Immunostaining of Dystrophin and Utrophin in Skeletal Muscle of Dystrophinopathies
KO SAHASHI, Tohru IBI, Hiroshi SUOH, Naoki NAKAO, Michinari TASHIRO, Kiminori MARUI, Kiichi ARAHATA* and Hideo SUGITA*

Immunostaining of biopsied skeletal muscle of 4 Duchenne (DMD), 12 Becker muscular dystrophy (BMD) and 3 DMD carriers' was performed using monoclonal antibodies against dystrophin and utrophin. In DMD, dystrophin-negative staining was observed except for revertant fibers which showed different stain patterns for each antibody. In 7 BMDs, there was faint/patchy stain in cases of deletion between exons 45–52, while in one case there was deletion between exons 12–17 and no stain was noted relevant to the deletion site. Moreover, in 2 cases of undetectable deletion, antibodies which recognize a terminal portion of the C-terminal domain revealed the absent stain. In DMD, the utrophin-positive fibers corresponded to dystrophin-negative fibers. In BMD, this relationship did not necessarily occur in each fiber. In DMD carriers, a cluster of dystrophin-negative fibers which was positive for utrophin were prominent. In dystrophinopathy, the immunostaining of dystrophin and utrophin is useful, in combination with dystrophin gene analysis to make a definite diagnosis.

(Key words: progressive muscular dystrophy, histochemistry, sarcolemma, skeletal muscle, myopathy)

Introduction
Duchenne (DMD) and Becker muscular dystrophy (BMD) are allied neuromuscular disorders with sex-linked recessive trait caused by an abnormality of dystrophin, a muscle cytoskeletal protein (1) with a molecular mass (MM) of dystrophin ~420 kDa. The abnormality appears in the size and/or relative abundance (2–6) of dystrophin; thus, dystrophin in DMD is deficient, and dystrophin in many patients with BMD consists of a smaller MM. But it has been reported that dystrophin in the majority of BMD is detectable on SDS-PAGE as a single smaller MM (2), or as two different MMs (4, 6) consisting of the dystrophin ~390 kDa and ~230 kDa. Utrophin (DRP), originally discovered in the process of cloning dystrophin, is one of the dystrophin-related-peptides with MM ~395 kDa which is encoded in the chromosome 6q24 and a cytoskeletal protein with homology of 80% at the C-terminal of dystrophin (7). We performed specific immunostaining analyses of biopsied skeletal muscle in DMD, BMD and definite carriers of DMD using poly- and monoclonal antibodies (pAb and mAb, respectively) against dystrophin and utrophin.

Materials and Methods
Frozen biopsied muscle samples were obtained with appropriate informed consent from 19 patients (4 DMDs, 3 definite DMD carriers and 12 BMDs) at Aichi Medical University. The clinical diagnosis of DMD and BMD was made on the basis of onset and progression of illness, high creatine kinase in serum (CKemia) and routine histochemical study of the biopsied muscles. Four child DMD patients (mean age 6.5±2.1 years old) could walk alone and no mental deficiency was recognized. Three definite manifesting DMD carriers showed pseudo-hypertrophic calf muscles and high CKemia. In 12 BMDs, the age of patients ranged from 8 to 58 years old (mean age 32.6±14.1 years old). All except for patient (38 years old) could walk or run. Pseudohypertrophic calf-muscles (10/12) and moderate high-CKemia (12/12) maximally up to 30 times the normal upper value were found; cardiomyopathy was observed in 7 patients, and mental retardation and diabetes mellitus in spite of well preserved muscle strength were found in 2 child siblings.

By the multiplex PCR method of dystrophin gene in periph-
eral blood cells and muscle using Chamberlin's and Begg's primers (Fig. 1), deletion was detected in 2 DMD and 8 BMD patients. In DMD, the deletion site was between exons 45 and 52. In BMD, deletion in 7 cases was localized between exons 45 and 52, which mainly locate at the region of rod domain (major hot spots): In 1 case, a deletion between exons 12 and 17 at an N-terminal portion of the rod domain was found; in 4 cases, including siblings, no deletion was detected.

A few frozen samples were stored at -80°C in a deep freezer or liquid nitrogen tank. Samples were sliced for immunostaining into 8 μm-thick frozen sections, using a cryostat.

**Anti-dystrophin antibodies for immunostaining**

A 60 kDa pAb (donated by Prof. Miike of Kumamoto University) and 5 mAbs were used for the dystrophin stain. Locations which anti-dystrophin antibodies recognize were on the order of Dys 3, 2-5E2, 60 kDa, Dys 1, 4-4C5 and Dys 2 consecutively from N-terminal to C-terminal portions (Fig. 1).

For the mAbs, 2-5E2 [clone No. 2-5E2] and 4-4C5 [clone No. 4-4C5 (8)], which were raised against an N-terminal portion of triple helical segments (the amino-acid sequence between 440th and 489th) and a portion of the C-terminal domain (the amino-acid sequence between 3,495th and 3,544th), respectively, were obtained from Fujirebio Inc., Tokyo, Japan. The mAbs, Dys1 [clone No. Dy4/6D3 (9)] raised against a central portion of triple helical segments (the amino-acid sequence between 1,181th and 1,388th), Dys2 (clone No. Dy8/6C5) raised against a terminal portion of the C-terminal domain (the amino-acid sequence between 3,669th and 3,685th), and Dys3 (clone No. Dys10/12B2) raised against a N-terminal portion of triple helical segments (the amino-acid sequence between 321th and 494th), were purchased from Novocastra Laboratories Ltd., Newcastle, UK.

Anti-utrophin (DRP) pAb was donated by Dr. Ishiura of Tokyo University, and the mAb was purchased from Novocastra Laboratories Ltd. These anti-utrophin antibodies had no cross-reactivity to dystrophin.

**Routine histopathology and histochemistry of frozen sections**

Hematoxylin and eosin stain, modified Gomori trichrome method, and stains of NADH-TR, succinic dehydrogenase, routine-myosin ATPase, cytochrome c oxidase, desmin, and developing myosin heavy chain were performed by the conventional methods.

**Immunostaining**

Frozen sections were preincubated in phosphate-buffered saline (PBS) containing 1% normal horse serum, washed once in PBS and then incubated with the anti-dystrophin mAbs and anti-utrophin antibodies finally diluted in PBS (anti-dystrophin pAb & mAbs 60 kDa 1:200; Dys1 1:500; Dys2 1:5,000; Dys3 1:2,000; 2-5E2 1:4,000; 4-4C5 1:5,000; anti-utrophin pAb 1:200, mAb 1:20,000) overnight in a cold room (4°C). Afterwards, sections were rinsed three times and gently blotted onto filter paper. In order to detect dystrophin-anti-dystrophin immune complexes, affinity-purified horse anti-mouse IgG and
goat anti-rabbit IgG conjugated to avidin-DH biotinylated horseradish peroxidase (ABC method) were applied, and then rinsed three times in PBS. Sections were mounted in Vectastain. The control staining was performed using conventional methods including application of non-immune serum without antibodies. In addition, biopsied muscles of patients without detectable neuromuscular diseases were used for the negative and positive controls. All stained sections were observed, and were photographed by Nikon light microscopy (Nikon, Tokyo, Japan).

**Results**

Routine histopathology and histochemistry

The number of muscle fibers was relatively well preserved. Hypertrophic and splitting fibers, moderately increased internal

<table>
<thead>
<tr>
<th>Case</th>
<th>Del. Exon</th>
<th>Dys3</th>
<th>5E2</th>
<th>60kd</th>
<th>Dys1</th>
<th>4C5</th>
<th>Dys2</th>
<th>% (Freq)</th>
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<tr>
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<td>45</td>
<td>++</td>
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<td>-</td>
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<td>+</td>
<td>+</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>49–52</td>
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<td>+</td>
<td>0.18</td>
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</table>

ND: not detected.
nuclei and large opaque fibers were observed in DMD and BMD, and were consistent with the myopathic change of dystrophinopathy.

By NADH-TR, an oxidative enzyme activity which was characterized by mottled appearance, was noted. Cytochrome c oxidase-negative fibers were not significantly increased.

Connective tissue elements were increased in peri- and endomysium.

**Immunostaining**

**DMD**

Immunostaining of anti-dystrophin antibodies is shown in

![Immunostaining images of DMD, BMD, and DMD carrier]
Immunostain and Dystrophinopathy

Figs. 1 and 2.

Staining of all antibodies used here was totally deficient. Very few dystrophin-positive fiber clusters ("so-called" revertant fibers (10)) appeared between 0.1 to 0.7% of fibers examined under random fields. In revertant fibers, staining differences between antibodies were noted in each case and in each fiber (Table 1). Revertant positive fibers to Dys1, which recognizes the central portion of the rod domain were much less frequent, while those positive to Dys2, which recognizes the C-terminal portion, were more common (Fig. 1).

Immunostaining of anti-utrophin antibodies is shown in Fig. 3. All fibers were fairly well stained, but regenerating fibers with a smaller diameter, which were desmin-positive, were more strongly stained.

DMD Carriers

Negative fibers against anti-dystrophin antibodies were frequently present in clusters (Fig. 3). The number of dystrophin-negative fibers seemed to gradually decrease by age.

Relationships between negative and positive reactions against anti-dystrophin and anti-utrophin antibodies were noted in most fibers.

BMD

Results of immunostaining of anti-dystrophin antibodies is shown in Table 2. In 7 BMD patients with deletions between exons 45 and 52 (major hot spot), the staining showed a faint/patchy pattern by a pAb and all mAbs examined (Fig. 4).

In one BMD patient with a deletion between exons 12 and 17 (minor hot spot), the staining showed the total absence in Dys3 and 2-SE2, which are relevant to a deleted portion of dystrophin gene, instead of fairly preserved faint/patchy patterns as seen in 60 kDa, Dys1, 4-4C5 and Dys2 (Fig. 4).

In 4 patients with no detectable deleted dystrophin gene, the staining patterns were subdivided into two groups, two patients showing a faint/patchy pattern in a pAb and all mAbs, and the other two siblings exhibiting the absent pattern in 4-4C5 and Dys2, instead of the faint/patchy pattern in 60 kDa, Dys3, 2-SE2 and Dys1 (Fig. 4).

Immunostaining of anti-utrophin antibodies was positive in regenerating fibers and in a few non-regenerating dystrophin-negative fibers in some cases (Fig. 3).

Discussion

DMD and BMD are allied disorders of a dystrophin abnormality, dystrophinopathy. The precise function of dystrophin remains controversial, but it is known to have a calcium-related mechanism, because of its localization on the surface membranes of the following two systemic tissues: 1) contractile cells such as skeletal, cardiac and smooth muscle cells and myoepithelial cells; and 2) neurons and synapses. On the other hand, it is also reported that functional dystrophin contributes to stability based on experiments using hypo-osmotic shock to determine stress resistance and a mouse model (mdx) for human disease (11).

Abnormality in dystrophinopathy becomes manifest in the size and/or relative abundance (2-6) of dystrophin; thus, dystrophin in DMD is deficient, while dystrophin in many patients with BMD can be observed in less abundance, mostly with a smaller molecular mass. In addition, it has been pointed out that dystrophin in the majority (80%) of BMD can be detected as a single smaller molecular mass (2), or sometimes as two different molecular masses (4, 6) consisting of ~390 kDa and ~230 kDa.

The immunostaining of mAbs and pAb of dystrophin has shown a negative pattern in DMD except for the "so-called" revertant fibers (8). On immunostaining of DMD, the dystrophin staining pattern was consistent with that in the previous reports (12), but the fiber to fiber differing patterns in revertant fibers of DMD probably means that a new conversion (new mutation) from an out-frame deletion to an in-frame deletion might occur differently in each revertant fiber. Utrophin in DMD was thought to provide a mechanism for functional membrane support closely related to dystrophin deficiency.

On immunostaining of DMD carriers, the number of negative fibers in anti-dystrophin antibodies gradually decreased

<table>
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<th>SE2</th>
<th>60kd</th>
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</table>

ND: not detected.
with age. This finding may indicate that fibers deficient in dystrophin are lost with aging, or that normal dystrophin is supplied from the dystrophin-positive fibers. In addition, the fact that negative and/or positive reactions of dystrophin or utrophin did not always correspond might be simply independent of dystrophin or utrophin genes.

On immunostaining of 7 BMD using 5 mAbs and 1 pAb recognizing through the N- to C-terminal domains, faint/patchy immunostaining was noted in all patients with a deletion at the major hot spot region between exons 45 and 52. The data in
these cases indicate that both molecular terminals of dystrophin were probably preserved less abundantly. In one patient with deletion at the minor hot spot region between exons 12 and 17, the immunostaining of dystrophin was totally devoid of reaction for two mAbs (2-5E2 and Dys3) which recognize the upstream region of the rod domain, and are relevant to the deletion site between exons 12 and 17.

In 4 BMD patients without detectable deletion, two immunostaining patterns of dystrophin were observed; two cases faint/patchy in pAb and all mAbs and the other 2 cases deficient in 2 mAbs (4-4C5 and Dys2). The mAbs 4-4C5 and Dys2 recognize the very terminal portion of C-terminal domain. Recently, it was reported that a defect of either cysteine-rich domain or a part of C-terminal domain binding dystrophin-associated glycoprotein (DAP) causes serious illness (13). Therefore, it is thought that the latter siblings might have a smaller deletion or the nucleotide substitution within the C-terminal domain except for the DAP binding site. On the other hand, their muscle weakness was mild in spite of the association with mental retardation and juvenile diabetes mellitus. Their immunostaining pattern suggests that the tissue distribution of abnormal dystrophin was different from organ to organ, since muscle weakness was mild compared with the other clinical features (mental deficiency and diabetes mellitus).

In utrophin staining of BMD, the regenerating fibers were strongly positive. This fact implies that utrophin appears at the early developing stage of muscle fiber, and gradually disappears with development of dystrophin. In conclusion, dystrophin is the most important sarcolemmal protein. When it is totally or partially absent in DMD, DMD carriers and BMD, then utrophin, one of the membrane constituents, may play an essential role in compensating for the dystrophin deficiency.

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References