Selective Vacuolar Degeneration in Dystrophin-Deficient Canine Purkinje Fibers Despite Preservation of Dystrophin-Associated Proteins With Overexpression of Dp71

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Background—Respiratory support therapy significantly improves life span in patients with Duchenne muscular dystrophy; cardiac-related fatalities, including lethal arrhythmias, then become a crucial issue. It is therefore important to more thoroughly understand cardiac involvement, especially pathology of the conduction system, in the larger Duchenne muscular dystrophy animal models such as dystrophic dogs.

Methods and Results—When 10 dogs with canine X-linked muscular dystrophy in Japan (CXMDJ) were examined at the age of 1 to 13 months, dystrophic changes of the ventricular myocardium were not evident; however, Purkinje fibers showed remarkable vacuolar degeneration as early as 4 months of age. The degeneration of CXMDJ Purkinje fibers was coincident with overexpression of Dp71 at the sarcolemma and translocation of \( \mu \)-calpain to the cell periphery near the sarcolemma or in the vacuoles. Immunoblotting of the microdissected fraction showed that \( \mu \)-calpain–sensitive proteins such as desmin and cardiac troponin-I or -T were selectively degraded in the CXMDJ Purkinje fibers. Utrophin was highly upregulated in the earlier stage of CXMDJ Purkinje fibers, but the expression was dislocated when vacuolar degeneration was recognized at 4 months of age. Nevertheless, the expression of dystrophin-associated proteins \( \alpha \)-, \( \beta \)-, \( \gamma \)-, and \( \delta \)-sarcoglycans and \( \beta \)-dystroglycan was well maintained at the sarcolemma of Purkinje fibers.

Conclusions—Selective vacuolar degeneration of Purkinje fibers was found in the early stages of dystrophin deficiency. Dislocation of utrophin besides upregulation of Dp71 can be involved with this pathology. The degeneration of Purkinje fibers can be associated with the distinct deep Q waves in ECG and fatal arrhythmia seen in dystrophin deficiency. (Circulation. 2008;117:2437-2448.)

Key Words: cardiomyopathy ■ conduction ■ dystrophin ■ Purkinje fibers ■ utrophin

Duchenne muscular dystrophy (DMD) is a lethal X-linked disorder caused by mutations in the \( DMD \) gene.\(^1\) Seven promoters in the huge \( DMD \) gene drive tissue-specific expression of 427-kDa full-length dystrophins and various C-terminal isoforms such as Dp71.\(^2\) The full-length muscle-type dystrophin is located at the inner surface of the sarcolemma with dystrophin-associated proteins (DAPs) and forms the dystrophin-glycoprotein complex linking the intracellular actin cytoskeleton of myofibers to the extracellular matrix.\(^3\) A lack of dystrophin prevents the assembly of DAPs on the sarcolemma and leads to muscle degeneration. Utrophin, an autosomal homologue of dystrophin, is upregulated in dystrophin-deficient muscles of DMD and the animal models.\(^4\) It has been considered that utrophin overexpression at the sarcolemma compensates for the lack of dystrophin in dystrophic muscle.\(^5\)

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In humans, the loss of dystrophin accompanied by a deficiency of dystrophin-glycoprotein complex at the sarcolemma leads to progressive degeneration not only in skeletal muscle but also in cardiac muscle. The subsequent respiratory or cardiac failure causes death at an early age, but recent progress in the use of respirators has reduced the rate of death due to respiratory failure and improved the prognosis. Consequently, cardiac death has become a serious problem. The cardiac involvement in DMD is characterized by cardiomyopathy, cardiac arrhythmias, and distinctive ECG findings.
Progressive myocardial fibrosis primarily in the posterobasal region of the left ventricle (LV) results in dilated cardiomyopathy. Various arrhythmias such as ventricular tachycardia, atrioventricular block, bundle-branch block, and fascicular block are often observed in DMD. In DMD patients, the prevalence of lethal arrhythmias has been considered to be lower than that in myotonic dystrophy or Emery-Dreifuss muscular dystrophy, but some studies showed that sudden death accounts for 12.1% of all deaths of DMD patients and that ventricular arrhythmia is an important cause of death. A distinctive ECG pattern of deep narrow Q waves in leads I, aVL, and V5 through V6 or II, III, and aVF has been detected and is ascribed to myocardial fibrosis in the posterobasal region of the LV.

The X-chromosome–linked muscular dystrophy (mdx) mouse and the golden retriever muscular dystrophy dog (GRMD) lack dystrophin and serve as models for DMD. The phenotypic expression of GRMD is more similar to DMD than that of the mdx mouse, and GRMD shows progressive cardiomyopathy and deep, narrow Q waves in leads II, III, and aVF comparable to cardiac involvement in human subjects. However, it is very difficult to maintain a GRMD colony because of their severe phenotypes. On the other hand, interbreeding of GRMD with small dogs results in mild phenotypes of the disease. Thus, we have developed a beagle-based colony of medium-sized dogs and named it canine X-linked muscular dystrophy in Japan (CXMDJ). Beagle-based dystrophic dogs show mild phenotypes not only in skeletal muscle but also in cardiac muscle. Moreover, we found that the distinctive deep Q waves precede the LV posterobasal lesion on echocardiography and histopathology in CXMDJ. The histopathological changes in the conduction system have not been investigated fully, especially in the early stages of the disease, although the expressions of dystrophin and DAPs have been examined in normal systems.

The molecular mechanisms of the dystrophin-deficient heart are still unclear. Two main possibilities exist relative to the functional roles of dystrophin. One hypothesis is that dystrophin maintains the structural integrity of the sarcolemma and confers mechanical strength during muscle contraction. The membrane tears due to dystrophin deficiency may increase the permeability to Ca2+, which might trigger protease activity. The second hypothesis is proposed because dystrophin and DAPs anchored neuronal nitric oxide synthase, aquaporin-4, and Na+/K+ pump in the sarcolemma, and L-type Ca2+ channels and stretch-activated channels were also regulated by expression of dystrophin and DAPs. The expression and functions of these membrane-associated proteins were altered in the absence of dystrophin, which may lead to dysfunction of muscle fibers. Either stretch-activated channels or the enhancement of the open probability of leak channels was involved in dystrophin-deficient muscle, resulting in elevation of the Ca2+ influx. Elevated [Ca2+]i acts on the autophagy cascade, leading to myofilament destruction and/or muscle cell death by activation of calcium-dependent cysteine proteases, the calpains.

The main isoforms of the calpain family, m- and μ-calpain, are activated in vitro in the presence of millimolar and micromolar concentrations of Ca2+, respectively. In dystrophin-deficient skeletal muscle, calpains are increased and activated at the pathological stage.

In this study, we examined the conduction system in the CXMDJ heart. We show here for the first time that dystrophin deficiency results in selective degeneration of Purkinje fibers. Furthermore, activation of μ-calpain and dislocation of utrophin with Dp71 overexpression were observed in the early stage of CXMDJ, Purkinje fibers.

Methods

Animals

We determined the serum creatine kinase levels and genotypes of CXMDJ, and normal littermate pups soon after birth. Moreover, we routinely examined the dogs for clinical manifestations including gait and mobility disturbances, involvement of limb, temporal and tongue muscles, dysphagia, and drooling according to our clinical grading scale. In this study, we used third-generation CXMDJ dogs (n = 10) and normal third-generation dogs (n = 7) from 1 to 13 months of age, but we mainly examined 4 CXMDJ and 2 normal littermate dogs at 4 months of age and a pair of CXMDJ and normal littermate dogs at 1 and 2 months of age in the time course analysis because the number of CXMDJ dogs available is limited. The phenotype of these affected dogs has been described previously. All experimental animals were part of the CXMDJ breeding colony at the General Animal Research Facility, National Institute of Neuroscience, National Center of Neurology and Psychiatry (Tokyo, Japan) or the Chugai Research Institute for Medical Science, Inc (Nagano, Japan).

The dogs were cared for and treated in accordance with the guidelines provided by the Ethics Committee for the Treatment of Laboratory Animals of the National Institute of Neuroscience or the Ethics Committee for Treatment of Laboratory Animal of Chugai Pharmaceutical Co, Ltd (Tokyo, Japan). These studies were also approved by the Ethics Committee for the Treatment of Laboratory Middle-Sized Animals of the National Institute of Neuroscience (approval Nos. 13-03, 14-03, 15-03, 16-03, 17-03, and 18-03). Skilled experimental animal technologists, who have special knowledge of methods to prevent unnecessary excessive pain, handled the dogs and assisted in the experiments.

Light Microscopy

Pathological changes in the heart were analyzed within 1 week of the performance of ECG and echocardiography. After a dog was given an overdose of intravenous pentobarbital, the whole heart was removed. To prepare specimens for immunologic analysis and electron microscopy, the block of the endocardial sides containing Purkinje fibers was dissected out from the interventricular septum and anterior, posterior, and lateral walls of the LV at the level of papillary muscles. The remaining heart was immediately fixed in 15% buffered formalin for histological analysis. Formalin-fixed hearts were dissected into separate blocks containing ventricular myocardium and or conduction systems, ie, the sinus node, atrioventricular node, bundle of His, left and right bundle branches, and Purkinje fibers from the LV or right atrium, as described elsewhere. Each piece of tissue was embedded in paraffin, and 10-μm sections were stained with hematoxylin and eosin. Photographs were taken with a DAS Mikroskop Leits DMRB microscope (Leica, Wetzlar, Germany) with the use of a digital still camera system HC-2500 (Fujifilm, Tokyo, Japan).

Electron Microscopy

Muscles from the LV were fixed in 2% glutaraldehyde, postfixed in osmium tetroxide, dehydrated in a graded ethanol series, and then embedded in Epon. Ultrathin sections stained with uranyl acetate and lead citrate were examined by H-7000 transmission electron microscopy (Hitachi High Technologies, Tokyo, Japan).
Immunohistochemistry

Seven-micrometer transverse cryosections of frozen LV muscles were fixed in acetone, blocked with 5% goat serum, and incubated with mouse monoclonal antibodies against various epitopes of dystrophin: MANEX1a recognizing amino acids 3 to 10 of muscle-type dystrophin molecule (generous gift of Dr G.E. Morris, Wolfson Centre for Inherited Neuromuscular Disease), NCL-DYSB recognizing amino acids 321 to 494 (Novocastra Laboratories, Newcastle, UK), NCL-DYS1 recognizing amino acids 1181 to 1388 (Novocastra), MANDYS8 recognizing amino acids 1431 to 1505 (Sigma-Aldrich, St Louis, Mo), F22.9C5 recognizing amino acids 1840 to 2266 (Alexis, Lausen, Switzerland), or NCL-DYS2 recognizing amino acids 3669 to 3685 (Novocastra); mouse monoclonal antibodies against β-dystroglycan (NCL-b-DG, Novocastra), β-sarcoglycan (NCL-b-SG, Novocastra), γ-sarcoglycan (NCL-g-SG, Novocastra), or δ-sarcoglycan (DSG1), rabbit polyclonal antibodies against utrophin (UT-2), α-sarcoglycan (α-SG2), or μ-calpain (anti-calpain 1 large subunit domain IV; Sigma-Aldrich). The primary antibodies were labeled with fluorescein-conjugated goat anti-mouse or anti-rabbit immunoglobulin G (Molecular Probes, Eugene, Ore), and signals were recorded photographically with a confocal laser scanning microscope (TCS-SP, Leica).

Immunoblot Analysis

For immunoblot analysis of ventricular myocardium or Purkinje fibers, laser capture microdissection was performed with the use of an LM 200 system (Arcturus, Mountain View, Calif), as described elsewhere with some modifications. Fifty-micrometer cryosections were prepared from frozen LV muscles and dehydrated in graded ethanol and xylene. Eight hundred 15-μm diameter microdissection spots were collected from ventricular myocardium or Purkinje fibers onto each CapSure Macro LCM Cap (Arcturus). Tissues captured on Caps were suspended in 20 μL of SDS-PAGE lysis buffer (10% SDS, 70 mmol/L Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 10 mmol/L EDTA) at 80°C for 15 minutes. Ten-microliter aliquots were separated in each lane on 7.5% or 9% SDS-PAGE and electrotransferred onto a polyvinylidene fluoride membrane (Immobilon; Millipore, Billerica, Mass), as previously described. Equality of the protein concentrations was confirmed by Coomassie blue staining, and myosin heavy chain was used as the loading control. After blocking by 2% casein in lysis buffer (10% SDS, 70 mmol/L Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 10 mmol/L EDTA) at 80°C for 15 minutes. Ten-microliter aliquots were separated in each lane on 7.5% or 9% SDS-PAGE and electrotransferred onto a polyvinylidene fluoride membrane (Immobilon; Millipore, Billerica, Mass), as previously described. The protein concentrations was confirmed by Coomassie blue staining, and myosin heavy chain was used as the loading control. After blocking by 2% casein in lysis buffer (10% SDS, 70 mmol/L Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 10 mmol/L EDTA) at 80°C for 15 minutes. Ten-microliter aliquots were separated in each lane on 7.5% or 9% SDS-PAGE and electrotransferred onto a polyvinylidene fluoride membrane (Immobilon; Millipore, Billerica, Mass), as previously described.

Results

Selective Degeneration of Purkinje Fibers in CXMD1

We investigated morphological abnormalities in the cardiac conduction system of CXMD1. In all CXMD1 dogs, the sinus node, atrioventricular node, and bundle of His showed no degenerative changes (Figure 1A). Many irregular vacuoles were observed, but only in Purkinje fibers (Figure 1A and 1B). Remarkable vacuolar degeneration was consistently observed in CXMD1 Purkinje fibers in dogs at 4 months old (Figure 1C), whereas degeneration and fibrosis were absent in the ventricular myocardium (Table). These results imply that Purkinje fibers are susceptible to dystrophin deficiency, which causes selective and progressive vacuolar degeneration in an early stage of the disease.

Using transmission electron microscopy, we examined the contents of the vacuoles of CXMD1 Purkinje fibers. On electron microscopy, almost all myofibrils and intracellular organelles had disappeared, and disrupted myofibrils and fragmented mitochondria were observed in the vicinity of the vacuoles (Figure 2). These results suggest that the vacuoles are the ruins of myofibrillar structures. In CXMD1 ventricular myocardium, which seemed healthy on the light microscopic level, mildly disrupted myofibrils and mitochondrial fragmentation were observed on electron microscopy (Figure 2).

Upregulation of Dystrophin Short-Isoform Dp71 in CXMD1 Purkinje Fibers

The causative mutation of CXMD1 is located in the 3’ splice site of intron 6 in the canine dystrophin gene. Thus, we examined whether the truncated C-terminal dystrophin isoforms were expressed in the Purkinje fibers. In control dogs, the sarcolemma of ventricular cardiomyocytes and Purkinje fibers was stained with all the anti-dystrophin antibodies used in this study: MANEX1a, NCL-DYSB, F22.9C5, NCL-DYS2 (Figure 3A), NCL-DYS1, and MANDYS8 (data not shown). The ventricular myocardium of CXMD1 lacked expression of the full-length dystrophin and its short isoforms, but the sarcolemma of CXMD1 Purkinje fibers was clearly stained with NCL-DYS2, which recognizes the C-terminal of full-length dystrophin and its isoforms (Figure 3A).

To confirm which dystrophin isoform is present in the Purkinje fibers, we selectively collected Purkinje fibers using laser capture microdissection. The result showed thick doublet bands around 71 to 75 kDa in CXMD1 Purkinje fibers and thin doublet bands in normal Purkinje fibers stained with NCL-DYS2 antibody (Figure 3B). These data suggest that the sarcolemma of CXMD1 Purkinje fibers lacked full-length dystrophin but showed overexpression of a C-terminal isoform of dystrophin, Dp71. Dp71 is well known to cause degenerative changes (Figure 1A). Many irregular vacuoles were observed, but only in Purkinje fibers (Figure 1A and 1B). Remarkable vacuolar degeneration was consistently observed in CXMD1 Purkinje fibers in dogs at 4 months old (Figure 1C), whereas degeneration and fibrosis were absent in the ventricular myocardium (Table). These results imply that Purkinje fibers are susceptible to dystrophin deficiency, which causes selective and progressive vacuolar degeneration in an early stage of the disease.

Expression of Utrophin and Preservation of DAPs in CXMD1 Purkinje Fibers

We found that the expression of Dp71 is certainly increased in CXMD1 Purkinje fibers. In the dystrophin-deficient mus-
cle, utrophin, an autosomal homologue of dystrophin, is compensatorily upregulated.\textsuperscript{4,5} We therefore investigated the expression of utrophin to assess the effect of overexpression of Dp71 at the sarcolemma in CXMD\textsubscript{J} Purkinje fibers. In control dogs, utrophin was present in intercalated disks and small vessels in the ventricular myocardium and also weakly expressed at the sarcolemma of Purkinje fibers (Figure 4A). In CXMD\textsubscript{J}, utrophin was present at the sarcolemma of ventricular cardiomyocytes, and, very interestingly, utrophin was upregulated in Purkinje fibers (Figure 4A). Immunoblot analysis also showed that utrophin was increased in the CXMD\textsubscript{J} Purkinje fibers when examined at 4 months of age (Figure 4B), although we further evaluated utrophin expression along with the time in subsequent sections.

Reduction of all of the DAPs has been reported in dystrophin-deficient muscle.\textsuperscript{3} We also examined distribution of the DAPs β-dystroglycan and α-, β-, γ-, and δ-sarcoglycans in CXMD\textsubscript{J} Purkinje fibers. In CXMD\textsubscript{J}, expression of α-sarcoglycan was well maintained in Purkinje fibers but reduced in the ventricular myocardium (Figure 4A). The same tendency was observed in other DAP expressions (data not shown). The immunoblot analysis showed that the expression level of α-sarcoglycan was preserved in the micro-dissected CXMD\textsubscript{J} Purkinje fibers but not in the CXMD\textsubscript{J} myocardium (Figure 4B).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Histopathology of hearts of CXMD\textsubscript{J} dogs. A, Hematoxylin and eosin–stained sections from the heart of a 13-month-old affected dog (III-D12MA in the Table) show representative histopathological findings in hearts of CXMD\textsubscript{J}. RA indicates right atrium; LVAW, LV anterior wall; LVPW, LV posterior wall; SN, sinus node; AVN, atrioventricular node; His, bundle of His; and PF, Purkinje fibers. Bar=100 μm. The conduction system is illustrated in the center. LBB indicates left bundle branches; RBB, right bundle branches. B, Purkinje fibers from a normal 12-month-old dog (III-D23MN in the Table) and the affected dog shown in A. Bar=25 μm. C, Purkinje fibers from 1-, 2-, and 4-month-old affected dogs (III-203 MA, III-202MA, and III-E02MA, respectively, in the Table). Bar=25 μm.}
\end{figure}

Activation and Accumulation of \(\mu\)-Calpain in CXMD\textsubscript{J} Purkinje Fibers

Upregulated utrophin and well-maintained DAPs do not guarantee the integrity of the sarcolemma in dystrophin-deficient Purkinje fibers. We asked why the myofibrils were severely disrupted in the CXMD\textsubscript{J} Purkinje fibers. One hypothesis was that an alteration of expression or function of several membrane-associated molecules such as Ca\textsuperscript{2+} channels results in activation of 1 or more calcium-dependent proteases such as \(\mu\)-calpain.\textsuperscript{21,24} We therefore investigated
the expression of \( \mu \)-calpain in CXMD\(_j\) Purkinje fibers. Immunohistochemical analysis showed that the expression of \( \mu \)-calpain was accumulated only in CXMD\(_j\) Purkinje fibers (Figure 5A). Furthermore, \( \mu \)-calpain localized at the cell periphery near the sarcolemma in nonvacuolated Purkinje fibers (Figure 5A) and at the vacuoles in vacuolated Purkinje fibers (Figure 5B) in the same CXMD\(_j\) heart. These results imply that \( \mu \)-calpain localization is altered with the progression of degeneration. We examined \( \mu \)-calpain protein levels in the microdissected Purkinje fibers and myocardium by immunoblot analysis. The result showed that \( \mu \)-calpain expression was significantly increased in CXMD\(_j\) Purkinje fibers but not in CXMD\(_j\) ventricular myocardium at 4 months of age (Figure 5C).

### Time Course of Dp71 Expression, \( \mu \)-Calpain Translocation, and Urophin Expression in CXMD\(_j\) Purkinje Fibers

We next investigated the time course of expressions of Dp71, \( \mu \)-calpain, and utrophin in 1-, 2-, and 4-month-old CXMD\(_j\) dogs to understand the relationship among them. In immunohistochemical and immunoblot analyses, the expression of Dp71 was clearly increased in 4-month-old CXMD\(_j\) Purkinje fibers (Figure 6A and 6B). Similarly, the subsarcolemmal accumulation of \( \mu \)-calpain was also observed in 4-month-old CXMD\(_j\) Purkinje fibers in the immunohistochemical analysis (Figure 6A), but the concentration of \( \mu \)-calpain was not changed in the immunoblot analysis (Figure 6B). These data indicate the subsarcolemmal translocation of \( \mu \)-calpain. The degeneration of CXMD\(_j\) Purkinje fibers was consistently observed at the age of 4 months, as described (Figure 1C). Therefore, overexpression of Dp71 and translocation of \( \mu \)-calpain may be involved in the degeneration of CXMD\(_j\) Purkinje fibers. In turn, utrophin expression was gradually reduced with time in both normal and CXMD\(_j\) ventricular myocardium (Figure 6A and 6C), but the reduction was more evident in CXMD\(_j\) Purkinje fibers (Figure 6C). The sharp reduction of utrophin might be related to upregulation of Dp71 in CXMD\(_j\) Purkinje fibers at this period.

<table>
<thead>
<tr>
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<th>Age, mo</th>
<th>Fibrosis of LV Wall</th>
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</tr>
<tr>
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</table>

The extent of histopathological changes of the heart is shown as −, none or within normal range; −/+ , minimal; +, mild; ++, moderate; and ++++, severe.

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**Figure 2.** Ultrastructure of Purkinje fibers (top panels) and ventricular myocardium (bottom panels) from normal (III-403MN in the Table) and affected (III-E07MA in the Table) 4-month-old dogs. N indicates nucleus; MF, myofibril. Arrowheads indicate mitochondria. Bar=1 \( \mu \text{m} \).
Figure 3. Expression of dystrophin and dystrophin C-terminal isoforms in LV of normal and CXMD_2 dogs. A, Immunohistochemical staining using several anti-dystrophin antibodies of the LV of normal (III-E01MN in the Table) and affected (III-E02MA in the Table) 4-month-old dogs. Serial cryosections from the LV were stained with MANEX1a, NCL-DYSB, F22.9C5, and NCL-DYS2 antibodies, which recognize different epitopes of dystrophin (see Methods and schema). P indicates Purkinje fibers. Bar=100 μm. The bottom schema shows full-length dystrophin and C-terminal short isoforms with the positions of epitopes recognized by each anti-dystrophin antibody (bars). B, Immunoblot analysis using the anti-dystrophin C-terminal antibody NCL-DYS2. Samples from the dogs shown in A were separated by SDS-PAGE on 9% acrylamide gel. Extracts from affected Purkinje fibers showed thick doublet bands around 71 to 75 kDa. Those bands could be Dp71 with the upper band being phosphorylated. Myosin heavy chain (MyHC) was used as the loading control. C, Immunoblot analyses of the samples described in B using the anti-dystrophin C-terminal antibodies anti-Ex71, p34a, and NCL-DYS2. Myosin heavy chain was used as the loading control. Each antibody recognizes a different epitope on the dystrophin C-terminal (see Methods and schema). Extracts from affected Purkinje fibers reacted with all antibodies around 71 to 75 kDa. The bottom schema shows the positions of dystrophin C-terminal epitopes recognized by each anti-dystrophin C-terminal antibody (bars).
Degradation of μ-Calpain–Sensitive Myofibrillar Proteins in Purkinje Fibers and Serum of CXMDj

To determine why μ-calpain upregulation leads to degeneration of CXMDj Purkinje fibers, we investigated the μ-calpain substrate. Desmin, cardiac troponin T, and cardiac troponin I have been identified as μ-calpain–sensitive myofibrillar proteins.36 Therefore, we investigated whether these molecules are degraded in CXMDj Purkinje fibers. Immuno blot analyses using microdissected samples indicated that desmin, cardiac troponin T, and cardiac troponin I were degraded in the Purkinje fibers of a CXMDj at 4 months of age (Figure 7A). Moreover, we found that serum cardiac troponin I was degraded in the previously reported 9-month-old CXMDj who died suddenly14 but not in an aged-matched normal dog (Figure 7B).

Discussion

Degeneration of Dystrophin-Deficient Purkinje Fibers Precedes Myocardial Lesion

We report that the characteristic vacuolar degeneration of dystrophin-deficient Purkinje fibers was consistently observed in CXMDj by 4 months of age. In normal Purkinje fibers, regular round vacuoles are often seen because the myofibrils in cardiac Purkinje fibers are relatively sparse and mainly arranged in thick layers variously near the cell membrane. Larger fibril-free sarcoplasmic regions, therefore,
look like vacuole structure in normal human and canine heart at the microscopic level. On the other hand, the vacuoles we found in CXMD1 were irregular, and the changes were extensive and progressive. In addition, electron microscopy revealed that dystrophin-deficient Purkinje fibers had severely disrupted myofibrils, suggesting that the vacuoles were the ruins of myofibrillar structures.

We have recently reported that the onset of degeneration of the myocardium in CXMD1 is not detected before 5 months of age. Given these results, it is obvious that the vacuolar degeneration of CXMD1 Purkinje fiber precedes the myocardial lesion. The pathological features of DMD cardiomyopathy have been well characterized, but reports of morphological changes in the conduction systems are very limited. To our knowledge, only 2 reports have described the degeneration of Purkinje fibers. However, these studies reported autopsies of DMD patients, and degeneration of the ventricular myocardium was also prominent. Thus, we are able to demonstrate for the first time that selective vacuolar degeneration of Purkinje fibers begins in the early stage of dystrophin deficiency.

**Overexpression of Dp71 Might Lead to Degeneration of Dystrophin-Deficient Purkinje Fibers Through Dislocation of Urophin**

Our study also showed that overexpression of Dp71 at the sarcolemma occurred concurrently with vacuolar degeneration of dystrophin-deficient Purkinje fibers. Indeed, previous transgenic mouse studies indicated that overexpression of Dp71 as well as Dp116 damaged or exacerbated normal or mdx mice skeletal muscle pathology. Dp71 and Dp116 are C-terminal isoforms of dystrophin and contain cysteine-rich and C-terminal domains that bind to DAP complexes, but both isoforms lack the rod and actin-binding domains of dystrophin. Therefore, Dp71 cannot link to the sarcolemma and actin cytoskeleton. Previous transgenic mouse studies demonstrated that muscle damage is caused by competition between Dp71 or Dp116 and dystrophin or utrophin at the sarcolemma. In our study, utrophin expression was upregulated in 1- and 2-month-old CXMD1 Purkinje fibers, but the upregulation decreased rapidly in 4-month-old CXMD1. The overexpression of Dp71 might be involved in the mechanism for the dislocation of utrophin, giving rise to Purkinje fiber degeneration.

**μ-Calpain Accumulation and Translocation Are Involved in Degeneration of Dystrophin-Deficient Purkinje Fibers**

Our study showed that expression of μ-calpain, one of the Ca²⁺-dependent cysteine proteases, accumulated significantly near the sarcolemma or in the rims of vacuoles and that μ-calpain–sensitive proteins were degraded in CXMD1 Purkinje fibers. Previous studies indicated an increase in Ca²⁺ permeability and subsequent μ-calpain activation and translocation at the sarcolemma in dystrophin-deficient muscle fibers and suggested that the activation of μ-calpain may

Figure 5. Expression of μ-calpain in LV of normal and CXMD1 dogs. A, Immunohistochemical staining using the anti-μ-calpain antibody (anti-calpain 1 large subunit domain IV) of the LV of normal (III-E01MN in the Table) and affected (III-E02MA in the Table) 4-month-old dogs. P indicates Purkinje fibers. Bar=100 μm. B, Hematoxylin and eosin staining (top panel) and immunohistochemical staining of μ-calpain (bottom panel) of vacuolated Purkinje fibers from the affected dog shown in A. Bar=25 μm. C, Immunoblot analysis of μ-calpain expression. Samples from the dogs shown in A were separated by SDS-PAGE on a 7.5% acrylamide gel. The antibody specific to μ-calpain recognized strong expression of the 82-kDa band in affected Purkinje fibers. Myosin heavy chain (MyHC) was used as the loading control.
Figure 6. Expression of Dp71, μ-calpain, and utrophin in the LV of CXMD_pups. A, Serial cryosections from the LV of 1-, 2-, and 4-month-old affected dogs (III-203MA, III-202MA, and III-E02MA in the Table, respectively) were stained with antibodies against dystrophin C-terminal (NCL-DYS2), μ-calpain (anti-calpain 1 large subunit domain IV), or utrophin (UT-2). P indicates Purkinje fibers. Bar=50 μm. B, Immunoblot analyses of Purkinje fibers for Dp71 and μ-calpain. Samples of Purkinje fibers of the affected dogs shown in A were separated by SDS-PAGE on a 9% acrylamide gel and stained with antibodies against dystrophin C-terminal (NCL-DYS2) or μ-calpain (anti-calpain 1 large subunit domain IV). Myosin heavy chain (MyHC) was used as the loading control. C, Immunoblot analysis using anti-utrophin antibody (UT-2). Samples from the ventricular myocardium and Purkinje fibers of the normal (III-204MN, III-E09MN, and III-E01MN in the Table, respectively) and affected 1-, 2-, and 4-month-old dogs shown in A were separated by SDS-PAGE on a 9% acrylamide gel. MyHC was used as the loading control.
Affect Purkinje fibers. It is well known that fewer myofibrils are present in dystrophin-deficient Purkinje fibers. It is not, however, clear why dystrophin deficiency preferentially affects Purkinje fibers. It is well known that fewer myofibrils contribute to the pathogenesis in fibrillating human atria. Moreover, it has been reported that proteolysis near the sarcolemmal ruptures increases the activity of calcium leak channels. It is therefore intriguing that accumulation of \( \mu \)-calpain was closely related to the appearance of vacuolar degeneration of CXMD\(_5\) Purkinje fibers. Translocation to the membrane might be one of the important steps in the regulation of enzymatic activities of calpains. A previous report showed that calpain is present in the cytosol in an unactivated at the cell membrane. Hence, our results of calpain–sensitive proteins (desmin, cardiac troponin T [cTnT], and cardiac troponin I [cTnI]) and internal control (\( \alpha \)-sarcromeric actin) in the microdissected myocardium and Purkinje fiber of the normal dog (III-2006FN in the Table) and CXMD\(_5\) (III-1903MA in the Table). Arrowheads indicate degradation forms of each molecule. B, Immunoblot analysis of cardiac troponin I in the serum of a 9-month-old CXMD\(_5\) who died a sudden death (III-D55MA in the Table). Arrowheads show degraded forms of cardiac troponin I. Albumin (Alb) and immunoglobulin G (IgG) are shown in a Coomassie Brilliant Blue staining of the same blotting membrane (bottom panel).

Degradation of Purkinje Fibers Might Be Associated With ECG Abnormalities or Crucial Ventricular Arrhythmias in Dystrophinopathies

We recently showed that characteristic deep Q waves were found in CXMD\(_5\), and this finding preceded posterobasal myocardial fibrosis. In normal LV, the initial QRS vector is oriented to the right, anteriorly, usually superiorly, and gives rise to small negative q waves. Septal q waves were prominent in dystrophin-deficient heart. Indeed, the deep septal q (distinct deep Q) waves are mainly developed not only in leads II, III, and aVF in CXMD\(_5\) and GRMD\(_1\) but also in leads I, aVL, and V\(_5\) to V\(_6\) in human DMD. The degeneration of Purkinje fibers might cause a delay of depolarization at the posterobasal region, resulting in distinct deep Q waves, although we need to carefully rule out degeneration of the myocardium itself at further molecular levels. It is noteworthy that 10 of 12 patients with muscular dystrophy, including 6 with DMD and Becker muscular dystrophy, had a prolonged His-ventricular interval, which indicates a delay of the His-Purkinje system.

We and other researchers have suggested that the lack of specific membrane proteins might modulate particular ionic current, resulting in ECG changes. It has been reported recently that the expression of the cardiac sodium channel Nav1.5 has been altered and sodium current decreased in the mdx/cTnT knock-in mouse and increased protein expression of the ryanodine receptor, implying the participation of the stretch-activated channels even in younger mdx mice. In addition, IP3-dependent calcium release has been shown to be involved in calcium imbalance in dystrophin-deficient cultured myotubes. Other than the physiological Ca\(^{2+}\) transient in Purkinje fibers, which lack a T-tubule system, abnormal Ca\(^{2+}\) channels possibly affect subsarcolemmal Ca\(^{2+}\) homeostasis in dystrophin-deficient Purkinje fibers.

In our dystrophic dog colony, one 9-month-old CXMD\(_5\) developed ventricular arrhythmia and experienced a sudden death, the details of which were described in our recent report. To evaluate the arrhythmias in CXMD\(_5\) colony, we performed Holter monitoring and found that 1 CXMD\(_5\) dog frequently showed nonsustained ventricular tachycardia (N.U. and N. Yugeta, DVM, PhD, unpublished data, 2003). In DMD, sudden death, presumed to be associated with ventricular arrhythmias, was not rare. Cardiac conduction blocks, such as atrioventricular block, bundle-branch block, and fascicular block, were a common complication in the
It is intriguing to note that a part of the His bundle and bundle branches as well as terminal Purkinje fibers is composed of cardiac Purkinje cells. Therefore, the conduction delay in the His-Purkinje system or conduction block due to the degeneration of Purkinje fibers may underlie the generation of ventricular arrhythmias in dystrophic deficiency. In addition, a case report describes sustained bundle generation of ventricular arrhythmias in dystrophin deficiency. Therefore, the degeneration of Purkinje fibers may underlie the delay in the His-Purkinje system or conduction block due to the degeneration of Purkinje fibers and development of arrhythmias.

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Disclosures
None.

References


38. Greenberg DS, Sunada Y, Campbell KP, Yaffe D, Nudel U. Exogenous dystrophin and dystrophin-associated proteins in myocardial development of Duchenne muscular dystrophy in Japan (CXMDJ), an appropriate dystrophin-deficient model of Duchenne muscular dystrophy, the Purkinje fibers showed remarkable vacuolar degeneration as early as 4 months of age despite no detectable fibrotic lesions in ventricular myocardium. The degeneration of CXMDJ, Purkinje fibers was coincident with overexpression of Dp71, a C-terminal truncated form of dystrophin, at the sarcolemma and translocation of calcium-dependent protease μ-calpain to the cell periphery near the sarcolemma or in the vacuoles. Utrophin, a homologue of dystrophin, was highly upregulated in the earlier stage of CXMDJ, Purkinje fibers, but the expression was dislocated when vacuolar degeneration was recognized at 4 months of age, despite preservation of dystrophin-associated proteins at the sarcolemma. The selective degeneration of Purkinje fibers may be associated with distinct deep Q waves on ECG and the fatal arrhythmia seen in dystrophin deficiency. Further clarification of the molecular mechanism of selective degeneration of Purkinje fibers may shed light on the development of new therapy for cardiac involvement in dystrophin-deficient muscular dystrophy.


**CLINICAL PERSPECTIVE**

In Duchenne muscular dystrophy, a devastating skeletal and cardiac muscle disorder caused by mutations in the dystrophin gene, cardiac failure such as dilated cardiomyopathy and arrhythmia needs to be overcome, although the respiratory management has significantly improved the life span of the patients. Among cardiac involvements, ECG findings such as distinctive deep Q waves have been noted and are ascribed to fibrosis in the posterobasal region of the left ventricle. When Urasawa et al (National Institute of Neuroscience, Tokyo, Japan) examined the hearts of dogs with canine X-linked muscular dystrophy in Japan (CXMDJ), an appropriate dystrophin-deficient model of Duchenne muscular dystrophic, the Purkinje fibers showed remarkable vacuolar degeneration as early as 4 months of age despite no detectable fibrotic lesions in ventricular myocardium. The degeneration of CXMDJ, Purkinje fibers was coincident with overexpression of Dp71, a C-terminal truncated form of dystrophin, at the sarcolemma and translocation of calcium-dependent protease μ-calpain to the cell periphery near the sarcolemma or in the vacuoles. Utrophin, a homologue of dystrophin, was highly upregulated in the earlier stage of CXMDJ, Purkinje fibers, but the expression was dislocated when vacuolar degeneration was recognized at 4 months of age, despite preservation of dystrophin-associated proteins at the sarcolemma. The selective degeneration of Purkinje fibers may be associated with distinct deep Q waves on ECG and the fatal arrhythmia seen in dystrophin deficiency. Further clarification of the molecular mechanism of selective degeneration of Purkinje fibers may shed light on the development of new therapy for cardiac involvement in dystrophin-deficient muscular dystrophy.
Selective Vacuolar Degeneration in Dystrophin-Deficient Canine Purkinje Fibers Despite Preservation of Dystrophin-Associated Proteins With Overexpression of Dp71

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