A SEVERE MUSCULAR DYSTROPHY PATIENT WITH AN INTERNALLY DELETED VERY SHORT (110 kD) DYSTROPHIN: PRESENCE OF THE BINDING SITE FOR DYSTROPHIN-ASSOCIATED GLYCOPROTEIN (DAG) MAY NOT BE ENOUGH FOR PHYSIOLOGICAL FUNCTION OF DYSTROPHIN

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(Received 2 September 1994; revised 17 November 1994: accepted 24 November 1994)

Abstract—We report a 4-yr and 5-month-old boy with severe clinical features of an early-onset Duchenne muscular dystrophy, who had a very short (110 kDa) dystrophin at the sarcolemma. The patient had a large deletion (exons 2-44) of the dystrophin gene which was predicted to cause a reading frame shift. Sequence analysis of dystrophin mRNA in muscle revealed an alternatively spliced gene product from exons 1 to 51 that caused restoration of the reading frame, in addition to an mRNA corresponding to the DNA deletion. A consistent result was obtained by immunocytochemical analysis of muscle; i.e. positive staining for dystrophin at the sarcolemma using antibodies against the C-terminus, cysteine-rich region and last three of 24 repeat units of the central rod-domain, but not for the remaining antibodies for dystrophin that recognize the N-terminal and proximal rod-domains. Immunostaining for dystrophin-associated glycoproteins (DAGs: 43 and 50 K) and merosin were preserved. Utrophin staining was positive but fainter than other DMD muscles. These results suggest that an extremely short dystrophin lacking the entire actin-binding site in the N-terminus cannot function properly even if the protein possesses the putative DAG-binding cysteine-rich and the C-terminal domains, and still has an ability to associate with sarcolemmal membrane.

Key words: Dystrophin, actin-binding domain, alternative splicing, dystrophin-associated glycoprotein (DAG), muscular dystrophy.

INTRODUCTION

Duchenne and Becker muscular dystrophies (DMD/BMD) are the most common X-linked diseases caused by mutations of the dystrophin gene [1,2]. In DMD muscle, dystrophin is barely detectable by both immunoblot and immunocytochemical analyses [3–7] while abnormal dystrophin with altered size and/or abundance is present in BMD muscle [8–11]. Such observations support the membrane hypothesis of DMD [12,13].

Recently, dystrophin has been shown to bind to the dystrophin-associated membrane glycoproteins (DAGs) through its C-terminal end [14], specifically through the cysteine-rich and the first half of the C-terminal domains [15]. Interactions between dystrophin and laminin (a major component of the basal lamina) through the laminin-binding 156 K DAG have also been suggested [16], and abnormal merosin (laminin-2 which has laminin M or α2 chain) expression is associated with muscular dystrophy in homozygous dy/dy mice which are known as a murine animal model for muscular dystrophy [17–19]. In DMD muscle, DAGs are either dramatically reduced [20] or reduced but
clearly positive [21], while merosin is unchanged [22]. Furthermore, missense mutations in the 50 K DAG (adhalin) gene at 17q12–21 has been found in a French family that shows DMD-like severe childhood autosomal recessive muscular dystrophy (SCARMD) [23]. On the other hand, SCARMD prevalent in North Africa has been mapped to chromosome 13q [24,25]. These results suggest the important role of dystrophin, DAGs and merosin interaction for producing the physiological function of dystrophin.

We have previously reported that the C-terminus of dystrophin is required for the physiological function of dystrophin, since the C-terminus was preserved in 30 BMD patients and 24 control skeletal muscles but not in 21 DMD muscle specimens [26]. However, several exceptional patients that did not fit our previous observation were subsequently reported [27–30]. Vainzof reported four out of 142 DMD patients who had positive dystrophin staining for the antibody against the C-terminus. Hoffman et al. reported a unique case of severe DMD, whose muscle contained substantial amounts of Becker-like abnormal dystrophin with no C-terminal domain but had variable sarcolemmal immunostaining using dystrophin antibodies against the rod and cysteine-rich domains. Récan et al. and Helliwell et al. also identified truncated dystrophin (270 and 225 kDa, respectively) lacking the cysteine-rich and C-terminal domains but which still retained sarcolemmal localization. These results suggest that dystrophin can associate with the sarcolemmal membrane without the cysteine-rich and C-terminal domains. Nonetheless, an important fact is that, from a clinical point of view, all of these exceptional dystrophic patients reported were diagnosed as having DMD but not milder BMD. These observations again support the hypothesis that the cysteine-rich and the C-terminal domains of dystrophin are crucial for its physiological function [26,31–33].

On the other hand, for the proper function of dystrophin, other functionally important domains have been pointed out besides the cysteine-rich and C-terminal domains [30,32–35]. In the present study, we report a patient with severe muscular dystrophy who has an extremely small (110 kDa) dystrophin that lacks the entire actin-binding site(s) in the N-terminal domain but still maintains the cysteine-rich and C-terminal domains at the sarcolemmal membrane.

**MATERIALS AND METHODS**

**Patient**

A 4-yr and 5-month-old boy was born to healthy parents as their third full-term pregnancy. There was no family history of neuromuscular diseases. The mother was aware of slightly decreased fetal movements during pregnancy. The delivery was spontaneous and the patient’s birth weight was 3230 g. The neonatal period was uneventful except for poor sucking activity. The patient could control his head and turn over at four months, sit alone at six months, but was unable to walk alone until 1 yr and 5 months. At age 8 months, an unexpectedly high serum creatine kinase (CK) activity was found (19,000–38,000 Ul−1) upon admission to the hospital for febrile convulsion. At age 1 yr and 9 months he was referred to the National Center of Neurology and Psychiatry for diagnostic muscle biopsy. His height and weight were below normal; BH 78 cm (−2 S.D.), BW 10.3 kg (−1.0 S.D.). On neurological examinations, he displayed mild hypotonia and weakness in his upper and lower proximal muscles without facial muscle involvement. Deep tendon reflexes were absent in the upper limbs and decreased in the lower limbs. He exhibited a positive Gowers’ maneuver, waddling gait and pseudohypertrophy of his calf muscles. Needle EMG revealed myogenic abnormalities and nerve conduction velocities were normal. CT scans and ultrasonography of muscle confirmed the muscle atrophy and pseudohypertrophy of calf muscles. Both serum CK (64,285 Ul−1; normal = 50–280) and aldolase (159.0 Ul−1; normal = 2.5–6.1) levels were elevated. At age 3 yr, he took more than 10 s to get up from the floor. Currently (4 yr and 5 months), although still able to walk without support, he cannot get up from the floor by himself. He has a progressive lordotic posture and has marked muscular wasting in the shoulder-girdle and upper arm muscles. Deep tendon reflexes are absent except in the Achilles reflexes. He has neither intellectual abnormality nor cardiac muscle involvement.
Muscle biopsy

A muscle biopsy specimen was obtained from the left biceps muscle for diagnostic purposes with informed consent at age 1 yr and 9 months. The muscle sample was flash-frozen in isopentane chilled with liquid nitrogen and was processed for a battery of histochemistry, immunocytochemistry and immunoblotting tests. DNA and mRNA analyses for dystrophin were performed using the peripheral blood lymphocytes and muscle samples as indicated below. Age-matched patients with other neuromuscular diseases were studied at the same time as the diseased controls.

Dystrophin tests

**Immunofluorescence.** We used antibodies raised against nine different regions of dystrophin as shown in Fig. 1. Six anti-dystrophin polyclonal antisera (D1-2a, 6-10, D8, D9, D10, D11,) were kindly provided by Drs. L. Kunkel and T. Byers. Three monoclonal anti-dystrophin antibodies (2-5E2, 3-4G4, 4-4C5) were raised against synthetic peptides corresponding to amino acids 440-489 (2-5E2), 2359-2408 (3-4G4) and 3495-3544 (4-4C5), respectively. Anti-LDP [36] for dystrophin-related protein, anti-DAGs [21,37] for 43 and 50 K, and anti-laminin M chain antibodies were also used [22]. Indirect immunofluorescence was done as previously described [10,26] on serial frozen sections. All fluorescent photographs were taken with Ektachrome 400HC under a Zeiss Axiophot microscope with epifluorescence.

**Immunoblotting.** Immunoblot analysis of dystrophin was done as previously described [5,38]. Ten 20-μm frozen sections of muscle were homogenized in SDS-sample buffer [2% SDS, 0.125 M Tris–HCl buffer (pH 6.8), 5% 2-mercaptoethanol] and boiled for 5 min. The final loading volume for SDS-polyacrylamide gel electrophoresis was adjusted after densitometric measurements of myosin heavy chain (MHC) (by TIAS Image Processor/NEC PC 9801RX) on the Coomassie blue stained gel.

**DNA analysis of the dystrophin gene.** DNA was isolated from peripheral blood lymphocytes and muscle samples as previously reported [32,39]. Multiplex PCR analysis for 27 dystrophin exons were performed using standard procedures [31,39–41].

Analysis of dystrophin mRNA.

RT-PCR and nested PCR (=nested RT-PCR) analysis. Total RNA was extracted from frozen muscle samples by acid guanidinium thiocyanate–phenol–chloroform methods [42]. Reverse transcription and nested PCR (nested RT-PCR) were done as described by Roberts et al. [43]. The first PCR amplification was done using primers sets of exon 1a (5′-CTTTCCCC-CTACAGGAACCTCAG-3′) and exon 51a (5′-GTCACCCACCTACCCCTTCTG-3′), and for the second amplification exon 1b (5′-CTGGGAGCCAATTACCCCTGG-3′) and exon 51b (5′-GTTAAGTTCTCTGCAAGCC-CGG-3′) or exon 46 (5′-GCAATGTATCT-GCTTCCCTC-3′) were used. These primer sets were expected to amplify the exons between 1 and 51.

**Sequence of the PCR amplified products.** The amplified nested RT-PCR products were separated by agarose gel electrophoresis and extracted sequentially with phenol, phenol-chloroform, chloroform, followed by ethanol precipitation at −20°C, and then dissolved in 1 × TE solution. The DNA samples were cloned into the pCR vector using the TA cloning Kit (Invitrogen, Inc. U.S.A.) and the purified plasmids were sequenced using an AutoRead sequencing Kit and A.L.F. Autosequencer (Pharmacia).

RESULTS

**Muscle histology and immunocytochemistry for dystrophin.** The patient’s muscle had severe dystrophic changes with occasional necrotic, hypercontracted and regenerating muscle fibers. There was a marked increase in the amount of endomysial and perimysial fibrous and fatty connective tissues. Immunofluorescent staining using antibodies against the N-terminal and first 20 repeat units in the rod-domain of dystrophin (D1–2a, 2–5E2, 3–4G4, D8) failed to show any positive staining (Fig. 1). In contrast, antibodies recognizing the last four repeats of the rod-domain, the cysteine-rich and the C-terminal domains (D9, D10, 4–4C5, D11) showed a Becker-like immunostaining pattern for dystrophin around each muscle fiber (Fig. 1). Age-matched control muscle exhibited normal staining for dystrophin with
Fig. 1. The bar at the top indicates the dystrophin molecule. The four main domains are: amino (N)-terminus (black box), central repeating (24 repeats are indicated) rod-domain, cysteine-rich region (diagonal lines) and C-terminal domain (open box). Localization of regions used to raise a battery of antibodies are shown over the bar with black lines. Immunoreactivities for anti-dystrophin antibodies are indicated with plus or minus below the bar. Localization of the DNA deletion (exons 2-44) is shown by the broken line with arrow heads on both sides. Immunofluorescence staining for dystrophin in the present patient (a-h) and an age-matched control (i-p) are shown. Note the negative immunostaining for antibodies that recognize epitopes within the DNA deletion and outside the deletion.
all of the antibodies tested. Twenty other DMD muscle samples examined in parallel had negative immunostaining for dystrophin against all the antibodies, with the exception of rare revertant fibers. Thus, the patient had a unique immunostaining pattern for this panel of dystrophin antibodies (Fig. 1).

Immunoblot analysis of dystrophin

By immunoblotting, an abnormally small dystrophin with a molecular weight of 110 kDa was detected using antibodies against the C-terminus (Fig. 2). Consistent with the immunofluorescence results, antibodies D1–2a, D8 and 3–4G4 did not detect the small dystrophin (data not shown).

Deletion analysis of the dystrophin gene

Polymerase chain reaction of the dystrophin gene was performed on DNA extracted from the diagnostic muscle biopsy. The patient had a large deletion spanning from exon 2 to 44 as shown in Fig. 3. This deletion was predicted to cause a reading frame shift.

Analysis of dystrophin transcripts in muscle

Nested RT-PCR amplification of the patient's muscle mRNA using primer sets of exons 1a/51a and 1b/51b produced three PCR products (Figs 4a and 5) and primers for exons 1b/46 produced a single band (Figs 4b and 5). As expected, no product was detected in control muscles (data not shown). Of these, both product 1 (1216 bp) and product 4 (468 bp) (Fig. 4a,b) were shown to have a deletion from exons 2 to 44, and thus exon 1 was directly spliced to exon 45 as predicted from the DNA deletion pattern (Fig. 5). This deletion results in the reading frameshift, changing a tyrosine (TAT) to an Amber stop codon (TGA) (Fig. 5). Although the product 2 (660 bp) band (Fig. 4a) was found to be a non-specific PCR product after sequencing, the product 3 (344 bp) band (Fig. 4a) was sequenced and shown to correspond to a transcript having a deletion from exons 2 to 50. This alternatively spliced gene product had an intact reading frame that produced no amino acid substitution and is expected to result in an internally deleted protein product.

Immunostaining for DAGs, utrophin and merosin

The immunostaining pattern for DAGs (43 and 50 K) was abnormal but the immunoreactivity was clearly preserved in our patient, although he had very severe clinical and histological changes (Fig. 6). Utrophin immunoreaction at the sarcolemma was positive but was fainter than other DMD muscles (Fig. 6). Merosin (laminin –2), known to be a major component of the basal lamina, immunostained normally in all muscles examined (Fig. 6).

DISCUSSION

We have described a 4-yr and 5-month-old male patient diagnosed as having DMD because of severe clinical symptoms including early-onset weakness in the proximal limbs, dystrophic histology of muscle and a frameshift deletion of the DMD gene (exons 2–44). Unlike most DMD patients, the boy has a normal amount of a very small (110 kDa) dystrophin protein detected by antibodies against the C-
terminal domain. Nested RT-PCR and subsequent sequence analysis revealed that the patient has at least two types of dystrophin mRNA in the muscle; i.e., a correctly spliced form of exon 1–45 (frameshift) and an alternatively spliced form of exon 1 connected to exon 51 (in-frame). The alternatively spliced dystrophin contains only the last four repeat units of the rod-domain and the cysteine-rich and C-terminal domains of dystrophin. Despite lacking three out of four dystrophin molecules at the N-terminal side, the small dystrophin of this patient localized correctly at the sarcolemmal membrane together with 43 and 50 K DAGs. Although this patient’s dystrophin has the putative cysteine-rich and C-terminal domains and localizes correctly at the sarcolemmal membrane together with DAGs, the protein seems to have no physiological function (or may even have negative effects?) because the patient is apparently so severely affected. Thus, it is an interesting question as to why this patient shows such a severe clinical phenotype. There are several possible explanations. First, it is possible that the abnormal dystrophin is actually an alternative isoform of dystrophin (besides the skeletal muscle form). Recently, different sized mRNA transcripts from the normal dystrophin gene have been reported.

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Fig. 3. Multiplex PCR analysis of genomic DNA, using dystrophin-specific 27 primer sets (Beggs, 1994:32). Note that the patient has missing PCR products corresponding to exons 2–44.

Fig. 4. Amplification products of skeletal muscle RNA from the patient using the nested RT-PCR method. First amplification has accomplished with the outer primer set exons 1a/51a, followed by the second PCR amplifications by the inner primer sets of exons 1b/51b (a) and 1b/46 (b). These primer sets are expected to amplify the exons between 1 and 51 in the patient but not in normal controls.
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Fig. 5. Sequence analysis of the amplified products of the muscle RNA obtained in Fig. 4. As expected from the DNA analysis, products 1 and 4 have a continuous sequence across the junction break point of exon 1/45 resulting in an Amber stop codon. In product 3, exon 1 has been directly spliced to exon 51, producing an in-frame alternatively spliced mRNA.

Product 2 is a non-specific amplification product.

[44-48]. These 4.5, 4.8 and 6.5 kb mRNAs encode the 75-80 and 116 K protein products named Dp71, Dp116 or apodystrophin. Under normal conditions, these protein products of the dystrophin gene are not detected in adult skeletal muscle but can be found in a variety of non-muscle tissues including brain and peripheral nerve. However, the abnormal dystrophin in our patient can be distinguished from these transcripts because of the differences in size of the protein and the presence of exon 1 of the dystrophin gene. Another possible explanation may be attributed to a defect of the actin-binding site(s) at the N-terminus of dystrophin [49-52]. The N-terminal domain is essential for proper function of dystrophin as suggested by Prior et al. [35]. We hypothesize that abnormal dystrophin molecules lacking the actin-binding site in the N-terminus can still localize at the plasma membrane through the DAG-binding site at the cysteine-rich and proximal part of the C-terminal domains. However, due to their inability to bind actin these molecules do not have physiological function. The N-terminal domain of human dystrophin has 240 amino acids and is 44% identical to the F-actin-binding domain of alpha-actinin. Hemmings suggested that the actin-binding site is located within residues 20-195, Levine showed two actin binding sites (residues 17-26, 128-156) and Way showed that DMD246 (the first 246 residues of dystrophin) binds F-actin in a strongly co-operative manner, but does not bind G-actin. In our patient, nested RT-PCR and subsequent sequence analyses revealed a deletion of exons 2-50. This result indicates that all of the putative actin-binding domain (residue 14240) of dystrophin is missing in the patient’s muscle.

There are several reported DMD cases that have dystrophin with defective N-terminal domains [30,34,53]. Although these cases have not been examined for muscle mRNA, they have DNA deletions including exons 3-19, 3-25, 3-31 and 3-42 (exon 2 is preserved in which several actin binding sites are located), and abnormal dystrophin with molecular weights of 340, 320, 290 kDa and not described, respectively. Our patient has a DNA deletion from exons 2 to 44, and has an
Fig. 6. Consecutive frozen sections of biopsied skeletal muscle from control (OND a,d,g,i), present case (b,e,h,k) and DMD (c,f,i,l) immunoreacted for 43 K DAG (a,b,c), 50 K DAG (d,e,f), utrophin (g,h,i) and merosin (j,k,l). Note the fainter staining for DAGs in DMD (c,f) than OND (a,d) and the patient (b,e). In contrast, utrophin is barely detectable in both OND (g) and the patient (h), but up-regulated in DMD (i). Merosin is normally immunostained among these diseases.

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additional alternatively spliced mRNA from exons 1 to 51 in the muscle, resulting in the production of a very small dystrophin protein (110 kDa).

We have previously reported milder BMD/DMD patients having a deletion of exons 2–7 who are expected to have no actin binding site [32], but the size of the dystrophin proteins were 370–380 kDa. This finding suggests that anti-parallel homo-tetramers [54] of dystrophin in these BMD/DMD patients may be able to interact with each other as shown in the model presented by Récan et al. [28]. In contrast, in the present case the membrane-associated dystrophin molecules seem too short to interact with each other because of the huge deletion.

Given the apparent clinical severity of our patient, another question is whether very small membrane bounded dystrophin can actively cause sarcolemmal instability by interacting with other membrane protein(s) in a deleterious fashion. We are currently producing a fusion protein of the truncated dystrophin to assess the biochemical properties of this molecule. Finally, decreased upregulation of utrophin, possibly due to the preferential binding of the small-sized dystrophin to the 43 K DAG through the cysteine-rich and the C-terminal domains, can possibly increase the clinical
severity if utrophin ameliorates muscle fiber breakdown in DMD patients as suggested by Matsunura et al. [55].

Acknowledgements—We wish to thank Drs Hideo Sugita (President, National Center of Neurology and Psychiatry, Japan), Shoichi Ishiura (Institute of Molecular Biology, University of Tokyo) and Yoshikuni Mizuno (Juntendo University Hospital) for their helpful discussions and advice. We also thank Drs Louis M. Kunkel and Timothy J. Byers for a generous supply of D1-2a, D8, D9, D10, D11 and 6-10 anti-dystrophin antibodies; Dr Eijiro Ozawa for anti-43 and 50 K antibodies to DAGs and Dr Eva Engvall for anti-merosin antibodies. This research has been supported by Research Grants from the Ministry of Health and Welfare, Japan, and from the Ministry of Education, Science and Culture, Japan. A.H.B. was supported in part by the Charles H. Hood Foundation, Boston, MA.

REFERENCES


30. Vainzof M, Takata R I, Passos-Bueno M R, Pavanello R C M, Zatz M. Is the maintenance of the C-terminus domain of dystrophin enough to ensure a

Note added in proof

After this manuscript was accepted, two experimental studies that support our hypothesis were published; i.e. in the transgenic mdx mice expressing Dp71, presence of Dp71 and DAGs in muscle was not sufficient to prevent muscle fiber damage, hence it follows that both the N- and C-terminals of dystrophin are possibly required for its normal function. Nature Genet 1994; 8: 333–339 and 340–344.