corp., Bedford, MA). The proteins were extracted from the membrane with the buffer containing 1% SDS, 1 mM TCEP (Pierce Biotechnology Inc., Rockford, IL) and 22 mM Tris-HCl (pH 6.7), and concentrated to one-third volume with a centrifugation evaporator.

Protein concentration was determined by Protein Assay Stain (Bio-Rad) or Coomassie Plus-200 Protein Assay Reagent (Pierce Biotechnology) using either BSA or γ -globulin as a standard.

Electrophoresis and Immunoblot Analysis

In SDS-PAGE, proteins were run on 5-10% or 5-15% precast gel (DRC, Tokyo, Japan). Subsequent transfer from the gel on to PVDF membranes (Millipore) was performed for 1 h at a current of 5 mA/cm² with a buffer similar to that of Kyhse-Anderson [24]. Following transfer, membranes were blocked with PBS including each 0.1% of casein and gelatin for 1 h at room temperature, and then incubated with primary antibodies followed by the respective horse-radish-peroxidase (HRP)-labeled secondary antibodies. Molecular masses were estimated with Biotinylated Standards kit (Bio-Rad) and HRP-labeled avidin. Bound antibodies were detected by chemiluminescence with Supersignal West Dura Extended Duration Substrate (Pierce Biotechnology). Signals were visualized with Luminoimager (Roche Diagnostics). Blot overlay assay for the

WGA-binding proteins was performed in accordance with the method of Yoshida and Ozawa [23]. Bound biotinyl WGA was detected with a combination of an RTU Vectastain Elite ABC reagent and a VIP-staining kit (Vector Labs).

Results

Immunohistochemical analysis of dysferlin in skeletal muscle fibers

We first analyzed the localization of dysferlin in skeletal muscles of Wistar rats and Balb/c mice by confocal laser-scanning microscopy. In both transverse and longitudinal cryosections, a mouse monoclonal antibody against dysferlin stained not only the sarcolemma but also the cytoplasm of muscle fibers (Fig. 1). In transverse sections, the cytoplasmic staining was observed as a granular staining pattern that appeared intense in some fibers. This staining pattern is similar to that previously reported [12]. In longitudinal sections, cytoplasmic localization was observed as cross-striated double bands under high magnification. We found no correlation between the intense cytoplasmic staining observed in some fibers and fiber type distribution (data not shown). We also examined the localization of dysferlin in skeletal muscles of SJL mice, which have an inframe deletion in the *dysferlin* gene. Dysferlin staining was significantly reduced from the sarcolemma and cytoplasm in both transverse and longitudinal sections of SJL mice skeletal muscle (Fig. 1). To investigate the specificity of the fluorescence signals observed, the antibody was also incubated with a polypeptide corresponding to its epitope. This resulted in a loss of any signal in the muscle sections (data not shown). These results indicate that dysferlin is localized not only on the sarcolemma but also within the cytoplasm of muscle fibers.

Subcellular distribution of dysferlin

Next, we examined the localization of dysferlin within muscle fibers by biochemical fractionation, using a previously established procedure [21] (Fig. 2A). This protocol allowed for the isolation of three distinct membrane fractions; a crude microsomal fraction enriched in sarcolemmal membranes (F2), and two internal membrane fractions: PF4 enriched in T-tubule and SR membranes, and PF8 an intracellular pool for glucose transporter4 (Glut4).

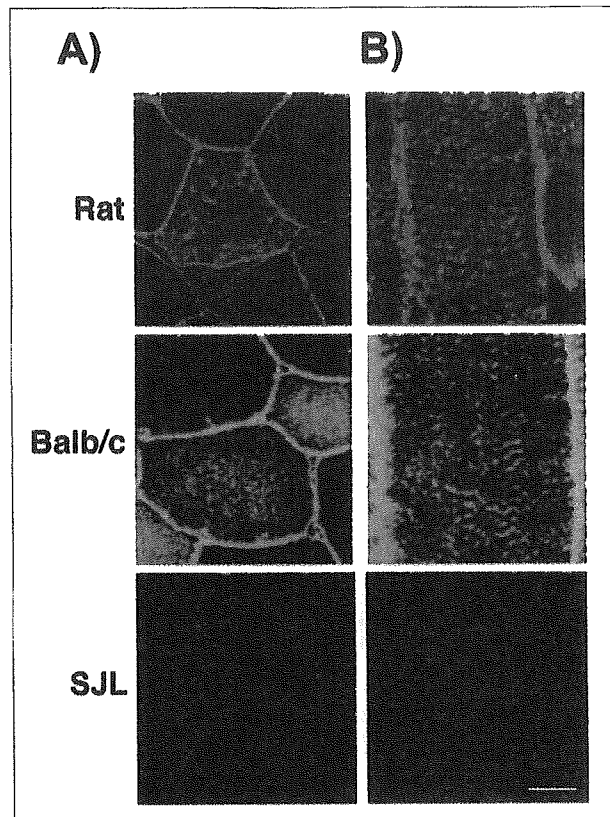


Fig. 1. Immunohistochemical analysis of dysferlin in skeletal muscle fibers. A) and B), immunofluorescent images of transverse and longitudinal sections (6 μ m), respectively, of the tibialis anterior (TA) muscles of Wistar rats, Balb/c and SJL mice stained with monoclonal antibody against dysferlin. Scale bar, 20 μ m.

Fractions obtained from Balb/c and SJL mice were analyzed for dysferlin and a number of other protein markers (Fig. 2B). NaK-ATPase, a sarcolemmal marker, was detected only in the F2 fraction. This fraction also included annexin A2 [13] and caveolin-3 [25], two proteins reported to interact with dysferlin at the sarcolemma. On the other hand, the PF4 fraction contained the intracellular membrane proteins DHPR, SERCA1, triadin and the ryanodine receptor, although some amounts of these were also detected in the F2 fraction. As shown in Fig. 2B, dysferlin was detected not only in the sarcolemma-enriched F2 fraction, but also in the PF4 fraction. Furthermore, the intensity of the dysferlin signal was found to be similar between F2 and PF4. This result shows that a substantial amount of dysferlin distinctly associates with non-sarcolemmal membranes.

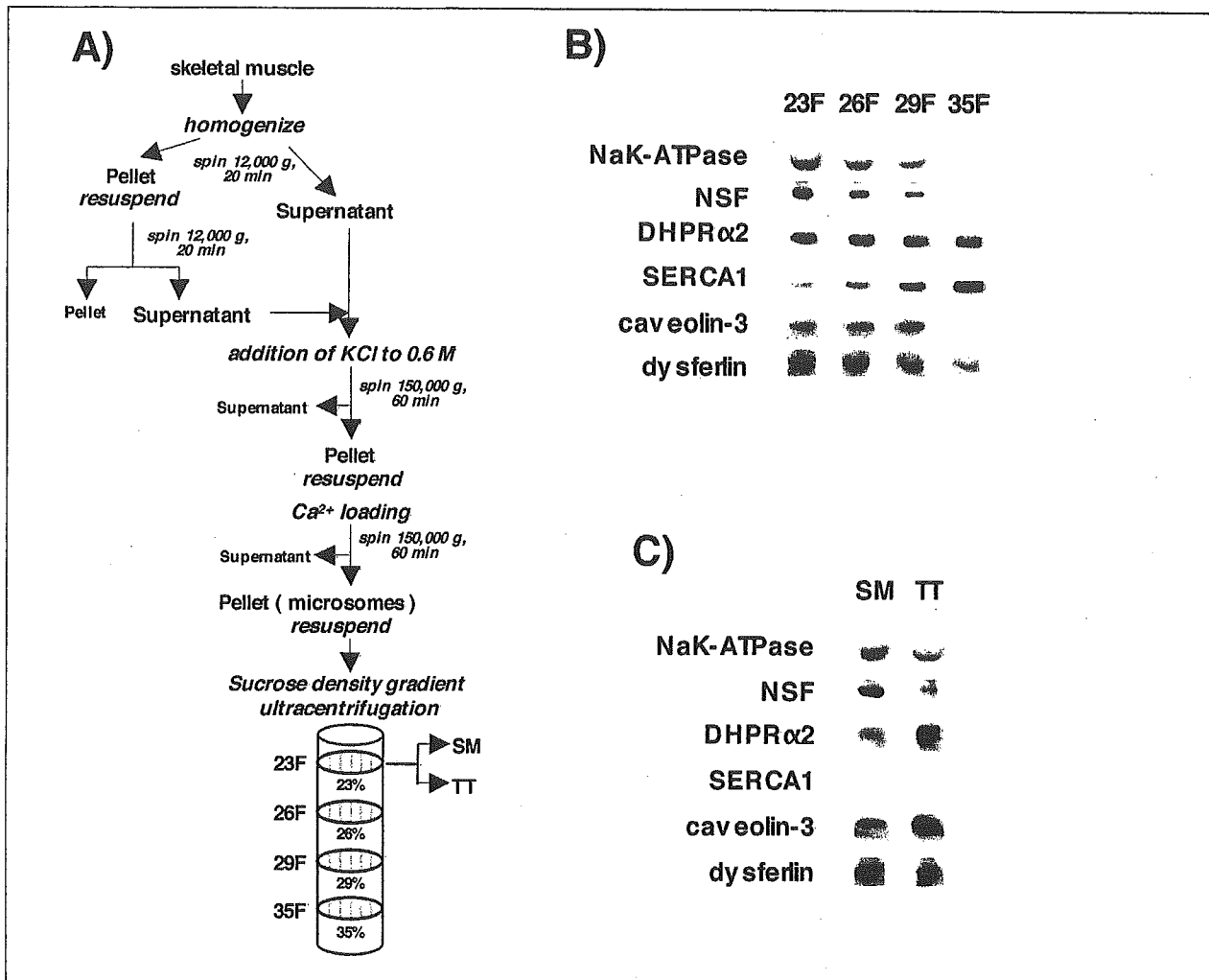


Fig. 3. Close association of dysferlin with T-tubules. A) Schematic representation of microsomal membrane fractionation protocol [22]. 23F; light microsomal fraction enriched in sarcolemmal and T-tubule membranes, 26F and 29F; fractions enriched in Glut4-containing intracellular vesicles, 35F; an SR-enriched fraction. SM and TT; the subfractions of 23F that were agglutinated and non-agglutinated with WGA, respectively. B) Immunoblot analysis of the four microsomal fractions with various antibodies. Membrane proteins in each fraction were loaded on the respective lanes in equal amounts, with the amounts changing depending on the antibody used (0.3 to 6 μ g). Dysferlin was detected in 23F and 26F, and to a lesser extent in 29F. C) Immunoblot analysis of SM and TT fractions with various antibodies. The amount of dysferlin was similar between both fractions.

collemmal fraction (SM) from a T-tubule-enriched membrane fraction (TT) [22]. This is based on the principle that WGA receptors present on T-tubules cannot interact with WGA, because of the inside-out orientation of the fragmented T-tubular membrane. As shown in Fig. 3C, NaK-ATPase and NSF were mainly detected in the SM fraction, while DHPR was mostly detected in the TT fraction. SERCA1 was notably absent from both fractions. Interestingly, dysferlin was recovered in similar amounts in both SM and TT, demonstrating that

in addition to sarcolemmal localization, dysferlin closely associates with T-tubule membranes. Caveolin-3 was also detected in both fractions but comparatively more in TT.

Interaction of dysferlin with DHPR

To date, dysferlin has been reported to interact with annexins A1 & A2 [13], caveolin-3 [25] and affixin [27]. The presence of dysferlin in T-tubule-enriched intracellular membrane fractions suggests the possibility of other dysferlin-interacting proteins.

To investigate this possibility, we solubilized crude microsomes with digitonin and fractionated them using WGA gel affinity chromatography as depicted in Fig. 4A. As expected, dystrophin [28] and DHPR [29] were detected in the bound fraction, and proteins such as SERCA1 and NSF were detected in the flow-through fraction (Fig. 4B). Interestingly, dysferlin was detected in both fractions, indicating that a portion of dysferlin was bound to WGA. Since the amino acid sequence of dysferlin precludes it as a glycoprotein [4,5], this finding suggested to us that a portion of dysferlin may bind to WGA probably through an association with WGA-binding protein(s). Caveolin-3 was also detected in both fractions.

To find out which WGA-binding protein(s) may associate with dysferlin, we immunoprecipitated dysferlin from the bound fraction and examined the pre-

cipitate for the presence of WGA-binding protein(s). Since the anti-dysferlin monoclonal antibody hitherto used was not suitable for immunoprecipitation, we raised an anti-dysferlin polyclonal antibody in rabbits as described under materials and methods. This polyclonal antibody specifically reacted with dysferlin in skeletal muscle membranes of mice (data not shown). Using this antibody, an immunoprecipitate was prepared from the WGA-bound fraction and analyzed by blot overlay assay with biotinyl WGA. As shown in Fig. 4C, positive bands were detected at approximately the 80-, 100- and 140-kDa positions. We predicted these to be putative dysferlin-interacting proteins.

DHPR, shown to bind to the WGA gel (Fig. 4B), is a protein complex composed of four subunits; $\alpha 1$, $\alpha 2$, β and γ . It reportedly binds to the gel through its

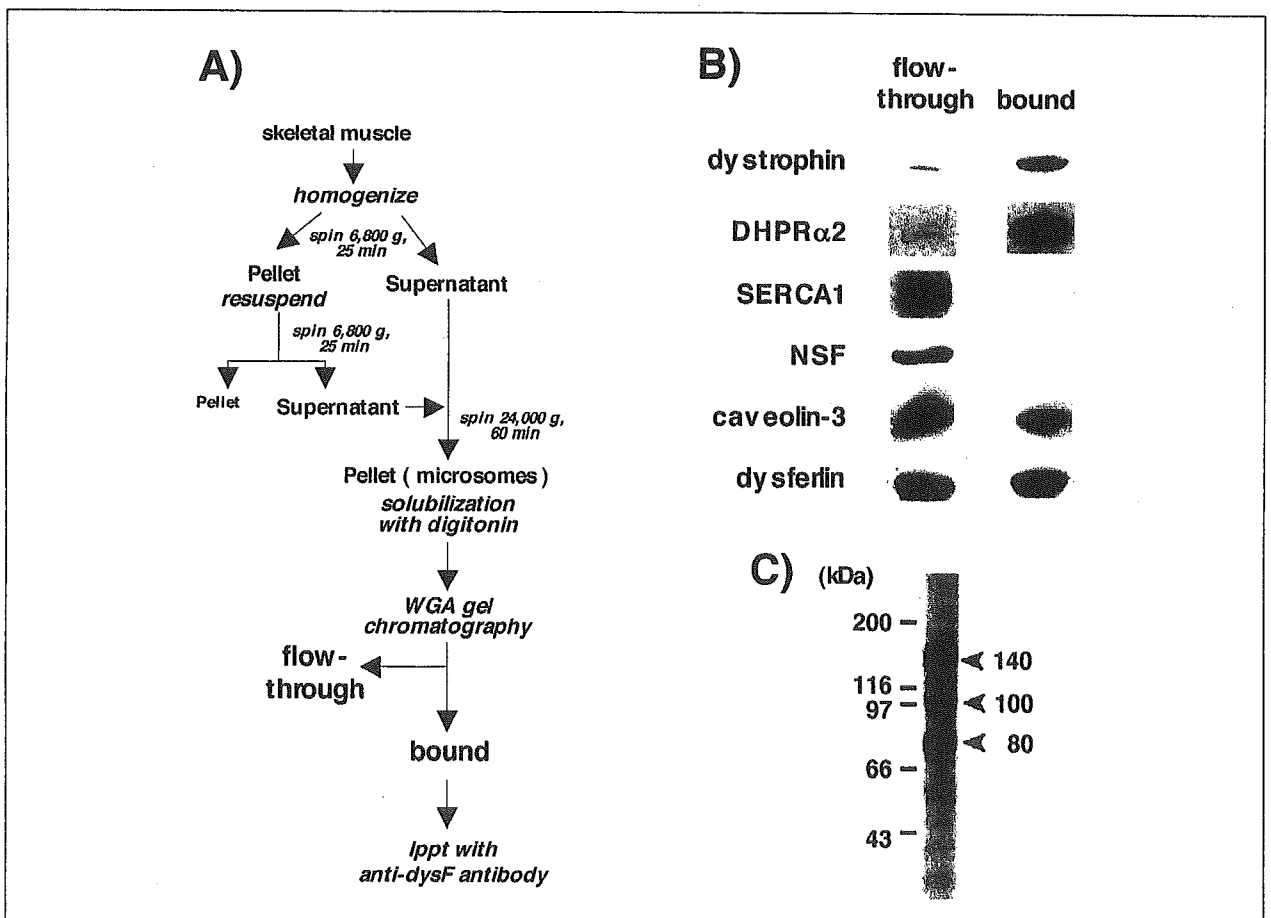


Fig. 4. Investigation of dysferlin-binding proteins. A) Schematic representation of crude microsome preparation and WGA gel chromatography of digitonin-solubilized microsomal proteins. B) Immunoblot analysis of the flow-through and bound fractions with various antibodies. The volume of both fractions was adjusted and aliquots loaded onto the gel. C) Blot overlay assay with biotinyl WGA of the immunoprecipitate prepared from the WGA-bound fraction with anti-dysferlin polyclonal antibody. Proteins of approximate molecular masses 140, 100 and 80 kDa were shown to react with WGA.

$\alpha 2$ subunit [29]. Its molecular mass of approximately 140 kDa under reduced conditions [29] is consistent with that of the largest dysferlin-interacting protein we detected (Fig. 4C). To test the possibility that dysferlin may associate with the WGA gel through an association with DHPR, we performed a series of immunoprecipitation experiments using the WGA-bound fraction. As shown in Fig. 5, DHPR was immunoprecipitated by an anti-dysferlin antibody, and conversely, dysferlin was immunoprecipitated by anti-DHPR $\alpha 1$ and $\alpha 2$ antibodies. This clearly demonstrates an interaction between dysferlin with DHPR. In another group of experiments we confirmed the presence of the dysferlin-interacting protein, caveolin-3 in immunoprecipitates prepared using the anti-dysferlin antibody. Additionally, analysis of immunoprecipitates prepared with both anti-DHPR $\alpha 1$ and $\alpha 2$ antibodies demonstrated the presence of caveolin-3, while anti-caveolin-3 antibody was found to reciprocally immunoprecipitate DHPR. These results suggest an association between DHPR and caveolin-3. Relative amounts of caveolin-3 in immunoprecipitation study indicated that only a portion of caveolin-3 might participate in the complex with dysferlin and DHPR.

We have not identified the 100- and 80-kDa WGA-binding proteins that were shown to interact with dysferlin, but their presence indicates that dysferlin may interact with more proteins than presently known.

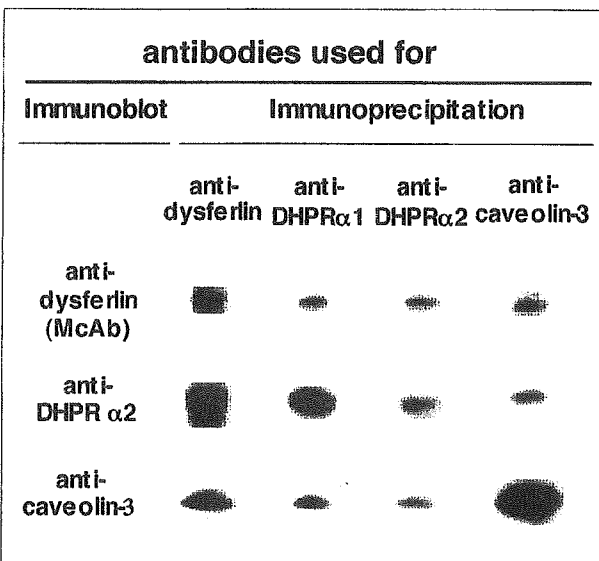


Fig. 5. Dysferlin, DHPR and caveolin-3 mutually interact. Immunoblot analysis of immunoprecipitates prepared from WGA-bound microsomal proteins. One μ l of immunoprecipitates was loaded on each lane.

Partial colocalization of dysferlin with DHPR

Based on our finding that dysferlin interacts with DHPR and caveolin-3, we expect that these proteins would colocalize in skeletal muscle fibers. Therefore we set out to verify this by double immunofluorescent staining. The anti-dysferlin monoclonal and anti-DHPR $\alpha 1$ antibodies both produced cross-striated bands of dysferlin and DHPR, respectively, in longitudinal sections of skeletal muscle fibers (Fig. 6). Analysis of double stained patterns revealed areas of colocalization between the two proteins.

Anti-caveolin-3 antibody showed a less organized cytoplasmic staining pattern in comparison with dysferlin and DHPR, and double staining did not show clear colocalization between caveolin-3 and either DHPR (data not shown) or dysferlin. This might be explained by the notion that only a small portion of caveolin-3 associated with DHPR and dysferlin, as suggested by immunoprecipitation.

We also investigated the localization of dysferlin in relation to other well characterized sarcomeric protein markers such α -actinin and desmin, two functionally different proteins associated with Z-

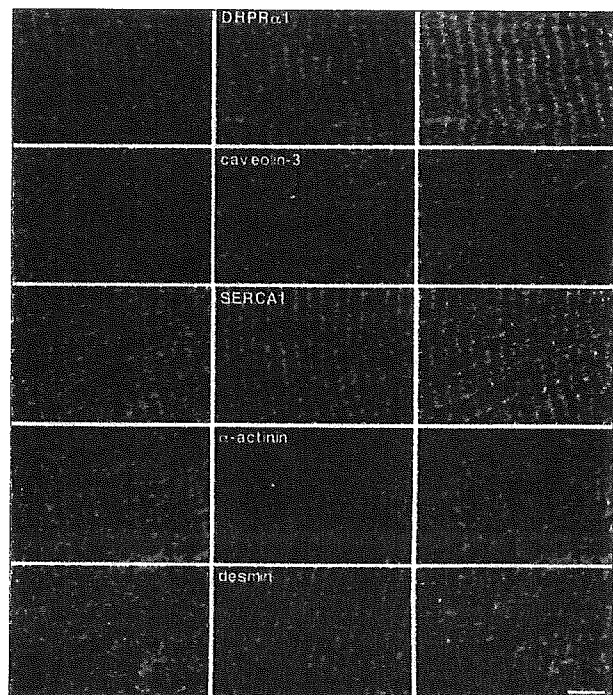


Fig. 6. Partial colocalization of dysferlin and DHPR. High-resolution images of 6 μ m longitudinal sections from the TA muscle of Wistar rats double stained with monoclonal antibody against dysferlin (red, left panels) and antibodies against DHPR $\alpha 1$, caveolin-3, SERCA1, α -actinin and desmin (green, center panels). Respective merged images are shown on the right panels. Scale bar, 5 μ m.

lines, and SERCA1, a protein responsible for the uptake of calcium into intracellular stores. Double staining of dysferlin with either α -actinin or desmin showed no areas of colocalization. Instead, striations produced by either α -actinin or desmin were labeled within the dysferlin doublet striated pattern. Results with SERCA1 were similar to that obtained with DHPR, with comparatively fewer areas of colocalization observed.

Discussion

While no comprehensive report has been published detailing the precise localization of dysferlin within the muscle fiber, a careful examination of literature shows a consensus indicating the presence of dysferlin at both the sarcolemma [12-16] and within the cytoplasm [12,16] of muscle fibers. In the present study we confirmed the presence of dysferlin at the sarcolemma initially by immunohistochemical observation (Fig. 1). Subsequent biochemical analysis revealed the presence of dysferlin, first in a crude microsomal fraction (Fig. 2), and then in a pure sarcolemmal fraction prepared from light microsomes (Fig. 3). We also observed dysferlin localization as granular cytoplasmic staining within muscle fibers by immunohistochemistry (Fig. 1). This pattern of cytoplasmic localization was of interest in view of the fact that dysferlin has a putative role in mediating intracellular vesicle fusion with the sarcolemma [12,13]. However, since not much is known about the nature of this intracellular localization, we investigated this further via a series of biochemical experiments.

Two significant findings were made in this study. The first of these was the observation that in addition to the pure sarcolemmal fraction, a portion of dysferlin was present in a T-tubule-enriched intracellular membrane fraction (Fig. 3). This was determined using a protocol of subcellular fractionation with further purification by WGA agglutination. T-tubules are specialized membrane invaginations of the sarcolemma and carry membrane depolarization deep into the fiber to trigger excitation-contraction coupling [30]. The protein and lipid composition of this system has been shown to be very distinct from that of the sarcolemma [30]. This suggests that the association of dysferlin with T-tubules may be independent of its localization at the sarcolemma, and is the first published data describing an association of dysferlin with non-sarcolemmal membranes.

Another significant finding was the identification of DHPR as a dysferlin-interacting protein complex. This result supports the finding of dysferlin present on T-tubules. The DHPR is responsible for the L-type Ca^{2+} current and serves as the voltage sensor for excitation contraction coupling [32]. It is composed of α 1, α 2, β and γ subunits [29]. The α 1 subunit is a transmembrane protein that contains the basic functional elements for the L-type Ca^{2+} channel while the β subunit is an intracellular protein essential for excitation contraction coupling. The functional significance of the α 2 and γ subunits is not clearly defined [32].

The question remains as to exactly how dysferlin associates with DHPR. Two possibilities exist as to how dysferlin may interact with DHPR. One involves a lateral association between dysferlin and DHPR in T-tubule membranes. The other involves an association between dysferlin, present in intracellular vesicles, and DHPR. Double immunostaining (Fig. 6) and membrane fractionation experiments indicate that the entire DHPR complex may not interact with dysferlin. Therefore, we are of the opinion that the interaction occurs between dysferlin and DHPR present on distinct membranes respectively.

It is not clear whether this association is direct or not, but it is interesting to assume that some other protein(s) may be involved in this association. The WGA-binding proteins of molecular masses 80 and 100 kDa shown to interact with dysferlin (Fig. 4C) might be involved in this association. Alternatively, caveolin-3, reported to be dysferlin-interacting protein [25] might be involved. In the present study, we observed by biochemical analysis that caveolin-3 associated with both membranes of T-tubules and the sarcolemma (Fig. 3). This distribution pattern was consistent with previous reports [33]. We confirmed by immunoprecipitations that dysferlin in fact interacts with caveolin-3 and in addition, we found that caveolin-3 interacts with DHPR. These results show that dysferlin, DHPR and caveolin-3 may participate in the formation of a large oligomeric complex. Caveolin-3 is a major protein component of cardiac and skeletal muscle caveolae [34], another system of specialized membrane invaginations of the sarcolemma implicated in a number of cellular processes including calcium homeostasis [35] and signal transduction [36]. It has been suggested that caveolin-3 associates mainly with T-tubules during myogenesis and has been proposed to play a role in the formation of T-tubules [37]. Indeed, caveolin-3 knockout mice show a phenotype

with T-tubule abnormalities [38], but most of caveolin-3 in the adult stage has been found at the sarcolemma by immunohistochemistry [37]. Caveolin-3 has also been reported to participate in an insulin-regulated transport mechanism in muscle [33]. Under activation, caveolin-3-containing vesicles traffic between intracellular sites and target sites on the sarcolemma and T-tubules. Not much is known about the precise nature of how caveolin-3 associates with T-tubules. However, based on our finding, it would be intriguing to assume that dysferlin might be involved in the fusion of caveolin-3-containing vesicles with T-tubules.

On the basis of immunohistochemical finding that dysferlin is present in the cytoplasm [12,16], it has been proposed that dysferlin-carrying vesicles participate in the injury-induced membrane repair of the sarcolemma [17]. However, our present findings suggest that the cytoplasmic staining does not always show the presence of such vesicles, since at least a portion was contributed by T-tubule-associated dysferlin. Evaluation of the proposed model, together with identification of the dysferlin-carrying membrane vesicles, should be encouraged by further studies.

In conclusion, we have shown by systematic subcellular membrane fractionation that dysferlin associates with both sarcolemma- and T-tubule-enriched membrane fractions. The latter was consistent with our demonstrated interaction of dysferlin with DHPR. We have also shown an association between DHPR and the dysferlin-interacting protein caveolin-3, raising the possibility that dysferlin may form an oligomeric complex. Two not yet identified WGA-binding proteins, 80 and 100 kDa, may participate in this complex. Our findings suggest that dysferlin might play role(s) other than the putative injury-induced membrane repair.

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Abbreviations

C. elegans, *Caenorhabditis elegans*; DHPR, the dihydropyridine receptor; EGTA, O, O'-bis(2-aminoethyl)ethyleneglycol-N, N, N', N'-tetraacetic acid; LGMD 2B, limb girdle muscular dystrophy 2B; NaK-ATPase, sodium potassium ATPase; RyR, ryanodine receptor; SR, the sarcoplasmic reticulum; T-tubules, transverse tubules; SERCA, sarco/endoplasmic reticulum calcium ATPase; WGA, wheat germ agglutinin.

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Major clinical and histopathological characteristics of canine X-linked muscular dystrophy in Japan, CXMD_J

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Canine X-linked muscular dystrophy (CXMD), which was found in a colony of golden retriever, is caused by a mutation in the *dystrophin* gene and it is a useful model of Duchenne muscular dystrophy (DMD). To investigate the pathogenesis and to develop therapy of DMD, we have established a beagle-based CXMD colony in Japan (CXMD_J) and examined their phenotypes. The mortality by 3 days of age in the third generation (G3) of CXMD_J dogs, 32.3%, was considerably higher than that in normal G3 littermates, 13.3%. Serum creatine kinase (CK) levels of G3 CXMD_J were significantly higher than that of normal male dogs with two peaks: at shortly after birth and around 2 months of age. Diaphragm muscle involvement occurred shortly after birth and was more severe than that of limb muscles. Stress during whelping might be associated with the neonatal death and respiratory muscle involvement. Gait disturbance was also noticed after 2 months of age. The involvement of limb and temporal muscles was observed from 2 months of age, which corresponded with the second peak of serum CK. Macroglossia, dysphagia, drooling and jaw joint contracture were overt from 4 months of age. We noticed severe macroglossia and hypertrophy of the sublingual muscles at the age of 12 months, and these were important features of this model, because dysphagia is one of major symptoms in older DMD patients. Overall, the phenotypes of CXMD_J were roughly identical to those of CXMD dogs in the literature. Beagle-based CXMD_J is smaller and easier to handle than golden retriever, therefore they are a useful model for DMD.

Key words: Canine X-linked muscular dystrophy; dystrophin; creatine kinase

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive disease characterized by skeletal muscle atrophy and weakness [1], and is

caused by mutations in the *dystrophin* gene [2]. DMD patients exhibit muscle weakness by 2 to 5 years of age, are unable to walk, and die by their twenties as a result of either respiratory or cardiac failure [1]. Recently the prognosis of patients suffering from DMD has been greatly improved by the use of respirators.

In research on the molecular pathogenesis of dystrophin deficiency, *mdx* mice have been frequently used as an experimental model, but the clinical symptoms of *mdx* mice are much milder than those of patients suffering from DMD [3]. Another model for DMD, canine X-linked muscular dystrophy (CXMD), which was found in a golden retriever, lacks dystrophin, and reveals clinical and pathological findings that are more similar to those of DMD [4-7]. Therefore, CXMD dogs are more appropriate than *mdx* mice as an animal model [5, 7, 8], although a severely affected animal model is difficult to raise and handle.

We established a beagle-based colony of CXMD dogs in Japan designated CXMD_J [9]. The first generations (G1) of carrier female dogs were produced by artificial insemination of a beagle bitch with frozen semen derived from a CXMD, and the second generation (G2) and the third generation (G3) of CXMD_J dogs were born to interbreed G1 and G2 carrier female dogs and beagle sires, respectively.

CXMD is a very important experimental model for the development of therapies for dystrophin deficiency. The efforts that have been made to

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explore experimental therapies in *mdx* mice may also be applicable to a large animal model such as CXMD in the future. These efforts include pharmaceuticals [10], gene transfer [11], or cell transplantation therapy [12]. Very recently hematopoietic cell transplantation has been tried using CXMD [13]. In this report, the major phenotypes of G3 CXMD_J dogs were investigated and compared with those of CXMD dogs. We found that CXMD_J dogs are a suitable model to develop therapies for DMD.

Materials and Methods

Animals

All dogs were part of the CXMD_J breeding colony at the General Animal Research Facility, National Institute of Neuroscience, National Center of Neurology and Psychiatry (Tokyo, Japan) or Chugai Research Institute for Medical Science, Inc. (Nagano, Japan) [9]. Seven G2 carrier females were mated with normal beagle sires or inseminated with fresh normal beagle semen. Ninety-nine G3 pups were produced by 12 whelpings of G2 carriers. The genotypes of the pups were analyzed by Snapback SSCP [14]. Forty-five male and female littermates were normal, 31 male pups were affected, and 23 female pups were carriers. We analyzed affected G3 males together with normal G3 male and female littermates, and carrier G3 females in this study.

A pregnant carrier G2 female was removed to stainless steel cage for whelping (1,580 x 1,580 x 930 mm) at 7 days before the expected whelping day. The carrier female nursed G3 pups after her whelping, and the weaning of pups was done at 6 weeks after birth. Affected G3 pups were then individually housed in the cages (1,700 x 960 x 1,570 mm), but the remaining pups were fed with 2 or 3 pups in the same largeness of cages up to around 3 months of age. All dogs were given a mixture of soaking pellet with synthetic powdered milk up to 3 months of age, and then given a pellet diet. They also had *ad libitum* access to filtered water. The room temperature was maintained at $22 \pm 2^\circ\text{C}$, and the relative humidity was 40-70%. A veterinarian examined the healthy care of the dogs once a week. All dogs in this experiment were treated in accordance with the guidelines provided by the Ethics Committee for Treatment of Laboratory Middle-Sized Animals of the

National Institute of Neuroscience, National Center of Neurology and Psychiatry (Tokyo, Japan) or the Ethics Committee for Treatment of Laboratory Animals of Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan).

Death by 3 days of age

The rates of death by 3 days of age were calculated in 31 affected G3 male pups and 45 normal G3 male and female pups, respectively.

Body weight

The body weight of normal ($n = 4$) and affected ($n = 8$) G3 males were measured at every second day from 1 to 21 days, and at 1.5, 2, 4, 6, 8, 10, and 12 months of age. Synthetic milk was given through tube for 14 days to four affected G3 pups whose body weight at 3 days of age was decreased than that of 1 day after birth.

Serum creatine kinase (CK) levels

Blood samples of normal male ($n = 5$), female ($n = 3$ before 2 months of age, $n = 2$ at 3 months of age), affected male ($n = 8$), and carrier female ($n = 4$) G3 dogs were obtained by cephalic or jugular venipuncture at 1 day, 3 weeks, and 1.5, 2, 3, 6, and 12 months of age. Serum CK levels were assayed using an automated colorimetric analyzer (FDC3500, FujiFilm Medical, Tokyo, Japan).

Clinical manifestations

We observed gait and mobility disturbances, involvement of limb, temporal, and tongue muscles, dysphagia, and drooling as clinical signs in normal ($n = 5$) and affected ($n = 5$) G3 males at the ages of 1, 2, 4, 6, and 12 months. The severity of each sign was classified according to a new grading scale for the CXMD_J colony (**Table**) based on both a previous report [15] and a clinical record prepared for an Australian CXMD colony by J.M. Howell et al. (personal communication). We also measured the maximum mouth opening to evaluate the jaw joint contracture. At least one veterinarian and two medical doctors who are very familiar with symptoms of dystrophic dogs did all clinical examinations.

Macroscopic and histopathological examinations

Muscle samples from normal ($n = 5$) and affected ($n = 5$) G3 males were obtained when

Table 1. Grading of clinical signs in CXMD_J dogs.

Gait disturbance	
Grade 1:	None
Grade 2:	Sitting with hind legs extended
Grade 3:	Bunny hops with hind legs
Grade 4:	Shuffling walk
Grade 5:	Unable to walk
Mobility disturbance	
Grade 1:	None
Grade 2:	lying down more than normal
Grade 3:	Cannot jump on hind legs
Grade 4:	Increasing difficulty moving around
Grade 5:	Unable to get up and move around
Limb or temporal muscle atrophy	
Grade 1:	None
Grade 2:	Suspect hardness
Grade 3:	Can feel hardness or apparently thin
Grade 4:	Between Grade 3 and 5
Grade 5:	Extremely thin or hard
Drooling	
Grade 1:	None
Grade 2:	Occasionally dribbles saliva when sitting
Grade 3:	Some drool when eating and drinking
Grade 4:	Strings of drool when eating or drinking
Grade 5:	Continuous drool
Macroglossia	
Grade 1:	None
Grade 2:	Slightly enlarged
Grade 3:	Extended outside dentition
Grade 4:	Enlarged and slightly thickened
Grade 5:	Enlarged and thickened
Dysphagia	
Grade 1:	None
Grade 2:	Takes time and effort in taking food
Grade 3:	Difficulty in taking food from plate
Grade 4:	Difficulty in chewing, swallowing or drinking
Grade 5:	Unable to eat

These grading scales were developed at our institute on the basis of a previous report [15] and a personal communication with J.M. Howell et al. in Australia.

the dogs died at 1 day of age or were sacrificed at the ages of 3 days and 2, 6, and 12 months by using an overdose of intravenous pentobarbital. Some portions of the diaphragm and anterior tibial muscles were snap-frozen in cooled isopentane, and cryostat sections (6 μ m) were stained with hematoxylin and eosin (H&E). The tongue, genioglossus, and geniohyoideus muscles were fixed in 15% neutral buffered formalin, embedded in paraffin, and 6 μ m sections were stained with H&E.

Statistical analysis

The data of body weight, serum CK level, and the maximum mouth opening were subjected to a one-way analysis of variance test. Fisher's protected least significant difference test was used for post-hoc comparisons. The statistical significance of the pup death rate was evaluated using the chi-square test. All values are expressed as mean \pm standard error (S.E.). A *p* value of less than 0.05 was considered to indicate statistical significance. All calculations were performed using StatView software (SAS Institute, Cary, NC, USA).

Results

Death by 3 days of age

Ten of 31 G3 CXMD_J pups died, a death rate of 32.3%. Among normal G3 male and female pups, six out of 45 (13.3%) died. The ratio of affected G3 pups was significantly higher than that of normal pups (*p* < 0.05). We did not find any dead pups from 4 days to 4 weeks of age during the lactation period.

Body weight

Body weight changes of G3 male dogs (4 normal and 4 affected without tube feeding) in the CXMD_J colony are shown in **Fig. 1A**. Affected G3 males showed retardation in increase of body weight after 6 months of age and the difference between them was statistically significant at the age of 10 and 12 months (**Fig. 1A**). While, the body weight of affected G3 male pups needed tube feeding was significantly lower than that of either normal or affected G3 male without tube feeding at 5, 7, and 9 days of age (**Fig. 1B**). The difference in body weight between normal and carrier G3 females was not seen from 1 day to 6 months of age (data not shown).

Serum CK levels

Changes of serum CK levels of dogs in the CXMD_J colony are shown in **Fig. 2**. The values of normal G3 males and females ranged from 80 to 4,000 IU/l up to 12 months of age. The difference in the CK levels between normal and affected G3 males was statistically significant at every age examined. The average serum CK level made a biphasic pattern: it was 120,000 IU/l at 1 day of age and then had decreased to 11,000 IU/l at 3

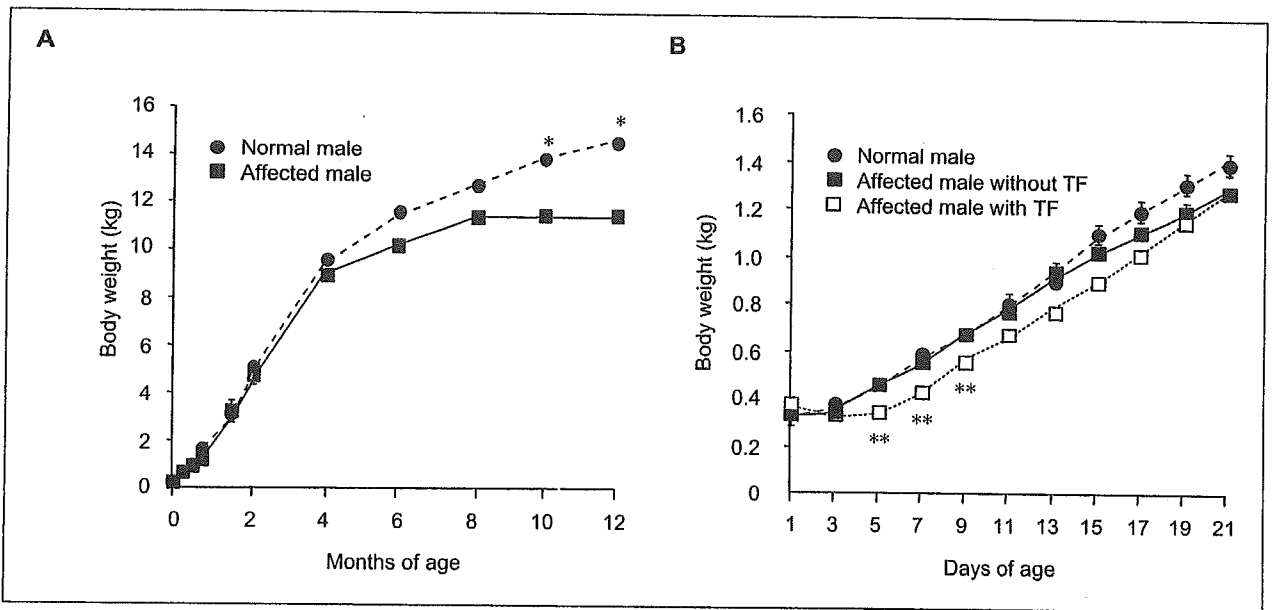


Figure 1. (A) Body weight changes up to 12 months of normal and affected G3 male dogs in CXMD_J colony. (B) Body weight changes of G3 male dogs of normal, affected without tube feeding (TF), and affected with TF up to 21 days. Bar: mean ± S.E.; ***p* < 0.01; **p* < 0.05.

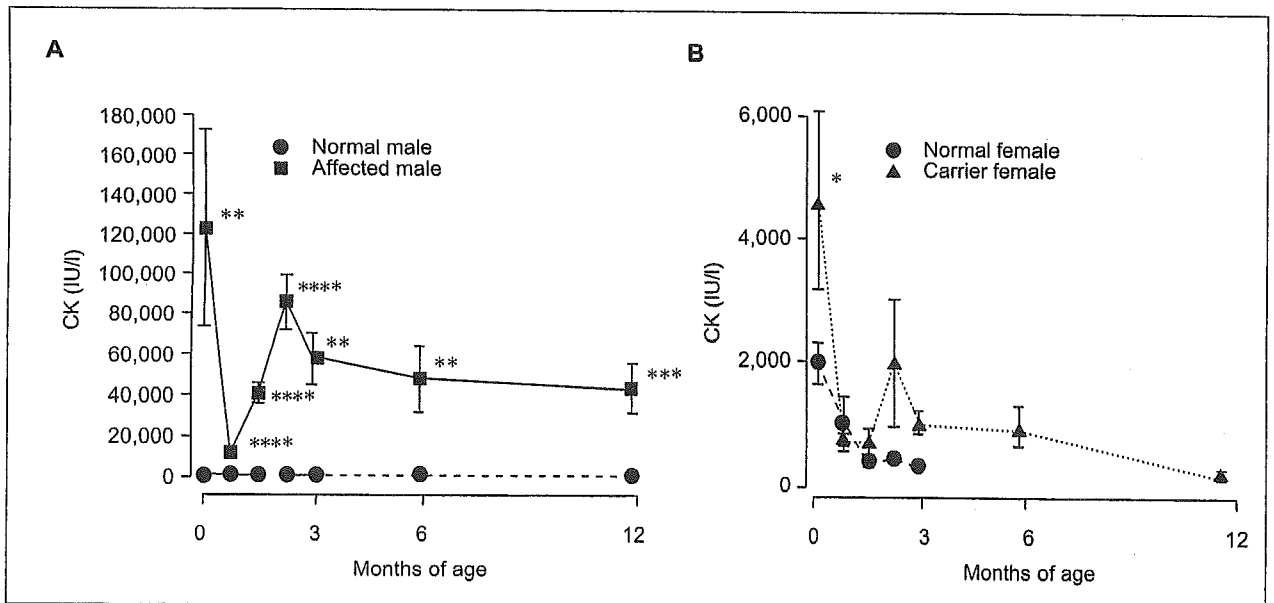


Figure 2. Serum CK levels of G3 dogs in CXMD_J colony in (A) normal and affected males and (B) normal and carrier females. Bar: mean ± S.E.; *****p* < 0.0001; ****p* < 0.001; ***p* < 0.01; **p* < 0.05.

weeks of age (Fig. 2A); however, the levels had increased again to 85,000 IU/l at 2 months of age, and then the values gradually decreased. Like affected G3 males, the serum CK levels in carrier G3 females, both at 1 day and 2 months of age, were higher than those in normal G3 females, and the difference at 1 day of age was statistically significant (Fig. 2B).

Clinical manifestations

In CXMD male dogs at 14 months of age, kyphosis and atrophy of temporal, truncal, and limb muscles were observed; many muscles, especially the proximal limb muscles, were firm on palpation [15]. A representative G3 CXMD_J male dog at 12 months of age had signs similar to those described above (Fig. 3). Moreover, CXMD dogs