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RAPID COMMUNICATION

Immunocytochemical Study of Dystrophin in Muscle Cultures from Patients with Duchenne Muscular Dystrophy and Unaffected Control Patients

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Using immunocytochemical methods, the localization of dystrophin, the gene product affected in Duchenne muscular dystrophy (DMD) in aneural, differentiating human muscle cultures, was studied. Dystrophin was not demonstrable in undifferentiated myoblasts from control patients and from two patients with DMD. After myoblast fusion, the protein was found in circumscribed sarcoplasmic patches, in the perinuclear area, and along the surface of all normal multinucleate myotubes, with more mature myotubes showing predominantly sarcolemmal distribution. There was no staining in myotubes from one DMD patient and only faint diffuse fluorescence in myotubes from the second affected boy, however. These data provide further evidence that dystrophin is a sarcolemma-associated protein, that it is developmentally regulated, and that it is absent or greatly reduced in quantity in skeletal muscle cultures from patients with DMD. (Am J Pathol 1988, 132:410–416)

"DYSTROPHIN" IS THE GENE product affected in Duchenne muscular dystrophy (DMD).[1] In patients with DMD, the protein is either lacking or greatly reduced in quantity in muscle.[2] Immunocytochemistry and subcellular fractionation studies of healthy muscle have localized dystrophin at the sarcolemma[3–5] and at the junctional portion of the T system.[6,7] Dystrophin was absent or greatly reduced in muscle from DMD patients by immunocytochemistry.

Immunocytochemistry was applied to cultured human muscle cells to study the expression, development, and localization of dystrophin during myogenesis in unaffected controls and in DMD patients. The localization of dystrophin was compared with that of two other proteins: fodrin, a spectrinlike protein associated with the plasmalemma,[8–10] which is present in both myoblasts and multinucleated myotubes, and muscle-specific myosin heavy chain (MHC), a sarcomeric protein expressed in myotubes but not in myoblasts.[11]

Materials and Methods

Source of Human Muscle

Muscle biopsies were obtained in the course of diagnostic evaluation with informed consent. Specimens were obtained from two Duchenne patients (ages 3 and 4 years) who met clinical, electromyographic, and histopathologic criteria for the diagnosis of DMD. Six biopsies were used as controls. Two came from a 41-year-old man and a 2-year-old boy who were diag-

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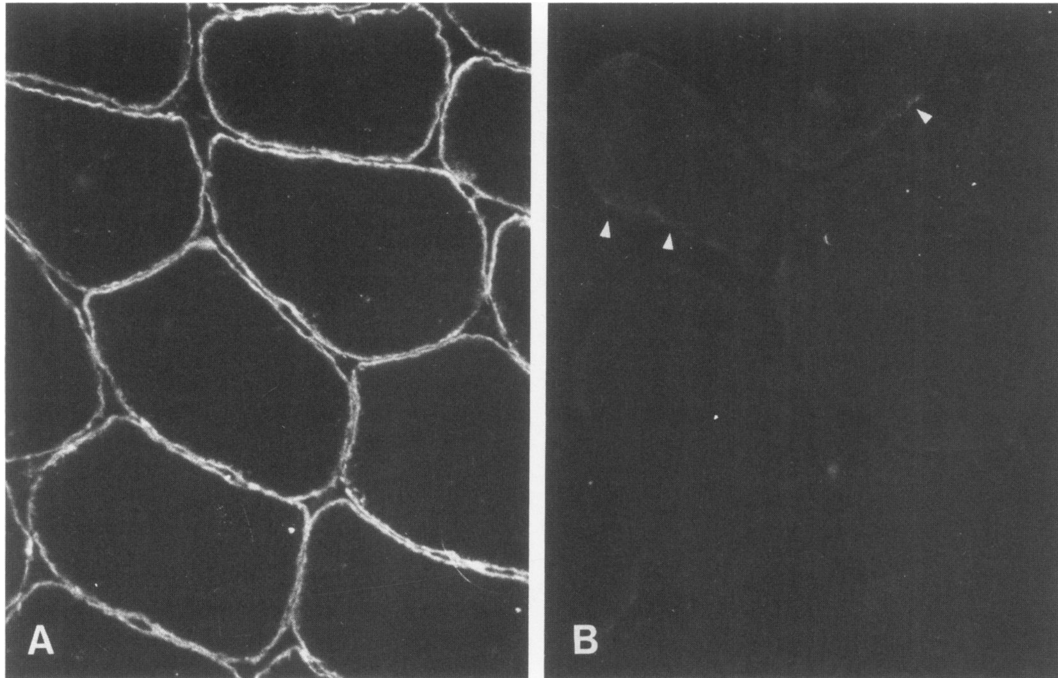

Figure 1—**A**—Frozen cross-section of normal human muscle immunostained with anti-dystrophin antibody. Dystrophin is localized at the sarcolemma. **B**—Cross-section of muscle from a patient with DMD. There is only faint patchy immunostain at the sarcolemma of a few fibers (arrowheads). (×400)

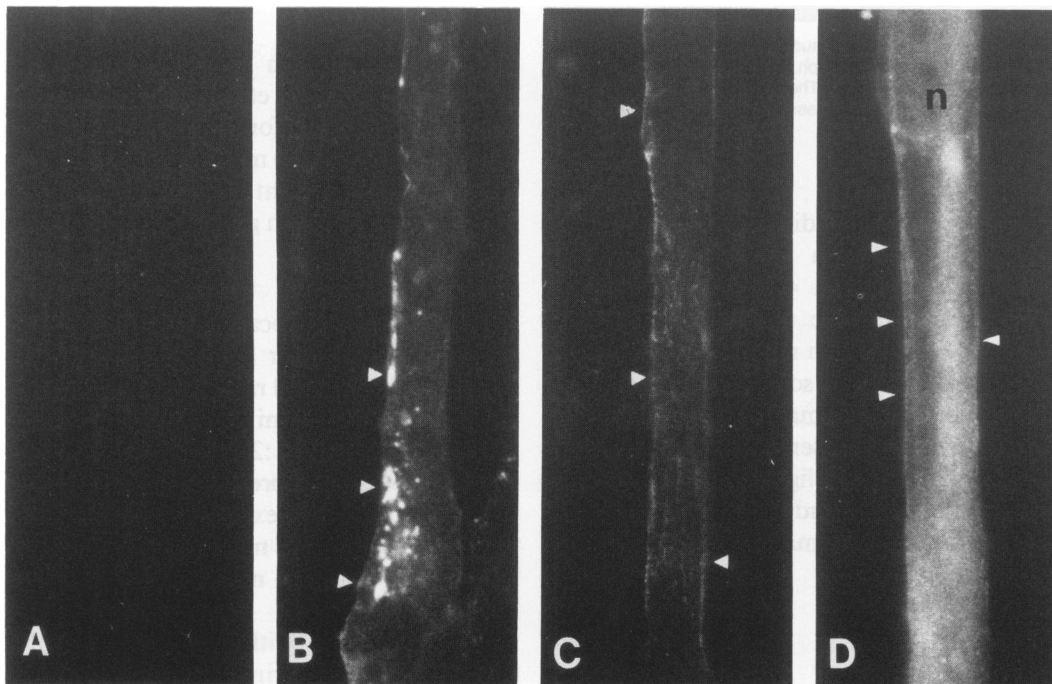

Figure 2—Myotubes in acetone-fixed control muscle cultures immunostained with anti-dystrophin antibody. **A**—Mononuclear myoblasts in a 3-day-old undifferentiated culture do not stain. **B**—Myotube in a 6-day-old differentiating culture shows patches of immunostaining near the sarcolemma (arrowheads). **C**—Myotube in a 10-day-old culture shows faint sarcolemmal immunostain (arrowheads). **D**—Myotube in a 14-day-old culture shows bright immunostain of the cell surface (arrowheads), obliterating a myonucleus (n). (×875)

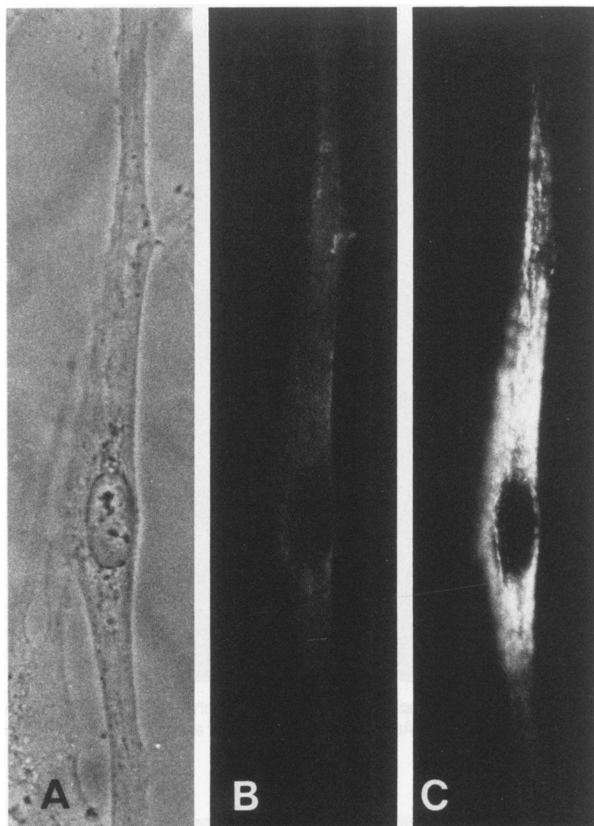

Figure 3—A differentiating (unfused) mononuclear muscle cell in a 10-day-old control culture. **A**—Phase micrograph. **B, C**—Paired immunolabeling with anti-dystrophin and anti-MHC. There is some dystrophin present at the cell surface (B), and the cell expresses muscle-specific MHC (C). (×875)

nosed as free of neuromuscular disease. Other control muscles were obtained from a 17-year-old woman with peripheral neuropathy, a 44-year-old woman with undefined proximal limb weakness, and two boys (ages 14 and 16 years) with minor histologic abnormalities. Before cultivation, some specimens were kept frozen at −160 C in minimal essential medium (MEM) with 20% fetal bovine serum (FBS) and 10% DMSO. When examined in the light microscope with phase optics, all cultures (including DMD cultures) appeared morphologically normal at myoblast and myotube stages.

Cultures

Muscle cultures were obtained from biopsies. The tissue was dissociated with trypsin and myogenic cell clones isolated were as described previously.[12,13] As the cells approached confluency, they were trypsinized and transferred to 35-mm dishes with coverslip inserts. The cultures were studied at the time intervals indicated in the text and legends. In some cultures, myotube formation was promoted by reducing the serum concentration to 5%. Tissue culture methods and media formulations have been described elsewhere.[13,14]

Fibroblast cultures were obtained from diagnostic skin punch biopsies deemed to be normal by morphologic and biochemical criteria. These studies were performed in cultures that had undergone approximately 6–10 population doublings *in vitro*. Methods and media formulations have been described elsewhere.[15]

Immunocytochemistry

Antibodies

Polyclonal anti-dystrophin antibodies directed against a 60 kd aminoacid sequence near the N-terminal portion of the molecule and showing a band at 400 kd in Western blots of skeletal muscle homogenates were used.[1] Anti-fodrin antibody was raised in rabbits against fodrin from pig brain purified and characterized as described previously.[8] Anti-MHC monoclonal antibody (MF20) against myosin from adult chicken skeletal muscle was characterized as described previously.[11]

Fixation

Cultures grown on 22-mm coverslips were fixed with 100% methanol, ethanol, or acetone at −10 C for 1 minute or with 1% formaldehyde (from paraformaldehyde) in PBS for 30 minutes. The PBS solution contained 0.1% detergent (NP40) to permeabilize the cells. Acetone fixation gave the best results.

Immunostaining

Dystrophin was localized by application of anti-dystrophin (1:1000 or 1:1500 in PBS for 1 hour), followed by biotinylated rabbit anti-sheep immunoglobulin (1:200 for 20 minutes) and fluorescein-conjugated streptavidin (1:250 for 20 minutes). A faint diffuse fluorescence present in all cells in all cultures was eliminated by exposing the diluted anti-dystrophin antibody to methanol-fixed monolayers of HeLa cells, which do not express dystrophin,[16,17] for 2 hours.

Paired labeling with combinations of anti-dystrophin and anti-fodrin, or anti-dystrophin and anti-MHC antibodies, was performed by applying a mixture of the two antibodies to be evaluated. Anti-dystrophin was tagged by the biotin-streptavidin method (see above). Anti-fodrin and anti-MHC were tagged with Texas red-conjugated goat, anti-rabbit, and rabbit anti-mouse antibodies (1:200), respectively. Negative control specimens included cultures pretreated

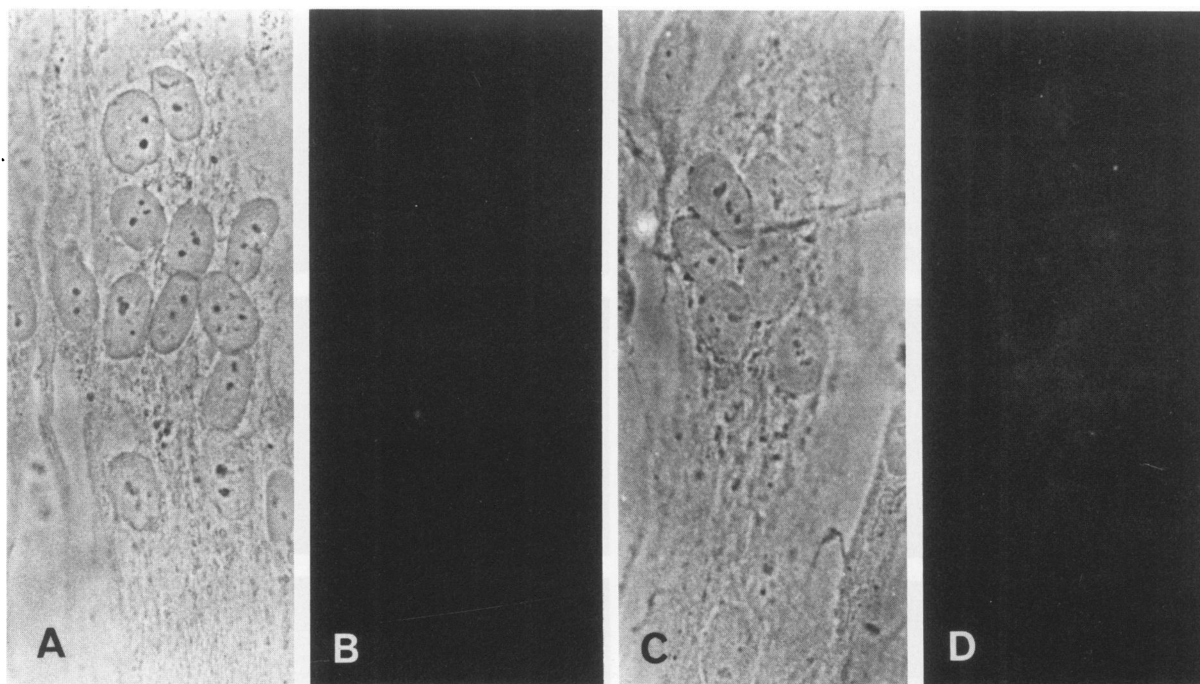

Figure 4—Myotubes in 14-day-old acetone-fixed cultures from two patients with DMD. A, C—Phase micrographs. B, D—Immunocytochemistry with anti-dystrophin antibody. There is no staining in a myotube from one DMD patient (B), and faint diffuse cytoplasmic fluorescence in a myotube from a second DMD patient (D). (×875)

with nonimmune serum and second antibodies, or treated with nonimmune serum, second antibody or fluorescein-conjugated streptavidin, or both.

Unfixed cultures were evaluated by addition of anti-dystrophin (1:1000) to the culture medium. The cultures were rinsed in PBS (5 × 5 minutes), then fixed in acetone and stained as described above.

All preparations were rinsed with PBS after each step (5 × 5 minutes), mounted in PBS-glycerin (8:2), and examined with epi-illumination, in a Zeiss microscope equipped with phase optics, an ultraviolet light source, and appropriate filters.

Results

As reported previously,[3] immunocytochemistry determined that dystrophin was localized at the plasma membrane of normal muscle fibers, where it formed a continuous lining (Figure 1A), whereas virtually no immunofluorescence was seen in DMD muscle biopsies (Figure 1B).

Early muscle cultures, 2–5 days after plating, consist predominantly of proliferating mononuclear cells. After 6–7 days in culture, as cells approach confluence, myoblasts form linear rows and begin to fuse into myotubes that contain two or more nuclei. After 8–10 days in culture, some myotubes have cross-striations.[12]

Using immunocytochemistry, dystrophin was undetectable in prefixed undifferentiated myoblasts from control cultures (Figure 2A). During the period of rapid myoblast fusion, circumscribed areas of fluorescence were seen in the sarcoplasm of control myotubes, some in the vicinity of myonuclei and others further away from the nuclei (Figure 2B). In more differentiated control cultures (10 days), the myotubes showed staining of the cell surface (Figure 2C). After 2 weeks in culture, normal myotubes showed even brighter immunostain at the surface (Figure 2D).

A few spindle-shaped mononuclear cells also showed some fluorescence (Figure 3A, B). These same cells stained intensely in paired labeling experiments with anti-MHC (Figure 3C). Muscle cultures from one boy with DMD showed no stain in myoblasts or myotubes (Figure 4 A and B), and only faint diffuse fluorescence was seen in myotubes from the second patient (Figure 4 C and D).

Paired labeling with anti-dystrophin and anti-fodrin antibody in control cultures and in cultures from the DMD patient lacking dystrophin showed that fodrin was present both in mononuclear myoblasts and in myotubes in both control subjects and patients (Figure 5 C and F). The apparent diffuse staining in the DMD myotube shown in Figure 5F was due to the fact that in this myotube both cell margins and upper cell surface were in the plane of focus. As mentioned

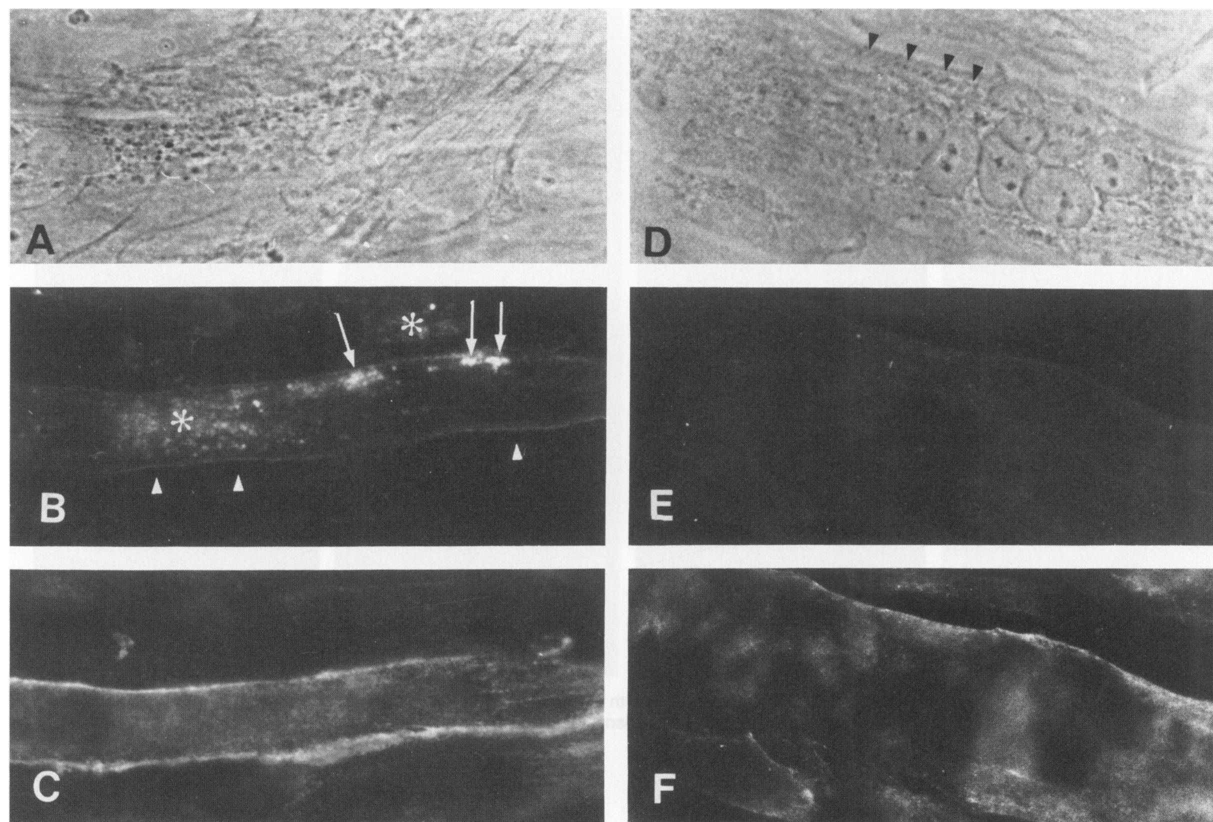

Figure 5—Myotubes in a 21-day-old control muscle culture (A, B, C) and a 21-day-old muscle culture from a DMD patient (D, E, F). Phase micrographs: **A, D**—Developing sarcomeres are indicated by arrowheads (D). **B, E**—Anti-dystrophin immunostain. **C, F**—Anti-fodrin immunostain. Paired immunolabeling with anti-dystrophin and anti-fodrin antibodies in normal myotube shows dystrophin staining in patches (arrows) and at the sarcolemma proper (arrowheads) of the control myotube (B), and distinct sarcolemmal staining with anti-fodrin (C). Some fluorescent spots in the perinuclear areas of the myotube and adjacent mononuclear cell (asterisks) represent orange autofluorescence of lipid droplets, often seen in older myotube cultures. Dystrophin is undetectable in the myotube from a DMD patient (E). Some faint (red) fluorescence of the myotube surface is due to fodrin staining not completely eliminated by the fluorescein filters. The localization of fodrin in the DMD myotube (F) is similar to that of the control myotube shown in panel C. (×875)

above, control myotubes were dystrophin-positive (Figure 5B), but no dystrophin was seen in DMD myotubes (Figure 5E).

Paired labeling of control cultures with anti-dystrophin and anti-MHC was also performed in frozen sections from pelleted cells: patches of dystrophin were present at the muscle cell surface of myotubes that expressed muscle-specific MHC, while no dystrophin was seen in MHC-negative cells (data not shown).

Skin fibroblasts from two healthy individuals showed no staining with anti-dystrophin except for a few spindle-shaped cells that showed bright cytoplasmic fluorescence (data not shown).

Discussion

During myogenesis *in vivo* and *in vitro*, mononuclear myogenic cells fuse spontaneously to form (postmitotic) multinucleated syncytia. This process coincides with the appearance of muscle-specific proteins.[18] Myogenesis is recapitulated during regeneration when "muscle satellite cells," wedged between the basal lamina and the muscle cell surface, are stimulated to divide and fuse to form new muscle.[19,20]

The expression and localization of dystrophin, a newly discovered muscle protein that is absent or decreased in DMD, was studied in developing human muscle *in vitro*. It was found that in normal muscle cultures dystrophin was expressed after myoblast fusion only. The protein first appeared in the cortical cytoplasm of myotubes as discrete round or elongated "packages" in perinuclear regions, a feature not observed in mature muscle. At later stages of development, dystrophin also was found at the sarcolemma of myotubes. Although undifferentiated myoblasts did not express dystrophin, a few spindle-shaped mononuclear cells did stain for dystrophin; however, these cells showed a good degree of differentiation, as indicated by the presence of muscle-type MHC. This finding indicates that myoblast fusion is not a prerequisite for the expression of dystrophin.

In 6–7-day-old cultures, many clearly circumscribed dystrophin-positive "spots" were seen in perinuclear areas and at the myotube surface, suggesting that dystrophin is "packaged" in the cytoplasm before becoming associated with the sarcolemma. Alternatively, the "vesicular" dystrophin-positive structures might represent the association of dystrophin with membranes of sarcoplasmic reticulum or developing T tubules. More detailed immunocytochemical studies at the ultrastructural level should clarify this issue. These data agree with other studies of normal cultures, which showed that dystrophin is absent in myoblasts but present in myotubes.[17,21–23]

Exposure of living, unfixed control muscle cultures to anti-dystrophin antibody did not produce immunocytochemical stain, indicating that there are no epitopes of dystrophin on the outer aspect of the sarcolemma. Sarcolemmal staining was found only in fixed, permeabilized myotubes.

Muscle cultures derived from two boys with DMD were studied: no staining for dystrophin was seen in myotubes from one patient and the stain was faint in the other. This observation is consistent with electrophoretic-immunochemical evidence that dystrophin is absent or greatly reduced in quantity in muscle from most DMD patients.[2]

Because the muscle cultures used in these studies were grown without nerve extract or embryo extract (which might contain "trophic" nerve factors), the expression of dystrophin does not require innervation or neural factors.

To see whether the expression of a sarcolemma-associated protein different from dystrophin was affected in DMD, paired labeling studies were performed for fodrin and dystrophin. No apparent dislocation of fodrin was found when dystrophin was absent, suggesting that fodrin is not affected in DMD. Also, lack of dystrophin did not seem to interfere with sarcomere formation, because cross-striated myotubes were seen in both aneural[24] and innervated muscle cultures from DMD patients.[25] The paired labeling studies also indicated that synthesis of dystrophin and MHC (a developmentally regulated sarcomeric protein) is expressed at approximately the same time.

The few dystrophin-containing cells seen in control skin fibroblast cultures are probably smooth muscle cells derived from blood vessels of the original skin biopsy, but the identity of these cells remains to be established.

This study shows that skeletal muscle cultures can be used to study factors that control the expression of dystrophin. This culture system also appears suitable for investigation of the functional importance of this protein and its interaction with other muscle proteins.

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