Electrophoretic Studies of Muscle Proteins in Duchenne Muscular Dystrophy and Other Neuromuscular Disorders

---With Special Reference to the Change of Dystrophin

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We studied total SDS-solubilized muscle proteins (TMP) of Duchenne muscular dystrophy (DMD) and other neuromuscular disorders, with special attention to the change of dystrophin suspected of being the product of DMD locus. SDS gel electrophoresis of DMD patients showed an absence of band 5 and an extreme faintness of band 2 with a decrease of band 4', 5', and 5''. Immuno blot analysis, using anti-dystrophin antibodies (anti-30 kd and anti-60 kd polyclonals), showed an absence of dystrophin in all 6 DMD cases. In other neuromuscular disorders, there was no change of TMP, and dystrophin was clearly detectable. To elucidate the degenerative mechanism of DMD muscle, further studies, including the problem of clarifying the physiological role of dystrophin, are necessary.

Key Words: Duchenne muscular dystrophy, Mdx mouse, Dystrophin, Immuno blotting, Immunostaining

Recent progress in molecular genetics has led to remarkable development in the study of genetic neuromuscular disorders such as Huntington's chorea (1), Lesch-Nyhan syndrome (2), familial amyloidotic polyneuropathy (3-5) and Duchenne muscular dystrophy (DMD) (6-8). Kunkel et al. (8, 9) revealed that the genetic locus of Duchenne muscular dystrophy (DMD) ranged over about 2,000 kilobases (kb) of the X chromosome short arm (Xp²¹), and that the deletion of part or all of about 60 exons contained therein became one of the major causes of DMD. These 60 exons correspond to 14 kb in mRNA length and have already been cloned as cDNA (9). Based upon these results, Wood et al. (10) conjectured that if mRNA of 14 kb transmits genetic information on single protein, that protein would have a molecular weight of 500 kilodaltons (kd). Accordingly, they made an analysis of DMD

patients' biopsy muscles by SDS gel electrophoresis (SDS-PAGE), attending to the change in intramuscular ultra-high molecular weight proteins and reported the absence of nebulin in all cases of DMD.

Regarding muscle structural proteins, comparatively low molecular weight proteins below the myosin heavy chain (MHC) have already been examined in detail (11-13) while molecular proteins higher than MHC have just recently started being studied. Here we describe our studies of skeletal muscle proteins, including dystrophin, for DMD and other neuromuscular diseases.

Subjects and Methods:

Biopsies were taken from the gastrocnemius, or biceps brachii muscles of 6 DMD patients (2-12 y), 1 female DMD (6 y), 3 congenital muscular dystrophy (non-Fukuyama type, 5 m-l y), 3

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myotonic dystrophy (20, 30, 48 y), 6 polymyositis (19-72 y), 1 distal myopathy with rimmed vacuoles (32 y), 1 myasthenia gravis (32 y), 3 Charcot-Marie-Tooth disease (30-51 y), 1 HTLV-1 associated myelopathy (60 y), 6 amyotrophic lateral sclerosis (62-72 y), and 6 controls (10-74 y). The present study was performed by the patients' agreement. The sample was divided into small muscle bundles that were soaked in a chilled relaxing solution (0.12 M KCl, 20 mM tris aminomethane, 20 mM maleic acid, 4 mM ATP, 4 mM EGTA, 4 mM MgCl₂, 0.1 mM phenyl methyl sulfonyl fluoride, 50% glycerine, pH 6.8). The single muscle fibers were then separated with electronmicroscopic tweezers under darkfield stereoscopic microscopy as described in a previous paper (13). About 20 isolated single fibers were homogenized in 15 μ l of proteolytic solution (2% SDS, 5% β -mercaptoethanol, 4 mM EDTA, 40 mM tris, 0.24 M glycine, 40% glycerine, 0.03% bromphenol blue, pH 8.5), incubated at 50°C for 20 min, and then subjected to SDS-PAGE with 5% single gel and 3-12% gradient gel. The electrophoretic gels were stained by silver staining method (14). Immuno-blotting analysis was performed using sheep anti-mouse dystrophin antibodies (anti-30 kd and anti-60 kd polyclonals) with biotinylated rabbit anti-sheep IgG secondary according to Hoffman's method (15, 16).

RESULTS

1) Control. On SDS-PAGE of TMP of normal controls, 6 major bands were observed at the molecular weight zone higher than MHC (Fig. 1). The molecular weights of bands 1, 2, 3, 4, 5, and 6 were estimated to be 2,500, 900, 700, 500, 400, and 250 kd respectively, by the mobility rate on SDS-PAGE. Bands 1, 3, and 6 were identified as connectin, nebulin, and filamin respectively, from comigrating with connectin and nebulin purified by Wang's method (17), and published patterns and molecular weight (18-21). The exact origin of bands 2 and 4 was unknown, though band 2 might be a degraded product of connectin. Besides these, several faint bands such as band 4' between band 4 and 5, band 5' and 5'' between band 5 and 6 were observed (Fig. 1). These bands were undetectable by the Coomassie blue and amido black staining technique.

Immuno blot analysis of TMP using sheep anti-

SDS PAGE of total SDS-solubilized proteins from Control Muscle.

Fig. 1. SDS gel pattern of total SDS-solubilized muscle proteins from control muscles. Protein bands with lower mobilities than myosin heavy chain (MHC) are labeled numerically from the top: bands 1, 3, and 6 are identified as connectin, nebulin, and filamin respectively from comigration of purified connectin and nebulin, and published patients. 5% polyacrylamide gel. N: purified nebulin, Mf: rabbit myofibrillar proteins, C: normal control.

dystrophin antibodies (30 kd and 60 kd) showed clear detection of dystrophin which was consist with band 5 on SDS-PAGE (Fig. 3).

2) DMD. SDS-PAGE showed an absence of band 5 in all 6 DMD patients. Besides this, the band 2 was extremely faint, with a decrease of bands 4', 5', and 5'' (Fig. 2). There were no apparent change of connectin, nebulin, and filamin. Immuno blot analysis, using sheep anti-dystrophin antibodies (anti-30 kd and anti-60 kd polyclonals), showed an

SDS PAGE of total SDS-solubilized proteins from DMD, ALS, and Control Muscle.

Fig. 2. SDS gel electrophoresis of total SDS-solubilized muscle proteins from Duchenne muscular dystrophy (DMD), amyotrophic lateral sclerosis (ALS), and control (C) muscle. Note the absence of band 5 in DMD patient. 3-12% gradient gel. C: connectin, N: nebulin, MHC: myosin heavy chain, α -A, α -actinin, Act: actin, TM: tropomyosin.

absence of dystrophin. In a female DMD patient, dystrophin was detected only at a trace level (Fig. 3).

3) Other neuromuscular disorders. These was no apparent change of TMP, and dystrophin was clearly detectable in all the patients examined with neuromuscular disorders other than DMD and female DMD.

DISCUSSION

Regarding myofibrillar proteins in DMD, some characteristic changes such as decreases in α -actinin, desmin, and troponin subunits have so far been reported (11-13) and these changes have been

Immuno blot analysis of DMD, female DMD, and control muscles using anti-dystrophin antibody

Fig. 3. Immuno blot analysis of DMD, female DMD, and control muscles using sheep anti-dystrophin antibody. Lanes 1-4: immuno blot using sheep anti-dystrophin antibody (60 kd polyclonals); lanes 5, 6: total SDS-solubilized muscle proteins stained with amido black (5), and silver (6). In normal controls, the immunoassay of the antigen using anti-dystrophin antibody gives a clear band at a position corresponding to an Mr of 400 kd (band 5). In contrast with this, dystrophin is not detected in DMD patient. In female DMD patient, dystrophin is detected only at a trace level.

attributed to the proteolytic action of endogenous protease (22-24). This offered a ground for the membrane abnormality theory of DMD (25-27). In 1987, Wood et al. (10) analysed myofibrillar proteins focusing on ultra-high molecular weight proteins and reported a complete disappearance or remarkable decrease of nebulin in all cases of DMD. However, the possibility that nebulin was the gene product of DMD was thereafter ruled out by Sugita et al. (28). In our studies, connectin and nebulin were well-preserved. Recently, Hoffman et al. (15, 16) produced specific antibodies against the proteins encoded by fragments of x-chromosome in mdx mice

which were very similar to the DMD gene in humans. These antibodies recognized a protein, dystrophin (Mr 400 kd) in normal skeletal and cardiac muscle of humans and mice. Moreover, they found that dystrophin was undetectable in skeletal muscle of DMD patients and mdx mice. Nowadays, dystrophin is strongly suggested to be the product of DMD locus. In our studies, dystrophin was undetectable in DMD muscles in contrast to the muscles of all other neuromuscular diseases. Our results were fundamentally identical with Hoffman's ones (15, 16). Through the accumulation of data of Becker and other types of muscular dystrophy, immuno blot analysis of TMP from biopsied muscles will contribute to make an exclusive diagnosis of DMD. On SDS-PAGE of TMP from DMD muscles, the outstanding finding was an absence of band 5 (dystrophin). Besides this, however, the decrease of bands 2, 4', 5', and 5'' was observed, though it might be a secondary change accompanied by muscle fiber degeneration. To elucidate the degenerative mechanism of DMD muscles, further studies, including the problem of clarifying the physiological role of dystrophin and the origin of band 2, 4', 5', and 5'' are necessary.

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