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Dystrophin and dystrophin-related protein in the central nervous system of normal controls and Duchenne muscular dystrophy

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Abstract To clarify the localization and characterization of dystrophin and dystrophin-related protein (DRP) in the human central nervous system (CNS), we carried out immunoblotting and immunostaining studies using three region-specific anti-dystrophin and one anti-DRP antibodies. With immunostaining, punctate immuno-reactivity of dystrophin was seen along the cell bodies and dendrites of the cerebral cortical neurons and cerebellar Purkinje cells in the normal controls autopsied. By contrast, dystrophin was not detected at all in the CNS of Duchenne muscular dystrophy (DMD) patients with intellectual disturbance. Immunoreactivity of DRP was observed in the vascular walls of both normal and DMD brains, but not in the neuronal cells. Compensatory increase of DRP was not noted in DMD brains. This study suggests that in DMD the brain-type dystrophin originally present in neurons is absent and may be related to the intellectual disturbance.

Key words Dystrophin · Dystrophin-related protein
Human brain · Duchenne muscular dystrophy

Introduction

Dystrophin is a protein product of the X-linked Duchenne and Becker muscular dystrophy (DMD,

BMD) gene [7, 8, 29]. Dystrophin is thought to be an important structural component of the membrane skeleton, providing resilience to the muscle plasma membrane during cycles of contraction and relaxation [16], but its exact role is still unknown. Dystrophin consists of four domains (actin-binding, spectrin-like rod, cysteine-rich, and C-terminal domains) [17], and at first dystrophin-associated glycoproteins in the plasma membrane were believed to be linked with the C-terminal domain [6]. Recently, however, the binding site was found to lie further inward than previously expected and to be confined to the cysteine-rich domain and the first half of the C-terminal domain [25]. Moreover, in animal models, dystrophin was recently found not only in muscles but also in the specific subsets of neuronal cells in the central nervous system (CNS), which does not undergo dynamic changes in shape [13, 19]. This result implies multiple roles for the dystrophin protein in tissues. After finding dystrophin protein coded by Xp21, the dystrophin-related protein (DRP) link to chromosome 6 was also found [14, 20]. In the skeletal muscles of DMD, BMD, and mdx mice that lack the dystrophin protein, a compensatory increase of DRP has been pointed out [22, 26, 27]. We previously reported on the localization of dystrophin in the murine brain [28]. In the present study, we investigated the localization and characterization of dystrophin and DRP in the human brain.

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Materials and methods

Autopsy materials were taken from the cerebrum (frontal lobe, parietal lobe) and cerebellum of five normal controls (42–74 years old) who died of non-neuromuscular diseases, and three DMD (17, 22, and 26 years old) with intellectual disturbance. The IQ scores of the three DMD patients were 61, 50, and below 50, respectively (tested with the Wechsler Intelligence Scale for Children-Revised or the Wechsler Adult Intelligence Scale). Autopsy was performed within 4 h after death, in cases of DMD the

specimens were collected within 2 h. For immunostaining, frozen sections cut 10 μ m thick were stained with the avidin-DH biotinylated horseradish peroxidase method as described previously [28]. For the primary incubation, three kinds of region-specific anti-dystrophin antibodies were used: two rabbit anti-dystrophin antibodies (antibody 6–10, and antibody IV), and mouse anti-dystrophin antibody (Dys-2; Novocastra Laboratories, UK). Antibody IV is a polyclonal antibody raised against 3495–3544 amino acids of dystrophin and is also well known to cross-react with DRP [12]. Antibody 6–10 is a polyclonal antibody produced in a rabbit immunized with a dystrophin polypeptide expressed in bacteria from dystrophin cDNA residues 6181–9544. It is known to be excellent for the detection of brain-type dystrophin [19]. Dys-2 is a monoclonal antibody raised against the 3669–3685 (C terminus). For the detection of DRP, antibody “LDP” was used. This polyclonal antibody was raised in the rabbit. The antigen is LDPDASGQPFHQAAAGEC predicted from B3 DRP cDNA which has no homology to the corresponding region of dystrophin except for the N- and C-terminal amino acids [26].

For immunoblot, tissues were homogenized in a buffer containing 2% SDS, 5% 2-mercaptoethanol, 4 mM EDTA, 1 mM 40 mM TRIS, 0.24 M glycine, 40% glycerine, and 0.001% bromophenol blue (pH 8.5). Ten microliters of sample buffer containing 100 μ g protein was loaded on 10-well 3.5–12% gradient SDS-polyacryl-amide gels (1 mm $\times$ 10 cm $\times$ 9 cm). After transfer into the nitrocellulose membrane, the specimen was blocked with 10% skim milk, and blots were stained with anti-dystrophin and anti-DRP antibodies at room temperature for 2 h. Dystrophin-anti-dystrophin and DRP-anti-DRP immune complexes were detected with affinity-purified secondary antibody conjugated to avidin-DH biotinylated horseradish peroxidase (Vectastain ABC kit and Elite ABC kit, Vector Laboratories, USA).

Results

With immunostain using antibody 6–10, localization of the antibody was noted to be punctated along the cell bodies and dendrites of the cerebral cortical neurons (Fig. 1 B, C) and cerebellar Purkinje cells (data not shown). With antibody “LDP”, small vascular walls and pia mater stained positive, but the neuronal cells did not (Fig. 1 E, F). With Dys-2 and antibody IV, the cerebral cortical neurons and Purkinje cells did not stain positively, but immunoreactivity was noted on the small arteries (Fig. 1 H, I, K, L). With antibody IV, pia mater also positively stained. On the other hand, in DMD there was no apparent dystrophin immunoreactivity in the neurons of the cerebrum and cerebellum with any anti-dystrophin antibody (Fig. 2 B, C). With antibody “LDP”, small arteries and pia mater of DMD brains stained positively (Fig. 2 E, F).

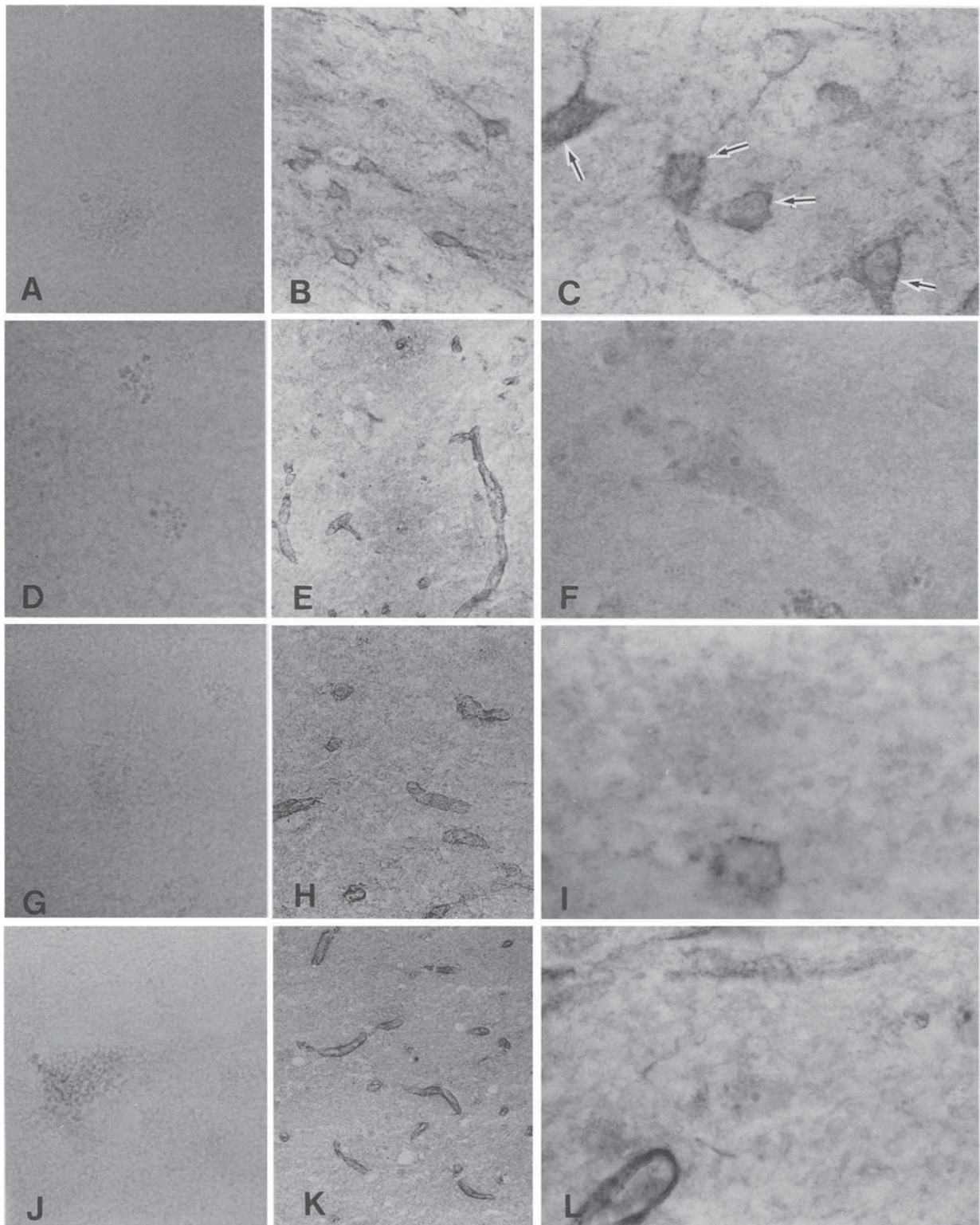
Upon immunoblot of normal control brains (cerebral cortex, cerebellum), antibody 6–10 revealed a clear dystrophin band at 427 kDa, but the dystrophin band was absent in DMD brains. With antibody “LDP”, DRP band was faintly noted at 427 kDa in both normal controls and DMD brains. Antibody VI revealed a clear 427-kDa band in normal control brains which was supposed to derive from both dystrophin and DRP; it also revealed a thin 427-kDa band in DMD brains, presumably deriving from DRP. Meanwhile, no dystrophin band was noted with Dys-2 in either normal controls or DMD brains.

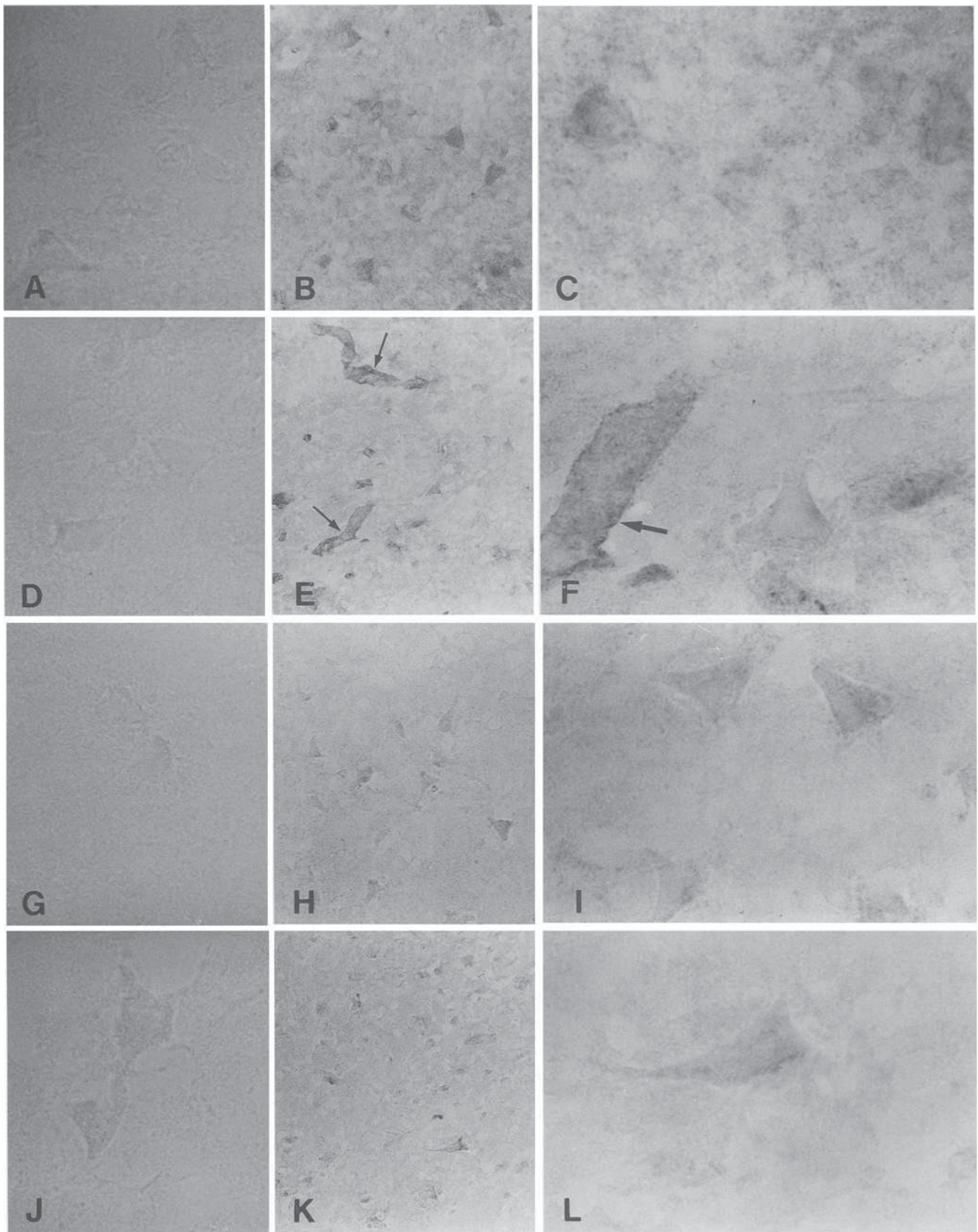
Discussion

The distribution of dystrophin in the CNS has been clarified in model animals [3, 4, 9, 19, 28] and recently in normal human [10], but not yet in DMD patients. In this study, we showed, using antibody 6–10, that dystrophin localized at the neuronal cells of normal human brains. By contrast, dystrophin was absent from the neuronal cells of DMD brains with intellectual disturbance. Although the pathological changes such as degeneration, necrosis, or loss of neuronal cells were absent from the DMD brains with classical histological techniques, the absence of dystrophin was thought to be a specific change linked to DMD gene abnormality and possibly related to intellectual disturbance frequently observed in DMD patients [1, 5, 21, 24, 30–32].

On the other hand, DRP is a chromosome 6-encoded protein with a size and C-terminal sequence similar to dystrophin, as identified by Love et al. [20]. DRP is thought to be a member of the spectrin/ α -actinin/dystrophin family of proteins, and may play a functional role similar to dystrophin. In the skeletal muscle of DMD and mdx mice, it is well known that a possible compensatory increase of DRP is induced by the absence of dystrophin [22, 26, 27]. In this study, immunoreactivity of DRP was observed in the small arteries, but not in the neuronal cells of both normal and DMD brains. Moreover, no possible compensatory increase of DRP was noted in the DMD brains. The immunostain findings of DRP were consistent with those of immunoblot. Takemitsu et al. [26], Pons et al. [23], and Nguyen thi Man et al. [22] reported that DRP was localized at the neuromuscular junction in the normal mouse, similar to the localization of the acetylcholine receptor, suggesting that DRP is one of the cytoskeletons which organize and stabilize the cytoplasmic domain of the acetylcholine receptor. The absence of DRP immunoreactivity in the cerebral cortical neurons may reflect a scant distribution of cholinergic neurons and acetylcholine receptors. Khurana et al. [15] reported that DRP was located at the inner plasma face of astrocytic foot processes at the abluminal aspect of the blood-brain barrier of model animals. However, we could not observe any labelling in glial cells with “LDP”, indicating that an immunoelectron microscopic study may be necessary. With Dys-2, the vascular walls in normal control brains were positively stained, but the

Fig. 1 Immunohistochemical localization of dystrophin and dystrophin-related protein (DRP) in the cerebral cortex (frontal lobe) of normal controls, labelled with preimmune serum (A, D, G and J), antibody 6–10 (B, C), antibody “LDP” (E, F), Dys-2 (H, I), and antibody IV (K, L). Note the punctate immunoreactivity along the cell bodies and dendrites of the cerebral cortical neurons (arrows). With antibody “LDP”, Dys-2, and antibody IV, small arteries were positively stained, but neuronal cells were negative. A, C, D, E, G, J, L $\times$ 785; B $\times$ 322; E, H, K $\times$ 238; I $\times$ 1185





neuronal cells were negative. As Dys-2 is specific against the C-terminal region of human muscle-type dystrophin, it presumably does not react with the brain-type dystrophin. Besides the known DMD gene products, the muscle- and brain-type dystrophin isoforms (427 kDa) translated from 14-kilobase mRNA, several dystrophin isoforms with lower molecular mass (70–78 kDa) were recently found [2, 11, 18]. In our study, clear bands were detected as doublet at around 71 kDa in both normal and DMD brains. However, similar bands of the same molecular mass appeared in the immunoblot using anti-DRP antibody and even preimmune serum. The 71-kDa bands in this study may not be dystrophin isoforms but nonspecific ones resistant to blocking. In this study, brain-type dystrophin was clearly localized in the neuronal cells of cerebral cortex and cerebellar Purkinje cells. Further study on the brains of DMD/BMD patients without mental retardation may help to clarify the physiological role of brain-type dystrophin.

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◀ **Fig. 2** Immunohistochemical localization of dystrophin and DRP in the cerebral cortex (frontal lobe) Duchenne muscular dystrophy (DMD), labelled with preimmune serum (**A, D, G** and **J**), antibody 6–10 (**B, C**), antibody “LDP” (**E, F**), Dys-2 (**H, I**), and antibody IV (**K, L**). With antidystrophin antibodies (antibody 6–10, Dys-2, and antibody IV), no apparent immunoreactivity was noted in DMD. With antibody “LDP”, the small arteries were positive (*arrows*), but the neuronal cells were negative. **A, C, D, E, G, I, J, L** $\times 786$; **B, E, H, K** $\times 238$

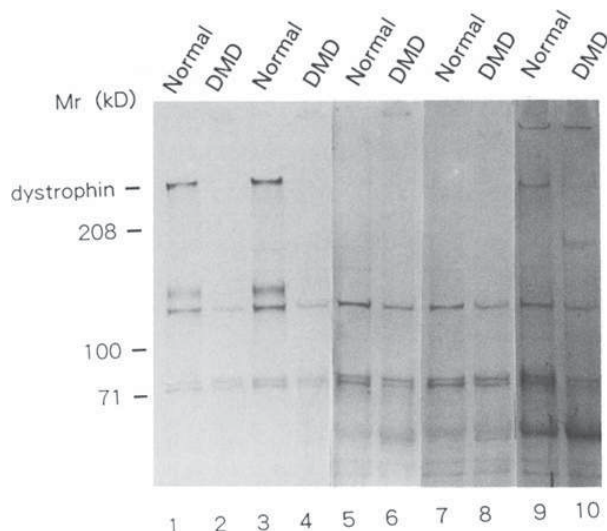


Fig. 3 Immunoblot of the brains from normal controls and DMD. Normal control, lanes 1, 3, 5, 7, 9; DMD, lanes 2, 4, 6, 8, 10. Lanes 1 and 2, frontal lobe; lanes 3 and 4, parietal lobe; lanes 5 to 10, frontal lobe. Blots were stained with antibody 6–10 (lanes 1 to 4), antibody “LDP” (lanes 5, 6), Dys-2 (lanes 7, 8), and antibody IV (lanes 9, 10). The dystrophin band is clear in the normal control brain (lanes 1, 3), but it is not detected at all in the DMD brain (lanes 2, 4). With antibody “LDP”, DRP band was noted very faintly at 427 kDa on the actual nitrocellulose membrane in both normal (lane 5) and DMD brain (lane 6). However, it is hard to confirm the DRP band on this photograph

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