Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 7154–7158, September 1989
Medical Sciences

Dystrophin diagnosis: Comparison of dystrophin abnormalities by immunofluorescence and immunoblot analyses

(Duchenne muscular dystrophy/Becker muscular dystrophy)

KIICHI ARAHATA*, ERIC P. HOFFMAN†, LOUIS M. KUNKEL†, SHOICHI ISHIURA*, TOSHIFUMI TSUKAHARA*, TADAYUKI ISHIHARA*, NOBUHIKO SUNOHARA*, IKUYA NONAKA*, EIJIRO OZAWA*, AND HIDEO SUGITA*

*National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187 Japan; and †Howard Hughes Medical Institute, Children's Hospital, Department of Pediatrics, Harvard Medical School, Boston, MA 02115

Communicated by Alexander G. Bearn, May 30, 1989

ABSTRACT **Immunoblot characterization and immunofluorescence localization of dystrophin are presented for 76 human patients with various neuromuscular diseases. Normal dystrophin (shown by immunoblotting) was invariably visualized as a continuous, peripheral membrane immunostaining of myofibers. Biochemical abnormalities of dystrophin (either lower or higher molecular weight dystrophin) resulted in patchy, discontinuous immunostaining, suggesting that the abnormal dystrophin proteins are not capable of creating a complete membrane cytoskeleton network. There was a very strong correlation of clinical diagnoses with the type of dystrophin abnormality; all Duchenne muscular dystrophy patient muscle contained no detectable dystrophin, Becker muscular dystrophy patient muscle had clearly abnormal dystrophin, and unrelated diseases showed normal dystrophin. However, a single patient of five carrying the diagnosis of Fukuyama dystrophy showed no detectable dystrophin and thus appeared to be a Duchenne dystrophy patient by the biochemical assays. We know of no other case of a patient with a disease thought to be unrelated to Duchenne/Becker dystrophy yet demonstrating dystrophin deficiency. Based on the data presented, we conclude that immunofluorescence is the best technique for the detection of female carriers of Duchenne dystrophy, whereas immunoblotting appears superior for the prognostic diagnosis of Becker muscular dystrophy.**

The underlying biochemical defects responsible for Duchenne and Becker muscular dystrophies are abnormalities of dystrophin, the protein product of the Duchenne muscular dystrophy gene (1–3). Dystrophin is thought to be a part of the membrane cytoskeleton in all myogenic cells (4–6). In cryostat cross sections of both normal muscle and muscle from patients with disorders unrelated to Duchenne/Becker dystrophy, dystrophin is visualized by immunofluorescence as a continuous, thin ring of staining at the periphery of every muscle fiber (7–9). By immunoblotting, dystrophin is detected as a large (≈400-kDa), low-abundance (<0.01% of total muscle protein) protein in normal muscle and in muscle from patients with unrelated disorders (1, 10). Both techniques have shown the specific absence of dystrophin in muscle from all patients with dystrophin dystrophy. The majority of patients with the clinically milder Becker dystrophy have been shown to have dystrophin of abnormal molecular weight (quality) and/or lower relative cellular abundance (quantity) compared to normal muscle (10, 11). Immunofluorescence studies of dystrophin localization in Becker dystrophy patients have found a disease-specific "patchy" immunofluorescent pattern (7, 8). Though dystrophin biochemical and immunofluorescence abnormalities have been reported for 68 Becker patients, no attempts have been made to correlate specific dystrophin biochemical abnormalities with cellular localization abnormalities in Becker patients. An additional diagnostic use of dystrophin analysis has been the delineation of female carriers (heterozygotes) for Duchenne dystrophy (12, 13).

Given the rapidly evolving importance of dystrophin testing in the clinical diagnosis of neuromuscular disease, it is necessary to evaluate the relative accuracies of immunoblot and immunofluorescence studies of dystrophin and to identify situations in which one type of assay system shows marked advantages over the other type. Finally, it is of basic biological interest to determine whether specific types of dystrophin abnormalities correlate with specific immunofluorescent staining patterns. To address these questions we have studied dystrophin by both immunoblot and immunofluorescence techniques in 76 patient muscle biopsy specimens.

MATERIALS AND METHODS

Patient Muscle Biopsies. All patient muscle samples were obtained as portions of the diagnostic biopsy specimens. The same muscle biopsy sample was used for both immunofluorescence analysis and immunoblot analysis, and both analyses were performed for each patient. Of the 76 samples studied, all were new, previously unpublished samples, except for those from 6 Becker dystrophy patients, which were included in a previous report (7). All clinical diagnoses were assigned in the National Institutes of Neuroscience, Japan, using standard criteria. Five of the 20 patients that were assigned a clinical diagnosis of Becker muscular dystrophy were under 5 years of age. Such young patients cannot be accurately assigned a definitive Becker dystrophy diagnosis on purely clinical grounds unless a previous X chromosome-linked history indicative of Becker dystrophy is available. Of these 5 young Becker patients, 2 had such a family history. The remaining 3 young patients were given a Becker dystrophy diagnosis based primarily on the dystrophin immunoblot analyses, which were done "blind" and have been shown to be very accurate in detecting Becker dystrophy patients at young ages (11). All 5 cases were also confirmed as Becker dystrophy by immunofluorescence analysis. Eleven of the 20 Becker patients studied had an X-linked family history.

Antisera. Each biopsy sample was tested with four different anti-dystrophin polyclonal antisera, two of which were raised in rabbits against synthetic peptides (immunofluorescence) and two of which were raised in sheep against fusion proteins (immunoblotting). For immunoblotting, affinity-purified sheep anti-30-kDa and anti-60-kDa mouse cardiac dystrophin antisera were used simultaneously (1). The mouse peptides correspond to amino acids 407–815 (60 kDa) and

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Abbreviation: CK, creatine kinase.

1181–1388 (30 kDa) of the human dystrophin sequence (5). The protocols used for 60-kDa and 30-kDa antigen production and purification, antisera production in sheep, and affinity purification of anti-dystrophin antibodies have been described (1).

For immunofluorescence, two rabbit polyclonal anti-human dystrophin antisera were used individually. One of these antisera (anti-DMDP) was raised against a 50-amino acid peptide corresponding to amino acids 440–489 of the human fetal skeletal muscle dystrophin sequence and has been described in detail (4). Here we refer to this antiserum as anti-DMDP II, due to its location towards the carboxyl terminus relative to the peptide described below. The peptide sequence to which anti-DMDP II is directed is contained within the 60-kDa dystrophin antigen described above.

The second antiserum used for immunofluorescence has not been described previously and was raised against a synthetic peptide representing residues 215–264 of the human amino acid sequence (5): PEDVDTTYPDKKSILMYITS-LFQTLPQQVSIEAIQEVEMLPRPPKVTKEE. Rabbits were immunized with this peptide and immune serum was produced as described (4). This antiserum is referred to as anti-DMDP I.

Immunofluorescence. Muscle biopsy specimens were processed for cryosectioning as described (14). Each coverslip processed for immunofluorescence contained three to seven experimental samples along with three controls: a normal muscle biopsy sample, a sample from a known Duchenne dystrophy patient, and a sample from a known Becker dystrophy patient. Each sample was tested with anti-DMDP I and anti-DMDP II (both diluted 1:300) on separate coverslips.

Immunoblotting. Immunoblot detection of dystrophin was as described (11).

RESULTS

Duchenne Muscular Dystrophy Patients. Eighteen patients, all male, were found to completely lack dystrophin by both immunofluorescence and immunoblotting. The clinical diagnosis of each of these patients was consistent with Duchenne dystrophy. Results for one of these patients are shown in Fig. 1.

Duchenne Muscular Dystrophy Carriers. Muscle biopsy samples from four obligate carriers were tested for dystrophin. All had creatine kinase (CK) levels at least four times the upper limit of normal, though none manifested any muscle weakness or atrophy. All four showed a mosaic pattern of dystrophin immunostaining, with completely positive fibers adjacent to completely negative fibers (Fig. 2). Each of these carriers showed apparently normal dystrophin by immunoblot, with no detectable reduction in dystrophin quantity.

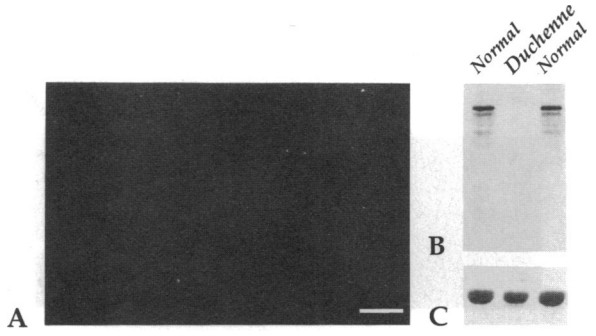

FIG. 1. Absence of dystrophin in a muscle biopsy sample from a patient with Duchenne muscular dystrophy, as shown by dystrophin immunostaining (anti-DMDP II) (*A*) and immunoblotting (*B*). Dystrophin is evident in normal muscle lanes of the immunoblot (*B*) as the expected protein of ≈400 kDa. Coomassie blue staining of myosin heavy chain after blot transfer serves as a control for the muscle protein content of each lane (*C*). (Bar in *A* = 25 μm.)

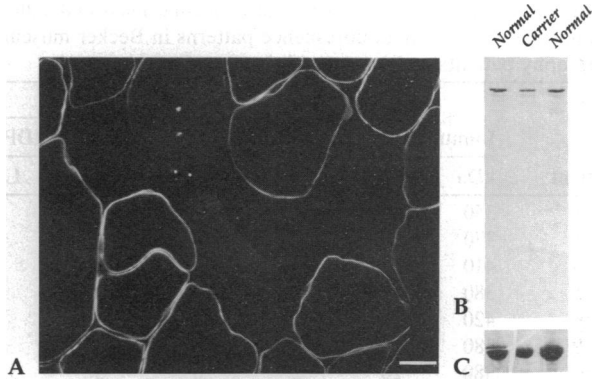

FIG. 2. Dystrophin in an asymptomatic, obligate carrier for Duchenne muscular dystrophy. Dystrophin immunofluorescence with anti-DMDP II (*A*) shows that both dystrophin-positive and dystrophin-negative fibers are present. Immunoblot detection of dystrophin in the same biopsy sample (*B*) shows no difference from the adjacent controls, after correction for the muscle protein content of the samples (*C*). (Bar in *A* = 25 μm.)

Becker Muscular Dystrophy Patients. Twenty patients, all male, who had a clinical phenotype consistent with Becker muscular dystrophy were tested. Dystrophin immunofluorescence invariably consisted of a patchy or discontinuous staining pattern around most fibers. The staining intensities in general were considerably fainter than those seen in normal muscle biopsy specimens. The dystrophin staining was quite variable between individual fibers within the same sample, with some completely negative fibers in all of the Becker samples tested. A subjective value of 0 to 5 was assigned to the brightest fluorescent and weakest fluorescent patch in each biopsy (Table 1). Individual samples often gave different staining intensities with the two antisera used.

Dystrophin immunoblotting of the same samples showed that all had dystrophin of abnormal molecular mass (Table 1). The largest dystrophin observed in these biopsies was only slightly larger than normal (420 kDa; normal = 400 kDa), while the smallest was 350 kDa. The relative quantities of dystrophin ranged from barely detectable (5% of normal) to 60% of normal.

For the comparison of the immunofluorescence results with those of the immunoblot experiments, all Becker patients are shown in Table 1 according to the percentage of dystrophin. From this table, it is evident that the dystrophin quantity determined by immunoblotting was not strongly correlated with the maximum strength of the patchy immunofluorescence signal. There was also no strong correlation between the intensity of the immunofluorescence signal and the molecular mass of dystrophin. As shown in Figs. 3 and 4, the immunofluorescent staining pattern of dystrophin also appeared to be independent of the size and relative abundance of dystrophin as determined by immunoblotting.

There were two additional patients identified whose dystrophin results were not entirely consistent (bottom of Table 1). The first of these patients, no. 25, was a 3-year-old boy who showed no dystrophin by immunoblot analysis or by immunofluorescent analysis with anti-DMDP II and was clinically diagnosed as a Duchenne patient. However, analysis of the same biopsy sample with anti-DMDP I showed a clear, strong, homogeneous staining of every fiber. One of every 20 Becker patients is expected to be have a molecular deletion encompassing the regions of dystrophin encoding the anti-DMDP II, 30-kDa, and 60-kDa antigens (11, 15). This

Table 1. Comparisons of dystrophin molecular mass and cellular abundance with immunofluorescence patterns in Becker muscular dystrophy patients and in two additional patients

			Immunofluorescence†			
	Immunoblot*		Anti-DMDP I		Anti-DMDP II	
Patient	kDa	%	High	Low	High	Low
31	350	5	0	0	1	0
11	370	10	0	0	1	0
13	410	10	4	0	3	0
15	380	10	3	0	3	0
16	420	10	3	0	3	0
19	380	10	4	0	4	0
5	380	20	2	0	3	0
8	390	20	3	0	2	0
17	420	20	3	0	3	0
18	380	20	3	0	3	0
32	390	20	1	0	1	0
2	380	30	1	0	3	0
3	360	30	2	0	2	0
6	370	30	2	0	3	0
57	420	30	4	0	3	0
1	380	40	1	0	3	0
4	380	40	2	0	2	0
7	360	50	3	0	2	0
24	370	50	2	0	3	0
9	380	60	2	0	3	0
25		0	3	3	0	0
29	360	10	0	0	0	0

Each patient exhibited a clinical phenotype consistent with Becker muscular dystrophy. Patient 25 (3 years old) was unique in that he showed no detectable dystrophin by immunoblotting or by immunofluorescence with anti-DMDP II yet showed a normal dystrophin immunostaining pattern with anti-DMDP I. While immunoblotting seemed more sensitive than immunofluorescence at detecting very low levels of dystrophin, patient 29 (7 years old) was unique in that no detectable dystrophin was seen by immunofluorescence with either antibody.

*Normal dystrophin molecular mass, 400 kDa; normal abundance, 100%.

†Immunofluorescence signal intensities are difficult to quantitate. To give an indication of the fluorescence intensities, a qualitative estimate was assigned for the most intensely staining regions (High) and the least intensely staining region (Low) of the plasma membrane in each Becker biopsy sample. This was accomplished by assigning relative fluorescent intensities on a scale of 0–5, with 0 being no detectable fluorescence signal, and 5 being the most intense signals observed in all normal samples, for both antisera used (anti-DMDP I and II). Normal muscle and muscle from patients with diseases unrelated to Duchenne/Becker dystrophy showed continuous peripheral immunostaining that ranged in relative intensity from 3 to 5. However, the immunostaining of Becker muscle fibers was invariably discontinuous. For this reason, both high and low relative intensities were tabulated for the individual fiber patches showing the highest and lowest fluorescence signal, respectively.

patient possibly represents such a Becker dystrophy patient. However, the immunofluorescence pattern of dystrophin in all other Becker patients studied to date has been patchy and discontinuous. Thus, if this patient is a true Becker dystrophy patient, his immunostaining pattern would be unique. The young age of the patient makes clinical confirmation of either Duchenne dystrophy or Becker dystrophy difficult.

The second patient, no. 29, was considered within the "severe-Becker" (11) category by immunoblot; he had a smaller dystrophin (360 kDa) of dramatically reduced quantity (10% of normal). This same patient was scored as dystrophin-deficient (Duchenne dystrophy) by immunofluorescence analysis. The results for this patient, and also the results for patients 11 and 31 (Table 1), suggest that immunoblotting is more sensitive than immunofluorescence for the detection of low amounts of dystrophin.

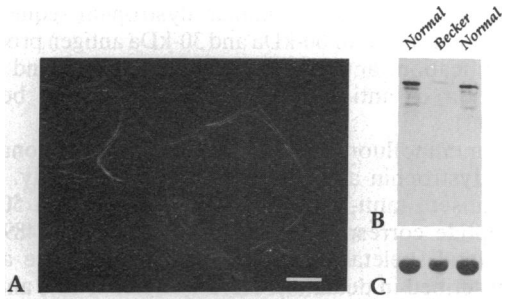

FIG. 3. High molecular weight, moderately abundant dystrophin in a Becker dystrophy patient. Shown is the immunofluorescent staining pattern with anti-DMDP II (*A*) in a muscle biopsy specimen of a Becker muscular dystrophy patient who exhibits high molecular mass (420-kDa) dystrophin in moderate quantity (≈30% of normal) (*B* and *C*). (Bar in *A* = 25 μm.)

Unrelated Disorders. A total of 32 patients were tested with disorders thought to be unrelated to Duchenne/Becker dystrophy on the basis of clinical information. Disorders represented and the number of patients tested were as follows: fascioscapulohumeral dystrophy, 3; limb-girdle dystrophy, 5; myotonic dystrophy, 3; Fukuyama muscular dystrophy, 5; distal muscular dystrophy (Miyoshi), 1; arthrogryposis multiplex congenita, 3; idiopathic scoliosis, 5; polymyositis, 2; malignant hyperthermia, 1; growth hormone deficiency, 1; spinal muscular atrophy, 3. All but one of these patients exhibited completely normal dystrophin by immunofluorescence (Fig. 5). When studied by immunoblotting, all these patients contained dystrophin of normal size, although one myotonic dystrophy patient and one limb-girdle dystrophy patient exhibited a reduced quantity of dystrophin (30% of normal).

A single patient, however, showed dystrophin deficiency by both immunofluorescence and immunoblotting, yet carried a clinical diagnosis of Fukuyama congenital muscular dystrophy (Fig. 6). This patient showed a clinical picture completely consistent with the diagnosis of Fukuyama dystrophy and was clinically indistinguishable from the other four Fukuyama dystrophy patients studied with normal dystrophin. However, a diagnosis of Fukuyama dystrophy is generally not considered definitive until corticogyral defects are verified by postmortem examination.

DISCUSSION

Dystrophin deficiency has been well documented as the underlying cause of Duchenne muscular dystrophy by both immunofluorescence and immunoblot analysis (10, 12).

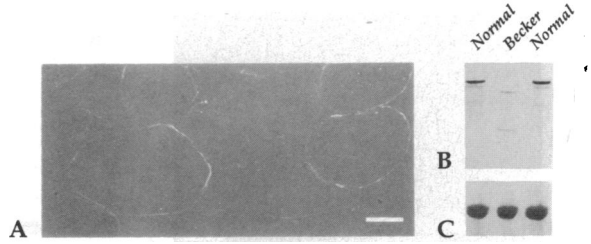

FIG. 4. Low molecular weight, low-quantity dystrophin immunofluorescence in a Becker dystrophy patient. Shown is the anti-DMDP II (*A*) immunofluorescence pattern corresponding to a low molecular mass (380-kDa) dystrophin at ≈10% normal quantity (*B* and *C*). Though less intense, the immunostaining pattern is quite similar to that seen in Fig. 3*A*. As this patient has only 10% the normal level of dystrophin in his muscle, he will probably follow a more severe clinical course (11). (Bar in *A* = 25 μm.)

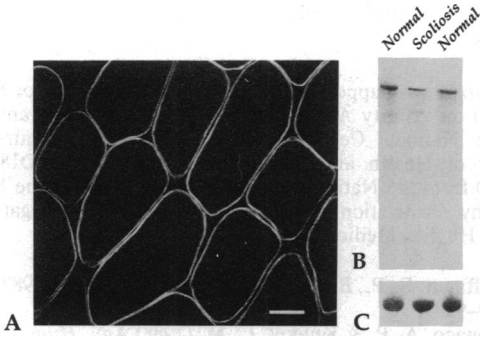

FIG. 5. Immunofluorescence and immunoblot characterization of dystrophin in a scoliosis patient. Dystrophin appears normal by both anti-DMDP II immunofluorescence (*A*) and immunoblot (*B*) analyses. The results shown were identical for all those patients having a disorder unrelated to Duchenne/Becker dystrophy. (Bar in *A* = 25 μm.)

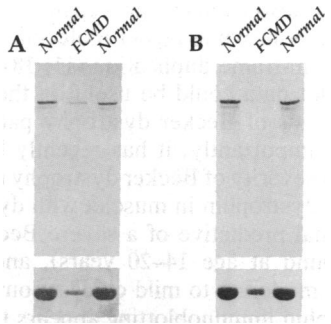

FIG. 6. Dystrophin immunoblot analysis in Fukuyama congenital muscular dystrophy (FCMD). Results are shown for two of the five FCMD patients studied. Four of the five exhibited completely normal dystrophin by both immunofluorescence and immunoblotting (example in *A*). A single patient, however, exhibited a complete lack of dystrophin by both immunofluorescence (data not shown) and immunoblotting (*B*).

Moreover, dystrophin deficiency has been shown to be specific for Duchenne muscular dystrophy by both techniques (10, 12) (Figs. 1 and 5). Indeed, adding the 18 Duchenne patients and the 32 patients with unrelated disorders reported in this paper to the previously reported patient studies brings the total number of patients studied to 160 patients with unrelated neuromuscular diseases (normal dystrophin) and 98 Duchenne dystrophy patients (dystrophin deficiency). Of all the patients studied, only a single diagnosed Duchenne patient has been found to have normal dystrophin (10), and this patient possibly has an autosomal recessive disorder (16). However, immunoblot and immunofluorescent data have not been reported together for any single patient. Here we report both immunoblot and immunofluorescent analyses on the same muscle biopsy samples from 76 patients.

Of the 32 patients carrying clinical diagnoses unrelated to Duchenne/Becker dystrophy, we found a single patient who exhibited dystrophin deficiency (Fig. 6). Such a case has not been reported previously. This patient was diagnosed with Fukuyama congenital muscular dystrophy, an autosomal recessive disorder endemic to Japan. Four additional Fukuyama dystrophy patients studied in this paper, and 5 studied in a previous publication (7), showed completely normal dystrophin. The single dystrophin-deficient Fukuyama dystrophy patient showed no clinical or histopathological variations from typical Fukuyama dystrophy; when studied at 8 months he had moderately delayed motor and mental development (DQ = 90), a slightly floppy appearance, and abnormal computerized tomography scans and electroencephalography indicative of an atrophic brain. We feel that there are three possible explanations for this finding. (*i*) Dystrophin deficiency (due to mutation of the dystrophin gene) can, in rare instances, result in a clinical presentation more severe than that normally expected in Duchenne dystrophy. (*ii*) In extremely rare cases (1 of 160), dystrophin deficiency can be associated with disorders unrelated to Duchenne dystrophy as a secondary effect of the primary disorder. (*iii*) This patient is suffering from a chance combination of Duchenne dystrophy and brain damage of unknown etiology and therefore might have multiple genetic abnormalities, one of which involves the dystrophin gene. Dystrophin gene and protein studies of this patient, and many additional Fukuyama-type patients, are needed to determine which of these possibilities is correct.

One goal of this study was to correlate the dystrophin immunofluorescence and immunoblot results for a large number of diagnostic muscle biopsies, and to identify diagnostic situations in which one technique might have advantages over the other. Although the two techniques were found to be equally highly accurate at delineating Duchenne dystrophy from unrelated disorders, differences were noted when they were applied to female carriers of Duchenne dystrophy and to Becker dystrophy patients. In the four asymptomatic obligate carriers studied in this report, all were correctly identified as carriers by immunofluorescence due to the characteristic mosaic staining pattern of myofibers (Fig. 2). These same four carriers, when tested "blind" by immunoblotting, were considered to have normal dystrophin and were therefore not identified as carriers. The superiority of immunofluorescence in identifying carriers is a consequence of the functional averaging of thousands of fibers in immunoblotting. Thus, while a single dystrophin-negative myofiber in 100 positive fibers can be identified by immunofluorescence, the 1% overall reduction in dystrophin content of the muscle is beyond the resolution of immunoblotting. It is important to determine whether immunofluorescence would be capable of detecting all female carriers. In this respect it should be noted that the four carriers tested in this report had high serum CK levels. Elevated CK levels are most likely the result of segmental dystrophin deficiency in individual myofibers. Thus, that these carriers had elevated CK levels indicates that dystrophin deficiency existed in some myofibers. Thus, one would *a priori* predict that only those carriers with high CK levels will be identifiable as carriers by immunofluorescence. In this regard it is useful to note that carriers have been reported with no dystrophin-deficient fibers (13). In addition, CK levels in carriers are known to decrease with advancing age, an effect likely to be the result of compensatory production of dystrophin by dystrophin-positive myonuclei in syncytial carrier myofibers (17). Given the highly variable nature of CK levels, it will be important to determine whether dystrophin immunofluorescence of potential carriers holds marked advantages over standard CK determinations.

This study was particularly informative at both the clinical diagnostic and the basic science level with regard to Becker muscular dystrophy. Both dystrophin immunoblotting and and immunofluorescence were highly accurate at delineating Becker muscular dystrophy patients; immunoblotting easily identified the lower molecular weight and lower quantities of dystrophin, while immunofluorescence found the patchy, heterogeneous membrane staining pattern of reduced intensity (7, 8, 10, 11). However, immunoblotting was capable of assigning a dystrophin molecular weight and relative quantity to each patient, whereas immunofluorescence was not. In a strictly diagnostic sense, it is of no great advantage to assign a specific dystrophin molecular weight to each Becker patient. However, immunoblot analysis of dystrophin is likely to be highly predictive of the underlying genetic defect in

Becker patients; lower molecular weight dystrophin implies an in-frame deletion, while higher molecular weight dystrophin implies an in-frame duplication (11, 18). Thus, dystrophin immunoblot data could be useful in the genetic counseling and analysis of Becker dystrophy patients and their families. More importantly, it has recently been suggested that the clinical severity of Becker dystrophy is dependent on the quantity of dystrophin in muscle, with dystrophin levels 5–15% of normal predictive of a severe Becker phenotype (wheelchair-bound at age 14–20 years), and levels >15% predictive of a moderate to mild clinical course (11). In this regard, dystrophin immunoblotting appears to be more sensitive than immunofluorescence at detecting low levels of dystrophin (5–15% range). Thus, it appears that immunoblotting is required for the prognostic categorization of patients into the severe Becker diagnostic category.

It is of interest to speculate as to why dystrophin of abnormal molecular weight causes a discontinuous, patchy distribution of dystrophin at the muscle fiber membrane. Dystrophin is thought to be a membrane cytoskeleton protein that is functional as an antiparallel homodimer (3). Dystrophin dimers are believed to be rodlike molecules ≈130 nm long, with the dimers forming some sort of network directly beneath the plasma membrane (3, 5, 19). The majority of gene mutations resulting in a Becker phenotype are the result of in-frame deletions in the central rod domain of dystrophin (15, 18). Thus, the dystrophin dimers present in most Becker patient muscle fibers are most likely shorter than normal. The immunostaining results suggest that dystrophin dimers of abnormal molecular length are incapable of forming a continuous network beneath the plasma membrane and instead form isolated patches of networked dystrophin dimers separated by patches of membrane with little or no underlying dystrophin.

In this study of 20 abnormal dystrophin proteins in 20 Becker patients, we found that the degree or extent of patchiness was not obviously correlated with either dystrophin molecular weight (higher vs. lower) or quantity (10–60% normal). We feel there are two possible explanations to rationalize these observations at the subcellular level. First, it is possible that all "abnormal" dystrophin is generally unstable and that the focal patches of dystrophin on the membrane are simply reflections of the sites of the greatest dystrophin synthesis, namely, the myofiber nuclei. This stochastic rationale implies that the membrane regions having the least underlying dystrophin are those areas furthest from the nearest nucleus. An alternative explanation (which assumes that dystrophin plays some role in membrane stability and/or function) is that dystrophin is initially laid down as a complete network beneath the plasma membrane. However, given dystrophin's compromised function due to its structural abnormalities, local regions of the plasma membrane experience instability and allow the influx of Ca^{2+}, which would further activate proteases. These proteases would degrade dystrophin at the site of the lesion, causing a region of membrane with no dystrophin staining. This scenario implies that such dystrophin-negative regions are transient and would be repaired over time. Immunofluorescence experiments using serial sections of Becker patient biopsy specimens should be able to distinguish between these possibilities.

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas, Ministry of Education; Grant 62A-1,2 from the National Center of Neurology and Psychiatry of the Ministry of Health and Welfare, Japan; Grants HD18658 and NS23740 from the National Institutes of Health; and the Muscular Dystrophy Association. L.M.K. is an associate investigator of the Howard Hughes Medical Institute.

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