Localisation and characterisation of dystrophin in the central nervous system of controls and patients with Duchenne muscular dystrophy

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Abstract

The aim was to localise and characterise dystrophin in various human tissues, especially in the CNS. Immunoblotting and immunostaining studies were carried out with eight region-specific dystrophin antibodies. In necropsy tissue from controls, dystrophin was noted as a doublet in immunoblots of striated muscle, and as a single band in those of smooth muscle and the CNS. With immunostaining, punctate immunoreactivity was seen on the cell bodies and dendrites of the cerebral cortical neurons and cerebellar Purkinje cells. By contrast, dystrophin was not detected in any tissues, including the cerebrum and cerebellum, of patients with Duchenne muscular dystrophy who had an intellectual disturbance.

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Materials and methods

Necropsy specimens of skeletal muscle, cardiac muscle, stomach, lung, liver, kidney, bladder, cerebrum (frontal lobe, parietal lobe, occipital lobe), cerebellum, and spinal cord were taken from normal controls (42 to 74 years old) who died of non-neuromuscular diseases, one patient with Kennedy-Alter-Sung disease (62 years old), one patient with myotonic dystrophy (54 years old), and three patients with Duchenne muscular dystrophy (17, 20 and 26 years old); these three patients were intellectually disturbed. Necropsy was performed within four hours of death. Specimens from patients with Duchenne muscular dystrophy were collected within two hours.

For immunoblotting procedures, tissues were homogenised in a buffer containing 2% sodium dodecyl sulphate (SDS) 5% β -mercaptoethanol, 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 40 mM Tris, 0.24 M glycine, 40% glycerine, and 0.001% bromphenol blue (pH 8.5). About 6 μ l of sample buffer containing 60 μ g protein for the striated muscle and 12 μ l (120 μ g protein) for the smooth muscle and brain were loaded on to 10-well 3.5 to 12% gradient SDS-polyacrylamide gels (1 mm $\times$ 10 cm $\times$ 9 cm). After transfer into the nitrocellulose membrane, the specimen was blocked with 10% skimmed milk and blots were stained with dystrophin antibody at room temperature for two hours. The dystrophin-antidystrophin immune complex was detected with affinity-purified secondary antibody conjugated to avidin-DH biotinylated horseradish peroxidase (Vecstain ABC kit and Elite ABC kit, Vector Laboratories, USA). Eight kinds of region-specific dystrophin antibodies were used: sheep dystrophin antibodies (60 kDa, 30 kDa polyclonals, the original antibodies of Hoffman *et al*²), rabbit dystrophin antibodies (P-20, DMD peptide IV, and antibody 6-10), and mouse dystrophin antibodies (DYS-1, DYS-2, and DYS-3; Novocastra Laboratories, UK). P-20 and DMD peptide IV were polyclonal antibodies raised against the 1750-2248 and 3495-3544 amino acids of dystrophin, respectively. Antibody 6-10 was a polyclonal antibody produced in a rabbit immunised with a dystrophin polypeptide expressed in bacteria from dystrophin cDNA

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Dystrophin is a large protein (molecular weight 427 kDa) that is absent or greatly reduced in patients with Duchenne muscular dystrophy.¹⁻³ Dystrophin was found by immunostaining to be localised on the surface membrane of striated and smooth muscle.⁴⁻⁸ In mice, dystrophin has recently been found not only in muscles but also in the CNS,⁹ which is unrelated to contraction and relaxation. This suggests that dystrophin is concerned with diversified physiological functions.

Even before dystrophin was identified, it was well known that children with Duchenne muscular dystrophy often had a slight or moderate mental retardation. The mean IQ is about 80 and remarkably similar in the many reported series.¹⁰⁻¹⁷ Although opinions have differed as to whether this is a primary phenomenon closely related to the course of the disease or a secondary effect caused by muscle dysfunction and lack of educational opportunity, the first idea is predominant currently.

To clarify the characteristics and localisation of dystrophin in various human tissues, especially in the CNS, a study was carried out with eight region-specific dystrophin antibodies.

Figure 1 Region-specific dystrophin antibodies used. A schematic of the 427 kDa dystrophin protein, with its four constituent domains is shown. Eight different antibodies were used each directed against a specific region of the dystrophin protein, as indicated; anti 6-10 = antibody 6-10; IV = DMD peptide IV.

residues 6181-9544. DYS-1, DYS-2, and DYS-3 were monoclonal antibodies raised against the 1181-1388 (mid-rod), 3669-3685 (C-terminal), and 321-494 (N terminal domain) amino acids of dystrophin (fig 1). For immunostaining, tissues were dissected out and frozen in isopentane cooled in liquid nitrogen. Frozen sections 10 μ m thick were cut and the avidin-DH biotinylated horseradish peroxidase method was performed as described previously.¹⁸ For the primary incubation, cooled acetone-fixed frozen sections were incubated with dystrophin antibodies at 4°C overnight; for secondary incubation, the sections were incubated in affinity-purified secondary antibodies conjugated to avidin-DH biotinylated horseradish peroxidase. Sections were examined with an Olympus photomicroscope.

Results

In tissue from five normal controls, DYS-1, DYS-3, 60 kDa, 30 kDa, P-20, antibody 6-10, and DMD peptide IV revealed a dystrophin band as pronounced as that of biopsied muscle at 427 kDa in skeletal and cardiac

Figure 3 Immunoblot of dystrophin in various tissues from normal controls (norm) and patients with Duchenne muscular dystrophy (DMD). Lanes 1 and 2, cardiac muscle (CM); lanes 3 and 4, cerebral cortex (CC); lanes 5 and 6, cerebellum (CB); lanes 7 and 8, stomach (ST); lanes 9 and 10, skeletal muscle (SM). Blots were stained with dystrophin antibody DYS-1. A dystrophin band was not detected in any tissues of patients with Duchenne muscular dystrophy

muscles, and a less intense dystrophin band in smooth muscle tissues such as the stomach and bladder, and also in the cerebrum (frontal, parietal, and occipital lobes) and cerebellum (fig 2). Only trace amounts were noted in the lung, kidney, liver, and spinal cord. A thin band close to 400 kDa was also found as a doublet with the 427 kDa upper principal band in skeletal and cardiac muscles tested with DYS-1, DYS-3, 60 kDa, 30 kDa, P-20, and antibody 6-10. On the other hand, only a single band was noted in smooth muscle, cerebrum, and cerebellum, at 427 kDa. These results agree fundamentally with those of Byers *et al*⁸ who used animal models, although they did not study the CNS. With DYS-2, that recognises the C-terminal region, dystrophin was noted as an apparent band in skeletal, cardiac, and smooth muscle, but not in tissues of the brain. On the other hand, because the polyclonal antibody DMD peptide IV cross reacts with a dystrophin-related protein,^{19,20} a 427 kDa band was noted in the brain as well as in striated and smooth muscles of the controls. In patients with Kennedy-Alter-Sung disease or myotonic dystrophy, dystrophin was also clearly detectable in skeletal, cardiac, and smooth muscles, and in the brain. By contrast, no dystrophin was detected by any antibodies in any tissues, including the cerebrum and cerebellum, of patients with Duchenne muscular dystrophy and with intellectual disturbance (fig 3).

With immunostaining, localisation of the antibody was noted on the surface membrane of skeletal, cardiac, and smooth muscles (fig 4) and punctate along the cell bodies and dendrites of the cerebral cortical neurons and cerebellar Purkinje cells (fig 5). The cerebral cortical neurons and Purkinje cells did not stain positive with DYS-2, but immunoreactivity was noted on the vascular wall. With DMD peptide IV, the neuroglia and vascular

Figure 2 Immunoblot analysis of dystrophin in various tissues of normal controls. Control in (lane 9) = control biopsied skeletal muscle; Lanes 1 to 9 were stained with DYS-1, and lanes 10 to 14 were stained with DYS-2. The location of dystrophin is indicated; the doublet appearance is typical of striated muscle (arrows). The very high molecular weight band (about 500 kDa) in skeletal muscle is thought to be nebulin as it was present in skeletal muscle but not in heart muscle.

Figure 4 Immunohistochemical localisation of dystrophin. Cryosections (10 μ m) of skeletal muscle from normal controls (A), skeletal muscle from patients with DMD (B), bladder from normal controls (C), bladder from patients with Duchenne muscular dystrophy (D) labelled with DYS-2. Note the intense dystrophin immunoreactivity of the surface membrane of skeletal and smooth muscle from normal controls (arrows), but no apparent immunoreactivity in tissues from patients with Duchenne muscular dystrophy. Original magnification: A and B $\times$ 157; C and D $\times$ 786.

wall stained positive. Immunoreactivity in tissue from patients with Kennedy–Alter–Sung disease or myotonic dystrophy was the same as in the normal controls. On the other hand, for patients with Duchenne muscular dystrophy there was no apparent immunoreactivity on the surface membrane of skeletal, cardiac, and smooth muscles, or on the neurons of the cerebrum and cerebellum (figs 4 and 5).

Discussion

We previously reported that, in control mice, dystrophin occurred not only in the skeletal muscle but also in the brain. The cell bodies of the neurons, especially the membranes, were immunostained, but in mdx mice, dystrophin was absent even in the brain.¹⁸ The results of immunoblotting on the brain with the same animal models have already been reported by Hoffmann *et al*,²¹ confirming the fact that the dystrophin band appears in the brains of control mice, but is absent from the brains of mdx mice. Because it is difficult to collect specimens, reports comparing normal human tissues with those of patients with Duchenne muscular dystrophy have not been available to date. In this study, tissues from necropsied normal controls, and from patients with Kennedy–Alter–Sung disease, myotonic dystrophy, and Duchenne muscular dystrophy were used. Results similar to those of experiments with animals were obtained by immunoblotting, and dystrophin was localised by immunostaining, as small spots on the cell bodies and dendrites of neurons in the brains of normal controls. Dystrophin was absent, however, from patients with Duchenne muscular dystrophy and with intellectual disturbance.

It has already been shown that the mRNA of dystrophin is present not only in human skeletal and cardiac muscle but also in small amounts in smooth muscle and brain.²² This agrees with the results of our study. By immunoblotting, dystrophin appeared as a doublet in the striated muscle of normal controls, and as a single band in smooth muscle and the brain. As Byers *et al* reported, the lower band of the doublet in striated muscle seemed to lack a portion of the C-terminal and may represent a dystrophin isoform.⁸ A dystrophin band appeared with DYS-1, DYS-3, 60 kDa, 30 kDa, P-20, antibody 6–10, and DMD peptide IV antibodies in the brain, but not with DYS-2 (recognising the C-terminal region of dystrophin in human skeletal muscle). This is assumed to be due to the fact that the four domains of dystrophin, especially in the C-terminal region, show differences in amino acid composition according to the type of tissue (skeletal muscle or brain).²³ Thus DYS-2 recognises dystrophin in skeletal, cardiac, and smooth muscle, but not in the brain. Besides the brain-type (427 kDa) dystrophin isoform, several dystrophin isoforms with lower molecular weight (70 to 78 kDa) were recently found.^{24–26} These proteins, so-called apodystrophins, were thought to be a major Duchenne muscular dystrophy gene product in the brain and non-muscle tissues. In our study, however, no clear band was detected at around 70 to 78 kDa in either control or Duchenne muscular dystrophy brains with DYS-2. This may be due to the fact that DYS-2 is a muscle-specific antibody and does not react with brain-type dystrophin or apodystrophins.

By immunoelectron microscopical study, Lidov *et al*⁹ reported on the localisation of dystrophin in the postsynaptic membrane of neurons in control mice. As for the human brain, no such findings have been reported, but the similarity between the results of Lidov *et al* and our immunostaining patterns for humans (as well as those of the rat brain (unpublished data)), indicates the presence of dystrophin even in the postsynaptic regions of human CNS cortical neurons. In patients with Duchenne muscular dystrophy, the reasons for the often subnormal IQ scores are not clear, but this study suggests that in Duchenne muscular dystrophy the brain-type dystrophin originally present in the neurons is lacking, possibly affecting CNS function. Thus as clarified in skeletal muscle of those with Duchenne muscular dystrophy and Becker muscular dystrophy,^{27–29} the relation of both quantitative and qualitative abnormalities of brain-type dystrophin to the degree of intellectual disturbance must be studied further to clarify its physiological functions.

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Figure 5 Immunohistochemical localisation of dystrophin in the CNS of tissues from normal controls and from patients with Duchenne muscular dystrophy. Normal control cerebral cortex (A-C), Duchenne muscular dystrophy cerebral cortex (D-F), normal control cerebellum (G, H), and Duchenne muscular dystrophy cerebellum (I, J), labelled with preimmune serum (A, D, G, and I) and antibody 6-10 (B, C, E, F, H, and J). Note the punctate immunoreactivity along the cell bodies and dendrites of the cerebral cortical neurons and the cerebellar Purkinje cells in tissue from normal controls (arrows). On the other hand, no immunoreactivity is apparent in tissues from patients with Duchenne muscular dystrophy. Original magnification: A, C, D, F, G, H, I, and J $\times 786$; B and E $\times 238$.

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