Expression and Localization of Dystrophin in Human Cardiac Purkinje Fibers

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**Background.** Mutations in the dystrophin gene produce clinical manifestations of disease in heart, brain, and skeletal muscle in patients with Duchenne and Beckers muscular dystrophy (DMD/BMD). Conduction disturbances and heart block contribute to cardiac decompensation in these patients, which suggests an important role for dystrophin in the cardiac conduction system. We therefore examined the messenger RNA (mRNA) expression and protein localization of dystrophin in normal human cardiac Purkinje fibers.

**Methods and Results.** Polymerase chain reaction amplification of isolated Purkinje fiber complementary DNA identified several alternatively spliced mRNA transcripts encoding for carboxy-terminal isoforms of the dystrophin protein. The predominant mRNA transcript detected was a splice form previously detected in the brain. Antipeptide antibodies specific for a carboxy-terminal dystrophin sequence were used for Western blot analysis and immunocytochemical localization. These antiseras detect ~400,000-d immuno-reactive band or bands on Western blot in normal heart and Purkinje fibers but not in DMD heart. Immunocytochemical staining showed that dystrophin was localized to the membrane surface of the Purkinje fiber.

**Conclusions.** These results suggest that dystrophin may be an important molecule for membrane function in the Purkinje conduction system of the heart and support the hypothesis that defective dystrophin expression contributes to the cardiac conduction disturbances seen in DMD/BMD. (*Circulation* 1992;86:147-153)

**KEY WORDS** • muscular dystrophy • heart block • arrhythmias • Duchenne

The cardiac conduction system frequently is affected in diseases that influence contractile function in heart and skeletal muscle. Heart block and arrhythmias are manifested in inherited genetic diseases such as myotonic dystrophy, Kearns-Sayre disease, hypertrophic cardiomyopathy, Emery-Dreifuss muscular dystrophy, facioscapulohumeral muscular dystrophy, and Duchenne and Becker muscular dystrophy (DMD/BMD). The proposed pathophysiological mechanisms that produce conduction defects in these patients remains descriptive, and the molecular basis for maintenance of a normal cardiod impulse remains poorly understood. Single-gene defects that produce abnormal cardiac conduction allow insight into cellular protein interactions associated with normal impulse generation and propagation.

DMD/BMD has been characterized by gene mutations that result in defective or absent expression of a membrane-associated protein called dystrophin. In addition to the skeletal and cardiac myopathies observed in DMD/BMD, these patients often develop atrial tachycardia and are prone to arrhythmias and complete heart block. Histological studies have shown that cardiac Purkinje fibers display pathological necrosis similar to the degenerative changes seen in the skeletal muscle of these patients. Therefore, normal dystrophin expression also appears to be required for the integrity and function of the conduction system of the heart.

To provide a basis for understanding the conduction defect in DMD/BMD, normal human Purkinje fibers were analyzed for the presence of dystrophin messenger RNA (mRNA) transcript(s) and protein. We found several alternatively spliced mRNA transcripts from the dystrophin gene expressed in Purkinje fibers. These different transcripts encode for multiple isoforms of the dystrophin protein, which primarily is localized to the Purkinje fiber cell membrane.

**Methods**

**Purkinje Fiber Isolation**

Human cardiac Purkinje fibers were isolated from 36 explanted hearts at the time of transplantation. The fibers were clipped as free intraventricular strands that cross the ventricular cavity, avoiding contamination with cardiac muscle cells. Purkinje fibers were either immediately frozen in liquid nitrogen or fixed in 4%
paraformaldehyde in phosphate-buffered saline (PBS) at 4°C overnight.

Polymerase Chain Reaction Analysis of Dystrophin Transcripts

Total RNA from human cardiac Purkinje fibers obtained from 15 explanted hearts was prepared by phenol/chloroform extraction and precipitation with isopropanol. The RNA was dissolved in water followed by a second ethanol precipitation. RNA 5 μg was then reverse-transcribed into complementary DNA (cDNA) as described previously and resuspended in 50 μl H2O. Polymerase chain reaction (PCR) was carried out using 40 ng cDNA, 200 μmol each dNTP and 2 U Taq polymerase in 10 mmol Tris-HCl (pH 8.3), 50 mmol KCl, and 1.5 mmol MgCl2, with 200 nmol of each primer in a 50-μl reaction volume. Reaction conditions were 94°C (30 seconds), 61°C (20 seconds) annealing, and 72°C (3 minutes) extension for 35 cycles. The primers used were designed to amplify three segments of the 3′ dystrophin mRNA transcript containing regions known to undergo alternative splicing. Oligonucleotides 25 nucleotides in length were prepared on an Applied Biosystems oligonucleotide synthesizer.

The dystrophin segments analyzed were as follows. Segment 1 was cDNA sequence 9,938–10,311 amplified with forward primer 5′-TCCCAAGACAGTTGGGT-GAAGTTGC-3′ and reverse primer 5′-TCTCTCC-3′. This segment contains a 167-base-pair (bp) sequence (10,016–10,182) that has been shown to be alternatively spliced in smooth muscle, resulting in a frame shift encoding for a truncated protein. Segment 2 was dystrophin cDNA human sequence 10,287–10,948 amplified with forward primer 5′-GCACCTCGACTACATCGAGAGAAG-3′ and reverse primer 5′-TTCCAGATTGTCTCATCCGATCTCC-3′. This segment contains a 330-bp sequence (located between bases 10,432 and 10,761) with four tandem exons of sizes 39 bp, 66 bp, 66 bp, and 159 bp, which are alternatively spliced in mouse and human heart and brain tissue. Segment 3 was human cDNA sequences 10,945–11,345 amplified with forward primer 5′-GGAAGACACAAATACAGCTGGAG-3′ and reverse primer 5′-GCTTCTTCTCATCTGTCAT-GACTG-3′ containing an alternatively spliced 32-bp exon (bases 11,223–11,254) that causes a frame shift that encodes for a unique C-terminal peptide sequence. PCR products were analyzed by 10 μl of the reaction on a 3% NuSieve gel and staining the electrophoretically separated DNA with ethidium bromide. The identity of the individual bands from each PCR reaction were verified by Southern blot transfer to GeneScreen (DuPont) and probed with 3′ γ-ATP (New England Nuclear) end-labeled internal oligonucleotides specific for the dystrophin transcript. The size of the bands detected on Southern blot were known alternatively spliced transcripts previously sequenced from heart and brain tissues.

Antipeptide Antibodies

Synthetic peptides corresponding to the deduced amino acid sequence for dystrophin were prepared by solid-phase synthesis. Two milligrams of a 10-amino-acid C-terminal dystrophin peptide (R-N-T-P-G-K-P-M-R-E) was coupled to 2 mg bovine serum albumin (BSA) with 1-ethyl-3-(3-dimethylaminopropyl) carbodi-imide hydrochloride (EDC, Pierce) and purified by gel filtration before injection. The BSA-peptide conjugate (200 μg) was then injected subdermally into New Zealand White rabbits with Freund's complete adjuvant (Pierce) and subsequent booster injections at 8-week intervals in Freund's incomplete adjuvant. Rabbit serum containing antidyshrophin antibodies was collected 14 days after the last booster injection.

Commercially available mouse monoclonal antidyshrophin antibodies (Novocastra, UK) also were used to confirm our antibody specificity. Anti-creatine kinase (MCK) antibodies used for control immunofluorescence staining have been characterized previously.

Western Blot Analysis

Purkinje fibers and cardiac ventricular tissues from 11 explanted hearts were solubilized in buffer (100 mmol Tris [pH 8.0], 10% sodium dodecyl sulfate, 10 mmol EDTA, and 100 mmol β-mercaptoethanol) at a ratio of 1:10 (wt/vol) using a Dounce homogenizer. The samples were boiled for 5 minutes and then cooled briefly on ice. The supernatant was collected after centrifugation at 3,500g for 15 minutes and stored at −20°C. Proteins were resolved electrophoretically by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 4.0% polyacrylamide gels with a bis-to-acrylamide ratio of 1:29 and a 3.0% stacking gel. The gels then were blotted onto a nitrocellulose membrane (0.2 mm, Schleicher and Schuell) for 2 hours at 220 mA constant current using a Tris (25 mmol)-glycine (190 mmol) transfer buffer containing 20% methanol. The membrane was blocked in Tris (10 mmol [pH 8.0])-buffered saline, 0.05% Tween-20 (TBST) containing 3% BSA for 15 minutes. Serum containing antidyshrophin antibodies was diluted 1:500 in TBST and incubated with the membrane for 1 hour at room temperature. The membrane then was washed three times for 5 minutes with TBST and incubated with alkaline phosphatase–labeled anti-rabbit immunoglobulin (IgG) antibodies (1:1,000 dilution in TBST; Sigma Chemical, St. Louis, Mo.) for 1 hour. The membrane then was washed three times for 5 minutes with TBST, and the immunoreactive bands were detected by incubation with nitroblue tetrazolium (0.2 mg/ml, Sigma Chemical) and bromochloroindolyl phosphate (0.1 mg/ml, Sigma Chemical) in buffer (0.1 mol/l Tris [pH 9.5], 0.1 mol/l NaCl, 5 mmol MgCl2) for 30 minutes.

Immunocytochemistry

Human cardiac Purkinje fibers from 10 explanted hearts were fixed in 4% paraformaldehyde and imbedded in paraffin and 5-μm sections were prepared for staining. The slides were blocked with 3% BSA in TBST for 15 minutes and then incubated with antidyshrophin antibody (1:100 dilution) for 2 hours at room temperature, washed three times for 5 minutes with TBST, and incubated with FITC-coupled anti-rabbit IgG secondary antibody at 1:128 (Sigma Chemical) for 1 hour. The slides then were washed three times with TBST for 5 minutes, and the fluorescence was visualized under ultraviolet light with a Zeiss Axioshot microscope.
FIGURE 1. Panel A: Polymerase chain reaction (PCR) amplification of three segments of 3′ complementary DNA from human cardiac Purkinje fibers detects multiple spliced transcripts. Top: Line diagram of exons that undergo alternative splicing. Left panel: Segment 1 (bases 9,938–10,311) amplification shows only the full-length 374-bp PCR product (top arrow), and the spliced product with a 159-bp exon excised is not detected (lower arrow). Middle panel: Segment 2 (bases 10,287–10,948) amplification detects the full-length 662-bp PCR product (top arrow) and several smaller bands, the lowest at 332 bp (bottom arrow). *PCR products in segment 2 determined by Southern blot to represent splice forms of the dystrophin gene (see panel B and Reference 15). The identity of the other two bands has not been confirmed, and they may represent nondystrophin amplification products.15 Right panel: Segment 3 (bases 10,945–11,345) amplification detects both the full-length form at 401 bp (top arrow) and the presence of the smaller spliced form at 369 bp (lower arrow) with a 32-bp exon excised. Seg, segment; ΦX174, ΦX174 DNA cut with HaeIII as a size standard. Panel B: Detailed representation of the multiple splice forms of dystrophin detected by amplification of segment 2 (see panel A). Southern blot confirmation of at least five spliced transcripts is demonstrated using a dystrophin-specific oligonucleotide internal to the primers used in the segment 2 PCR (left inset). These splice forms represent transcripts previously detected in murine and human brain and heart tissues14,15 and are shown schematically by their respective splicing patterns. The dystrophin complementary DNA coordinates for this region are along the top of the figure, and the four tandem alternatively spliced exons are depicted as rectangles with their base-pair size determined by sequence analysis.15 The size of each PCR fragment and the corresponding exon deletions (indicated as “deletion size”) are shown to the right of each splice figure. There is no cross hybridization to the PCR products derived from segments 1 and 3 (panel A), which were run in the agarose gel lanes flanking segment 2 on the Southern blot. The other fainter bands detected on the Southern blot are other dystrophin splice forms from segment 215 that do not appear to be significantly expressed in cardiac Purkinje fibers.
Results

Dystrophin mRNA and protein expression were analyzed in normal human cardiac Purkinje fibers as a first step in defining the potential role for dystrophin in the conduction system of the heart. Multiple alternatively spliced mRNA transcripts encoding for C-terminal isoforms of the dystrophin protein were detected by PCR from Purkinje fiber cDNA. Subsequent analysis for dystrophin protein expression and localization was confirmed by Western blot analysis and immunofluorescent staining of human cardiac Purkinje fibers. These results demonstrate the normal expression and localization of dystrophin isoforms in human cardiac Purkinje fibers.

Multiple mRNA Isoforms of Dystrophin Are Detected in Human Cardiac Purkinje Fibers

Previous studies have analyzed and sequenced alternatively spliced dystrophin mRNA via PCR amplification of selected regions of the transcript from tissues that express dystrophin.\textsuperscript{14,15} PCR amplification of dystrophin cDNA derived from human cardiac Purkinje fibers detected multiple PCR products (Figure 1A) that represent a number of alternatively spliced transcripts previously described in heart and brain.\textsuperscript{14,15} For example, Figure 1A shows that PCR amplification of segment 3 detected both a "full length" form (right panel, upper arrow) and a small amount of the alternatively spliced form that has a 32-bp exon deleted and is amplified as a smaller PCR product (right panel, lower arrow). In contrast, some previously described splice forms were not detected in cardiac Purkinje fibers. Such is the case for segment 1, which shows amplification of the full-length PCR product (left panel, upper arrow) but did not display amplification of a smaller spliced form detected previously in smooth muscle from the aorta (14; left panel, lower arrow).

The most complex splicing pattern is observed within segment 2, where PCR amplification detected a number of bands suggesting multiple splice forms in this region (Figure 1A, middle panel). To elucidate which of the bands in segment 2 corresponds to products derived from the dystrophin gene, Southern blot analysis was performed using a \textsuperscript{32}P-labeled internal oligonucleotide as a probe (cDNA sequence 10,391–10,415). Figure 1B (left inset) shows the Southern blot analysis of a gel containing the PCR products from segments 1–3 (Figure 1A reactions displayed as adjacent lanes on an agarose gel) and demonstrates hybridization to at least five bands in only the segment 2 lane, each of which represent a different splice form. There is no hybridization to the bands derived from segment 1 or 3 of the dystrophin transcript, illustrating the specificity of the oligonucleotide hybridization. Previous studies had demonstrated by direct-sequence analysis the existence of at least 11 different splice forms in this region of the dystrophin transcript formed by alternative splicing of four tandem exons in various combinations.\textsuperscript{15} The splicing format of the more predominant splice forms detected in cardiac Purkinje fibers is represented schematically in Figure 1B. The major splice form in cardiac Purkinje fibers appears as the lowest band on the ethidium-stained gel and Southern blot, which has 330 bp (all four exons) spliced out (Figures 1A and 1B).

Dystrophin Protein Is Localized at the Purkinje Fiber Cell Membrane

Isolated human Purkinje fiber homogenates were resolved by SDS-PAGE size separation followed by Western blot analysis. Figure 2 shows the pattern of immunostaining using antidystrophin antibodies. A specific \textasciitilde400,000-d protein band is detected in normal heart and cardiac Purkinje fibers but not in the heart of a dystrophin-deficient patient with DMD. The size and specificity of this immunoreactive band were verified with a commercially available mouse monoclonal antidystrophin antibody (Novocastra Labs). Specific dystrophin immunoreactivity was present in eight normal human and C57 mouse muscle tissues and was absent in dystrophin-deficient muscle tissue from four DMD patients and eight mdx mice (data not shown), consistent with previously reported data.\textsuperscript{19} Because of the large size of the dystrophin protein, resolution of the dystrophin isoforms detected in cardiac Purkinje fibers (predicted by the cDNA splice forms to vary by \textasciitilde1–10,000 d) was not possible in this gel system (Figures 1 and 2).

Immunocytochemical staining of dystrophin in tissue sections of isolated human cardiac Purkinje fibers...
FIGURE 3. Immunofluorescent staining of human cardiac Purkinje fibers. Panel A: Negative control; absent staining of Purkinje fibers with preimmune sera and FITC-coupled anti-rabbit immunoglobulin G. Panel B: Cytosolic control; positive staining using anti-creatine kinase antibodies shows a granular intracellular immunofluorescence with no background staining of the Purkinje fiber membrane. Panel C: Antidystrophin antibodies show immunofluorescent localization of dystrophin to the membrane of a Purkinje fiber cell shown in cross section. Some cytosolic staining also is apparent. Panel D: Low-power view of a cross section of a Purkinje fiber bundle with antidystrophin antibodies showing immunofluorescent dystrophin membrane staining present in all Purkinje fibers.
showed intense immunoreactive staining of the Purkinje membrane and, to a lesser extent, staining within the cytosol (Figures 3C and 3D). Two types of controls were used to assess the specificity of the immunostaining. First, the use of preimmune serum at an identical dilution showed no significant reaction product (Figure 3A). Second, a different antipeptide antisera directed against an unrelated protein (M-creatine kinase [M-CK]) shows a different pattern of staining, only within the cytosol of the Purkinje fiber (Figure 3B).

Discussion

Patients with DMD/BMD have a spectrum of clinical phenotypes that include skeletal myopathy, cardiomypathy, and mental retardation, and they have been characterized by absent or diminished expression of the dystrophin protein in skeletal muscle, heart, and brain. Histological Purkinje fiber necrosis and conduction abnormalities in patients with DMD/BMD suggest that dystrophin is important for the function and integrity of cardiac Purkinje fiber. Although the absence of dystrophin may not be the sole mechanism for cardiac conduction disturbances in these patients, it probably plays a major role. This is the first study defining the normal mRNA expression and protein localization of dystrophin in human cardiac Purkinje fibers, a subset of a variety of cell types that comprise the conduction system in the heart.

Cardiac Purkinje fibers display a number of differences in membrane anatomy, intracellular proteins, and cellular morphology that distinguish them from the surrounding myocardium. They are multinucleated cells that lack a T-tubule system, and they express an isoform of myosin heavy chain not found in the surrounding ventricular myocardial cells. It is unclear whether they are developmentally derived from mesodermal or ectodermal (neural crest) origin. Analysis of creatine kinase isoforms in cardiac Purkinje fibers shows that the differential expression of this enzyme displays some brainlike properties (M.D. Cortez, D.L. Friedman, T.S. Ma, H.T. Shih, P.R. Puleo, R. Roberts, M.B. Perryman; manuscript in preparation). Previous studies have shown that dystrophin mRNA is alternatively spliced at the 3' end encoding multiple protein isoforms, and that some of these isoforms are expressed in a tissue-specific and developmentally regulated pattern. Human cardiac Purkinje fibers express a splice form of dystrophin mRNA that has been shown to predominate in brain tissue. Brain dystrophin is found only at cortical postsynaptic neuronal junctions, in contrast with the ubiquitous membrane staining in skeletal muscle and heart tissues. It is tempting to speculate that dystrophin splice forms represent a variation in dystrophin function or a diversification in a region of the protein that associates with other cytoskeletal or integral membrane proteins. The expression of multiple isoforms of dystrophin in cardiac Purkinje fibers may reflect the specialized functional properties of this tissue.

The exact function of dystrophin is not understood. It is believed to be structural protein that is associated with a large integral membrane glycoprotein complex at the inner surface of the cell membrane. When dystrophin is absent in skeletal muscle, at least one of these glycoproteins is lost, and intracellular calcium levels are increased. These observations suggest that dystrophin may be linked to receptors or ion channels in the cell membrane. At least some of the elevated cytosolic calcium is known to be due to an increase in calcium leak channel activity at the membrane surface. An increase in stretch-inactivated calcium channels in dystrophic muscle also has been described. It is believed that elevated intracellular calcium activates proteolytic enzymes and phospholipases that degrade cellular phospholipids and produce lysophosphoglycerides, compounds that have significant electrophysiologic toxicity. Lyso phosphoglyceride production via elevated intracellular calcium in ischemic myocardium has been shown to cause membrane depolarization, automaticity, and changes in action potential configuration. This suggests that the conduction disturbances in DMD/BMD may be a combination of calcium ion disequilibrium and cellular necrosis. In the absence of dystrophin, expression of other dystrophin-like molecules may play a role in these abnormal physiological mechanisms.

To our knowledge, this is the first demonstration of a protein molecule normally expressed in the Purkinje system of the heart that is directly associated with conduction disease caused by a genetic defect. Further studies of the molecular complex associated with dystrophin at the Purkinje fiber cell membrane may provide insight into the physiological mechanisms responsible for normal membrane potentials and impulse propagation.

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