Immunocytochemical Study of Dystrophin Localization in Cone Cells of Mouse Retinas

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Purpose. Previously, the authors reported that dystrophin was observed under the rod cell membranes in rat retinas. However, it was not determined whether dystrophin is located in cone cells. In the current study, the authors clarify dystrophin localization in cone cells of mouse retinas.

Methods. Immunoblotting, confocal laser scanning microscopy, and immunoelectron microscopy were used to investigate retinal dystrophin with a monoclonal antibody raised against the human dystrophin C-terminus.

Results. Immunoblotting analysis showed some immunoreactive bands from retinal extracts. Confocal images indicated two different immunostaining patterns: One was a tiny dot, and the other was a larger, aggregated dot. Immunoelectron microscopy revealed that retinal dystrophin was localized in cone cells as well as in rod cells.


It is well known that dystrophin, a protein with high molecular weight of ~420 kDa, is absent in patients with Duchenne muscular dystrophy (DMD). This protein has been reported to be present under the sarcolemma of skeletal, cardiac, and smooth muscle fibers; therefore its function is assumed to stabilize their sarcolemmal structures. In addition, results of some studies show that the dystrophin is also located in other tissues including those of the central nervous system, peripheral nerves, and retinas.1 In recent decades, it had been accepted that neither visual functional disturbance nor morphologic abnormality was present in retinas of patients with DMD. However, some recent reports indicate that an ocular functional phenotype associated with DMD is characterized by abnormal findings in an electroretinogram (ERG), indicating a reduced amplitude of the b wave under conditions of dark adaptation.2,3 Accordingly, it has been suggested that abnormal signal transmission probably occurs in the outer plexiform layer. Results of immunohistochemical studies have also indicated a relationship between dystrophin and abnormal ERG, because dystrophin is expressed in the outer plexiform layer.4,5 Findings in our previous study showed dystrophin in the rat retina under the rod spherule membrane, determined by immunoelectron microscopy,6 but we did not investigate whether dystrophin locates in cone cells. In the current study, we examined mouse retinas immunocytochemically to clarify whether retinal dystrophin is localized in their cone pedicles.

MATERIALS AND METHODS. Animal and Tissue Preparation. Our investigation is compatible with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We used BALB/c mice anesthetized with ethyl ether and sodium pentobarbital and then perfused with 2% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3, through their hearts. Their eyeballs were immediately enucleated and divided into anterior and posterior hemispheres. The retinas were gently scraped off and immersed in the same fixative for 60 minutes. After treatment with 30% sucrose in PB, they were embedded in OCT compound, then frozen at −80°C.

Dystrophin Antibody. An antidystrophin monoclonal antibody (NCL-DYS2; Novocasta, Tyne, UK), raised against human dystrophin, recognizes the last 17 amino acids of the dystrophin C-terminus (SSGRNTPGKMPREDTM). It has been widely used to detect dystrophin localization in many kinds of animal tissues including retinas.3,6

Western Blot Analysis. Immunoblotting of dystrophin was performed as previously described.7 In brief, mouse retinas and skeletal muscles were separately homogenized and centrifuged at 10,000g for 60 minutes at 4°C. The supernatants were boiled with sample buffer containing 10% sodium dodecyl sulfate for 5 minutes, and then loaded on 5% sodium dodecyl sulfate–polyacrylamide gels for electrophoresis. After transfer on to polyvinylidene difluoride membranes, they were blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 60 minutes. Subsequently, they were incubated with antidystrophin antibody (×30), then with goat antimouse immunoglobulin G antibody conjugated to biotin (Amersham, Buckinghamshire, UK) for 60 minutes. After rinsing in the TBS-T solution three times, they were incubated with streptavidin conjugated with per-
oxidase (Amersham) for 60 minutes and finally visualized by an enhanced chemiluminescence substrate (Amersham).

**Immunofluorescence Staining for Confocal Laser Scanning Microscopy.** Immunocytochemical procedures were performed according to the description in our previous study.\(^5^6\) The frozen retinas embedded in OCT compound were sliced at a 10-μm thickness in a cryostat machine and their cryosections were put on gelatin-coated slide glasses. They were treated with 0.5% saponin in PB for 30 minutes to improve the penetration of antibodies, and in 10% normal goat serum for 30 minutes to block nonspecific binding. Then they were incubated with the monoclonal antidystrophin antibody at a 1:10 dilution for 60 minutes, followed by goat antinmouse immunoglobulin G antibody coupled with Texas Red (Molecular Probes, Eugene, OR) at a 1:200 dilution for 60 minutes. They were observed with a confocal laser scanning microscope (TCS 4D, Leica, Heidelberg, Germany). For immunocntrol specimens, cryosections were incubated with normal mouse serum or phosphate-buffered saline, instead of the primary antibody, and were prepared in the same way.

**Conventional Electron Microscopy.** In a routine manner some retinas were fixed with 2.5% glutaraldehyde in PB for 60 minutes and 1% osmium tetroxide in PB for 60 minutes. After being rinsed in phosphate-buffered saline, they were dehydrated in a graded series of ethanol concentrations and embedded in Quetol-812 (Nissin EM, Tokyo, Japan). Ultrathin sections were prepared, counterstained with uranyl acetate and lead citrate, and observed with a Hitachi H-600 electron microscope.

**Immunoelectron Microscopy.** The immunoperoxidase technique was used for immunoelectron microscopy. The 6- to 10-μm cryosections, which had been immunostained with an antidystrophin antibody, were subsequently incubated with rabbit antinmouse immunoglobulin G antibody conjugated with biotin and then horseradish peroxidase conjugated with streptavidin (Nichirei, Tokyo, Japan). After being rinsed in phosphate-buffered saline, they were fixed again by 0.25% glutaraldehyde in PB for 10 minutes. Then they were rendered visible by metal-enhanced DAB (Pierce, Rockford, IL) and treated with 1% osmium tetroxide in PB for 30 minutes. After routine dehydration in a graded series of ethanol concentrations and acetone, they were embedded in epoxy resin by the inverted gelatin capsule method. Finally, ultrathin sections, which had been counterstained by uranyl acetate and lead citrate, were observed with an electron microscope.

**RESULTS. Immunoblotting Analysis.** Immunoblotting analysis of extracts from BALB/c mouse retinas demonstrates molecular weight bands of approximately 260 kDa, 140 kDa, and 70 kDa, but not of 420 kDa. R = retina; SM = skeletal muscle.

**FIGURE 1.** Immunoblotting of skeletal muscle and retina extracts with antidystrophin antibody. The skeletal muscle lane clearly shows a 420-kDa molecular weight band (arrowhead), whereas other immunoreactive bands may indicate degenerative products. The retina lane shows molecular weight bands of ~260 kDa, 140 kDa, and 70 kDa, but not of 420 kDa. R = retina; SM = skeletal muscle.
FIGURE 2. Confocal images of dystrophin immunoreactivity in BALB/c mouse retinas. (a) Dystrophin is recognized as a dot pattern in the outer plexiform layer. Arrows indicate aggregated dots different from others. (b) Immunocontrol sections. (c) Obliquely cut sections clearly show large aggregated dots (arrowheads). INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer. Scale bar = 10 μm.

flat synapses to invaginated ones (Fig. 3b; left upper inset). The cone cell membrane facing the horizontal cell processes had no dystrophin immunoreactivity (Fig. 3b; right lower inset).

DISCUSSION. So far, three different promoters of retinal dystrophin have been identified, which encode 420-kDa, 260-kDa, and 70-kDa, molecular weight proteins. 1 The antidystrophin antibody we used could detect approximately 260-kDa, 140-kDa, and 70-kDa dystrophin isoforms but did not definitely recognize the 420-kDa dystrophin isoform. Accordingly, the mouse retina probably contains very little full-length dystrophin. The 260-kDa band is probably Dp260, named by D’Souza et al, 7 which is required to obtain normal results in an ERG. In addition, the approximate 140-kDa band was clearly recognized. Such a protein may be Dp140, a novel dystrophin isoform recently reported by Lidov et al. 8 According to their report, this dystrophin isoform is mainly expressed in the central nervous system and may play a key role in neurotransmissions. Therefore, it is reasonable to speculate that Dp140 plays an important function in mouse retinas. The 70-kDa band—probably Dp71—also plays a key role. These results suggest that multiple dystrophin isoforms are present in mouse retinas, although we should be careful that the peptide antibody’s lack of specificity could also produce a false immunolabeling.

Until a few years ago, there had been no reports on visual disturbance or morphologic abnormality in retinas of patients with DMD. Retinal dystrophin had been thought to locate at the postsynaptic membrane as it does in the brain. However, some investigators have recently reported that patients with DMD show a reduced b wave in the ERGs in the dark-adapted state—an abnormality that is presumed to arise at the level between photoreceptor cells and depolarizing bipolar cells. 2,3 Moreover, results of our previous study demonstrated that retinal dystrophin was localized just under some parts of the rod cell membranes. 5 Accordingly, it is probable that the lack of dystrophin is associated with the abnormal patterns found in ERGs.

Furthermore, there have been no reports on color-vision disturbance and abnormal ERG findings in cone cells in patients with DMD. 9 Accordingly, it

FIGURE 3. Electron micrographs of the cone synapses of BALB/c mice for pure morphology (a) and immunoperoxidase staining (b). (a) Asterisks indicate flat synapses, and large arrows indicate an invaginated synapse. (b) Dystrophin immunolocalization is demonstrated at flat synapses (arrow) and at invaginated synapses (arrowheads). Dystrophin immunolocalization (upper left inset) is continuously demonstrated from the flat synapses to the invaginated synapses. Invaginated synapse (lower right inset) at higher magnification. Arrowheads indicate dystrophin immunolocalization under the cone cell membrane facing the bipolar cell process. B = bipolar cell process; H = horizontal cell process. Scale bar = 0.2 μm.
was easy to conclude that no dystrophin was expressed in cone cells. However, in the current immunofluorescent preparations, two distinct immunostaining patterns were recognized: a small dot (~1 μm) was in the rod spherule and a large aggregation (~5 μm) was in the cone pedicle. It has been known that a cone pedicle has two kinds of synapses—flat and invaginated. In the immunoelectron micrographs, retinal dystrophin was localized at both synapses in cone pedicles. The flat synapse has no component of horizontal cells; therefore retinal dystrophin seems to be much less associated with horizontal cells than with bipolar cells. Why do no abnormal ERG responses arise in cone cells of patients with DMD? It may be because of the diversity of localizations and functions of dystrophin isoforms. However, very little is known about whether their localizations and functions are different in photoreceptor cells. Further study is needed to clarify the functional significance of the dystrophin located in cone cells.

In summary, it is clear that retinal dystrophin is localized in cone pedicles as well as in rod spherules. It is a pivotal component in photoreceptor cells and may be closely associated with signal transmission from photoreceptor cells to bipolar cells.

Key Words
bipolar cell, cone pedicle, dystrophin, electron microscopy, immunocytochemistry

References

Treatment of Bleb Leaks With Transforming Growth Factor-β in the Rabbit Model
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Purpose. The mechanism through which peribleb injection of autologous blood results in resolution of bleb leak in the rabbit model remains unclear. This study evaluates the clinical and histologic effects of peribleb injection of transforming growth factor-β (TGF-β) after leak induction in mitomycin-C-treated blebs.

Method. Posterior lip sclerectomies treated with mitomycin-C were created in New Zealand White rabbits. On postoperative day 7, a standardized stab incision was performed on all blebs, and the eyes were randomized to receive a peribleb injection of either TGF-β or of a balanced salt solution.

Results. Injection of TGF-β was associated with the resolution of bleb leak and maintenance of a functioning bleb in 50% (4 of 8) of treated eyes. The remaining TGF-β-treated eyes and control eyes demonstrated continued bleb leaks or bleb failures with intraocular pressure returning to preoperative levels. Histologic examination revealed increased peribleb cellularity and denser collagen deposition in the TGF-β-treated eyes compared with that observed in control eyes.

Conclusions. Peribleb TGF-β injections may contribute to healing bleb leaks, but the injections do not appear in this model to be as useful as whole-blood injections.

We have reported our experience using peribleb injections of autologous blood to treat refractory bleb