Am. J. Hum. Genet. 44:820–826, 1989

Dystrophin Analysis in Clonal Myoblasts Derived from a Duchenne Muscular Dystrophy Carrier

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Summary

Clonal myogenic cell cultures were established from a potential heterozygote for a mutant Duchenne muscular dystrophy (DMD) gene who was also heterozygous for isozymes of the X-linked enzyme glucose-6-phosphate dehydrogenase. Previous tissue culture studies of this muscle donor demonstrated equal proliferative capacity of myoblasts that had lyonized either the paternal or maternal X-chromosome, indicating that mutation of the DMD gene does not affect growth of myoblasts. If this muscle donor were a gonadal mosaic, this conclusion would be incorrect. In the present study, only those myogenic colonies expressing the glucose-6-phosphate dehydrogenase–A isozyme were found to express dystrophin, indicating that this woman was indeed a heterozygote for DMD. By documenting dystrophin deficiency in a specific population of myogenic cells from this woman, we verify our previous conclusion regarding the normal proliferative capacity of DMD myoblasts. Somatic cell testing of dystrophin expression may offer an alternative to established genetic carrier tests for those women in whom deletions of the DMD are not detectable, whose pedigree structure does not permit linkage analysis, or in whom standard phenotypic analyses are ambiguous.

Introduction

Duchenne muscular dystrophy (DMD) is considered one of the most important inborn disorders of man both because of its relatively high frequency in all populations and because of the emotional and economic burdens imposed by its inexorable clinical progression. The complete coding sequence (cDNA) for the X-chromosomal gene that, when defective, causes DMD has recently been determined (Monaco et al. 1986; Burghes et al. 1987; Cross et al. 1987; Koenig et al. 1987), and the normal protein product (dystrophin) of this gene has been identified (Hoffman et al. 1987*a;* Zubrzycka-Gaarn et al. 1988). Dystrophin deficiency has been documented as the primary biochemical defect in DMD-affected humans (Hoffman et al. 1988*a*), *mdx* mice (Hoffman et al. 1987*a,* 1988*b*), and CXMD dogs (Cooper et al. 1988). Dystrophin has been found to be associated with the cytoplasmic face of myofiber membrane systems (plasma membrane and t-tubules) (Hoffman et al. 1987*b;* Bonilla et al. 1988; Knudson et al. 1988; Sugita et al. 1988; Watkins et al. 1988) and is thought to be required for membrane integrity in mature myofibers.

The primary pathological manifestation of dystrophin deficiency is segmental necrosis of mature myofibers. Considerable evidence suggests, however, that the progressive weakness observed in muscle affected by DMD results from the gradual failure of myofiber regeneration, rather than from dysfunction of the myofiber itself (Walton and Adams 1956; Mastaglia and Kukulas 1969; Watkins and Cullen 1985). Muscle biopsies from DMD hemizygotes yield fewer myoblasts than do those from controls (Blau et al. 1983; Delaporte et al. 1984; Jasmin et al. 1984). In addition, myoblasts cultured from DMD hemizygotes undergo significantly fewer divisions in vitro before senescence than do those from controls (Blau et al. 1983). These observations have prompted the hypothesis that the DMD mutation directly inhibits the proliferative capacity of these cells. However, studies with clonal cell strains from muscle

Received January 27, 1989.

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0002-9297/89/4406-0007$02.00

donors thought to be somatic heterozygotes for DMD and also the X-linked marker enzyme, glucose-6-phosphate dehydrogenase (G6PD) have indicated that this hypothesis was incorrect, by demonstrating equal proliferative capacity of both lyonized populations of myoblasts. (Webster et al. 1986; Hurko et al. 1987). This conclusion is valid only if these donors were heterozygous for DMD in somatic as well as gametic cells. The primary purpose of these studies was to determine whether the woman whose cloned myoblasts had been extensively studied in our laboratory was indeed a somatic heterozygote for a DMD mutation (Hurko et al. 1987). In clonal myoblasts derived from this same individual, we studied the expression of the primary biochemical defect—i.e., dystrophin deficiency—responsible for DMD, and we demonstrated that she is a somatic mosaic for the DMD mutation. We thereby validate our earlier conclusion that myoblasts expressing the mutant allele of the DMD gene have a normal capacity for proliferation. In so doing, we establish the basis for a novel means of detection of DMD carriers by testing somatic cells for dystrophin protein.

Methods

Tissue Culture

A primary muscle culture was established by the dissociation technique of Yasin et al. (1977) and was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. Cultures were repeatedly divided at 50% confluence to prevent premature fusion into myotubes. One dilution plate was used to propagate the culture, and the others were used for analyses of proliferative capacity (Hurko et al. 1987) or for freezing in DMEM supplemented with 20% FCS and 10% dimethylsulfoxide. The studies described in the present report were performed on an aliquot of cells that had been frozen at passage 4. Clones were prepared by limiting dilution, and colonies were isolated with cloning cylinders. Clonal cultures were allowed to differentiate into myotubes by permitting growth to confluence and by replacement of the growth medium with DMEM supplemented with 2% horse serum.

G6PD Electrophoresis

Cell lysates were subjected to electrophoresis on cellulose acetate, and the position of the G6PD reaction product was compared with that of G6PD-A and -B standards in parallel lanes.

Western Blotting

For dystrophin analysis, cells were scraped from a culture dish, centrifuged, and then suspended in sample buffer (10% $NaDodSO_4$, 50 mM dithiothreitol, 10 mM EDTA, 0.1 M Tris, pH 8.0.) The protein concentration in the solubilized samples was about 6 mg/ml. Dystrophin was detected by immunoblot after electrophoresis on 3.5%–12.5% gradient $NaDodSO_4$-polyacrylamide gels as described elsewhere (Hoffman et al. 1987*a*). Molecular-weight standards were Coomassie blue–prestained proteins (Sigma) and myosin (M_r 205,000). Goat anti-human vimentin polyclonal antiserum was purchased from ICN Immuno-biologicals. Alkaline phosphatase second antibodies were purchased from Sigma.

Results

A muscle biopsy was obtained from the individual indicated in figure 1. This individual had three sons affected with DMD and was therefore an obligate germline heterozygote for a mutant DMD gene. As she had no previous family history for the disorder, however, it was impossible to determine whether she also car-

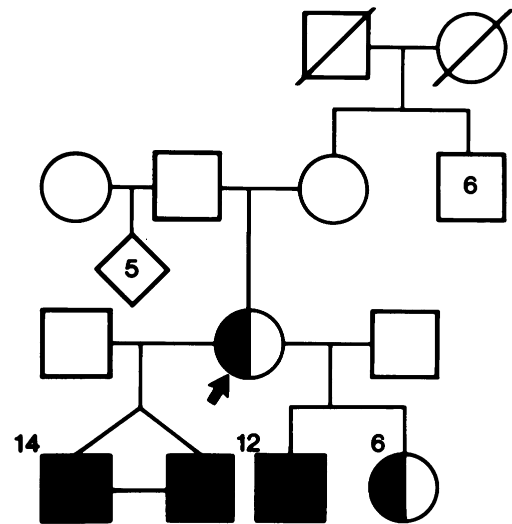

Figure 1 Pedigree of YL (arrow), a G6PD heterozygote from whose biopsied *vastus lateralis* cells were cultured. She was considered to be a DMD carrier because of three affected sons, two of whom were twins, and a daughter presumed to be a carrier because of high serum levels of creatine kinase. YL has no siblings or affected members in antecedent generations; her creatine kinase levels are normal; and her muscle biopsy demonstrated only minimal nonspecific abnormalities. By these criteria, she could be a gametic mosaic whose somatic cells did not contain the mutant DMD gene.

ried the DMD mutation in her somatic cells. This woman was also heterozygous for electrophoretic variants (A and B) of the X-linked marker enzyme, G6PD (Hurko et al. 1987). Given the stability of lyonization in a given somatic cell and its progeny (Lyon 1972), each clonal cell line should express one of the two G6PD variants depending on the active X chromosome of the parental cell. In addition, if the woman was indeed a somatic heterozygote for a mutant (inactive) DMD gene, then each clonal cell colony should either express dystrophin or not express it, again dependent on the specific active X chromosome in the progenitor cell.

A culture was established from an aliquot of cells frozen at passage 4 (10 population doublings in vitro) during the course of earlier studies on this woman (Hurko et al. 1987). Single cell–derived colonies were harvested individually after cloning by limiting dilution. Each colony was expanded to about 5×10^5 cells and then was divided into two sister cultures. One sister culture was harvested for electrophoretic determination of G6PD phenotype (fig. 2), while the other was placed into differentiation medium to allow formation of multinucleate myotubes. Cultures containing multinucleate myotubes (a minimum of three nuclei in a linear array) were scored as myogenic (fig. 3*A*). Those cultures not exhibiting extensive fusion into myotubes were scored as undifferentiated (fig. 3*B*); many of these probably represented fibroblast colonies. Each culture was harvested and scored for expression of dystrophin protein by immunoblot analysis (Towbin et al. 1979) (fig. 4), and the results were correlated with expression of a G6PD isozyme. In the 12 myogenic cultures that gave rise to a detectable G6PD signal, there was perfect concordance (*a*) between the expression of the G6PD-A isozyme and the presence of the dystrophin protein and (*b*) between the expression of the G6PD-B isozyme and the absence of dystrophin (table 1). Furthermore, with two exceptions, dystrophin was not detected in undifferentiated, putative fibroblast colonies. The two exceptional colonies most likely represented either vascular smooth muscle clones or myogenic colonies containing a low proportion of myotubes. These results

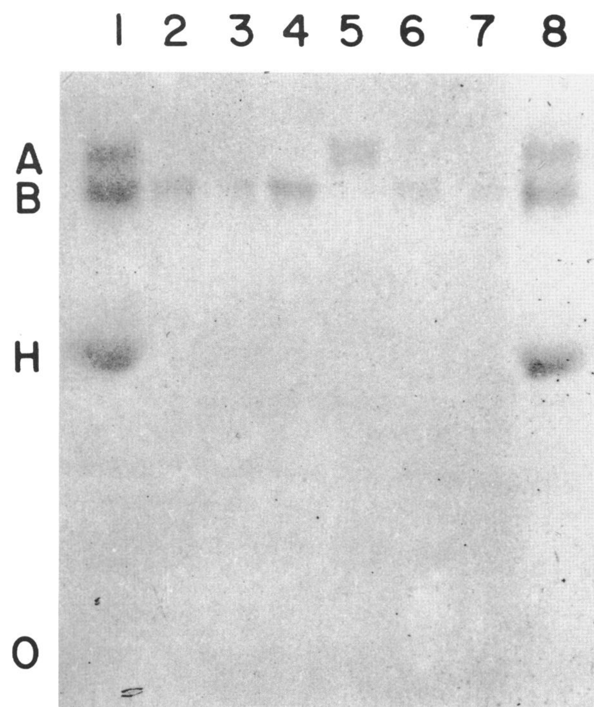

Figure 2 Cellulose acetate gels stained for G6PD activity. Lanes 1 and 8, Peripheral blood lysate from YVL, containing both isoforms. Lanes 2–7. Lysates of individual colonies of cloned muscle cells. A = G6PD-A; B = G6PD-B; O = origin; H = hemoglobin.

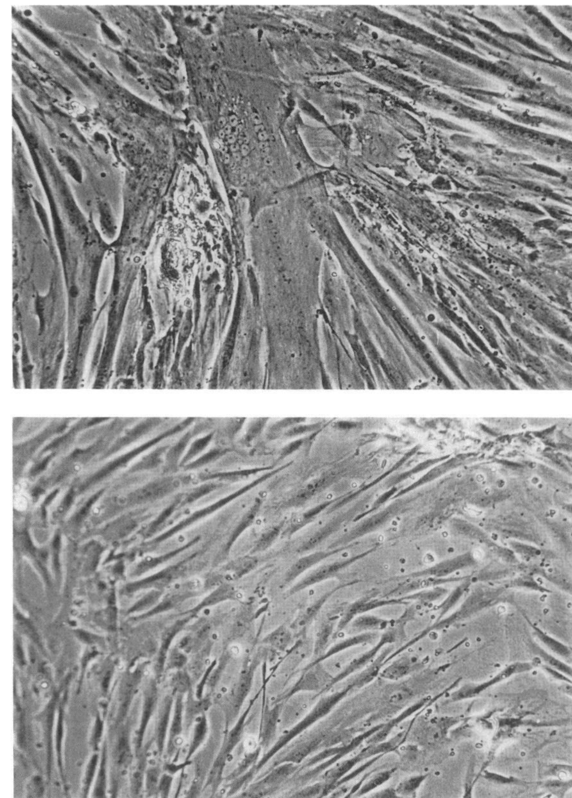

Figure 3 Phase micrographs of secondary cultures of human skeletal muscle growing in DMEM supplemented with 2% (w/v) horse serum. *A*, Differentiated myogenic cells that have fused to form multinucleated myotubes. *B*, Undifferentiated culture containing only mononucleated cells.

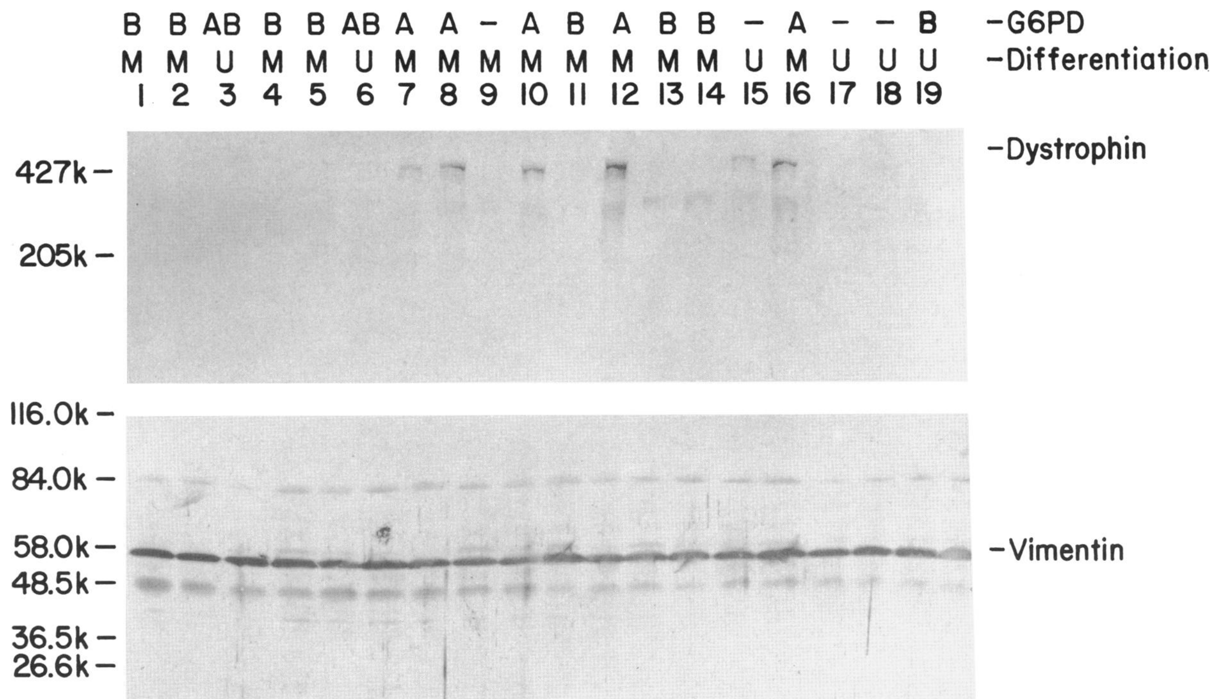

Figure 4 Immunoblot of total protein (50 μg) of the 19 clonal cell cultures derived from a putative DMD carrier biopsy. The protein product of the DMD gene (dystrophin; M_r 427,000) is shown, as well as a protein control (vimentin; M_r 52,000), developed on the same filter. Each lane is also labeled with the differentiation phenotype (M = multinucleated myotubes; U = undifferentiated, only mononuclear cells) and the G6PD phenotype (A = G6PD-A; B = G6PD-B; – = not detected).

verify that the woman tested was indeed a heterozygote for the mutant DMD gene, and demonstrate that carrier detection by dystrophin analysis of clonal myogenic lines is possible.

Discussion

Explanations for the increasingly restricted regeneration potential of skeletal muscle in DMD hemizygotes have included (*a*) putative primary defects of myoblasts (Blau et al. 1983; Delaporte et al. 1984), vascular smooth muscle (James 1962; Hoffman et al. 1988*b*), and neurons (McComas et al. 1971; Hoffman et al. 1988*b*) or (*b*) pathology—i.e., fibrosis (Watkins and Cullen 1985; Hoffman et al. 1987*a*), or proliferative senescence of myoblasts (Hurko et al. 1987)—that is secondary to dystrophin deficiency in myofibers. It appears likely that the loss of regenerative potential in muscle represents a failure of myoblasts, since these cells are responsible for regeneration. Such failure may not result necessarily from expression of the DMD gene in this cell type. Myoblasts derived from the muscle of DMD patients have been shown to have dramatically reduced proliferative potential (Blau et al. 1983), as well as other abnormalities (Delaporte et al. 1984; Jasmin et al. 1984). Such abnormalities could be the result of (*a*) primary expression of dystrophin deficiency in myoblasts, (*b*) exhaustion of the proliferative capacity of myoblasts,

Table 1

Dystrophin and G6PD Phenotypes of Myogenic and Fibroblast Colonies Cloned from a DMD-G6PD Double Heterozygote

Differentiation Phenotype	G6PD	No. of Colonies	Dystrophin Status
Myotubes	A	5	+
Myotubes	B	7	–
Myotubes	ND	1	–
Undifferentiated	A/B	1	–
Undifferentiated	A/B	1	±[a]
Undifferentiated	B	1	–
Undifferentiated	ND	2	–
Undifferentiated	ND	1	+

NOTE.—ND = not detected.
[a] This colony is shown in fig 2, lane 6.

owing to repeated cycles of degeneration and regeneration of myofibers, or (*c*) environmental factors in muscle tissue, resulting in metabolic poisoning of the constituent myoblasts. In this communication, we describe two distinct populations of myogenic cells matched perfectly for autosomal genes and for environmental variables differing only in the expression of dystrophin. These same two cell populations have been studied extensively with regard to their proliferative potential (Hurko et al. 1987). Our documentation of dystrophin deficiency in the G6PD-B population verifies our previous findings that there are no differences in proliferative capacity in dystrophin-deficient DMD and normal myoblasts. This observation, in combination with the failure to detect significant expression of dystrophin in undifferentiated myoblasts (Lev et al. 1987; table 1) suggests that the observed premature senescence of DMD myoblasts is not a primary consequence of dystrophin deficiency.

As our DMD carrier exhibited neither clinical symptoms nor elevations of creatine kinase in the blood, synthesis of dystrophin from myofiber nuclei that lyonized the abnormal X chromosome is apparently sufficient to protect most myofibers from degeneration. Presumably, those rare heterozygotes that are affected severely may have randomly lyonized a disproportionate number of normal X chromosomes (Gomez et al. 1977; Chutkow et al. 1987; Isaacs and Badenhorst 1987; Pena et al. 1987). In DMD carriers, there is an age-related decline in levels of creatine kinase in the serum. Hypothetically, replacement of dystrophin-deficient myofibrillar segments by dystrophin-positive myoblasts in a carrier could explain the age-dependent necrosis of myofibers and the subsequent release of creatine kinase into the blood. Future studies of carrier biopsies by immunofluorescence will test this hypothesis and permit us to determine whether carriers can be detected reliably by immunohistochemical staining of muscle sections for dystrophin.

The determination of dystrophin protein in cloned myoblasts offers a method potentially useful for the diagnosis of DMD carrier females. Because the gene encoding dystrophin is unusually large (Burmeister et al. 1988) and because the DMD mutation is genetically lethal for hemizygotes, there is considerable variability, at the level of nucleic acids, in mutations resulting in the DMD phenotype. For this reason, it is difficult to envision a practicable nucleic-acid assay for the diagnosis of DMD carriers or hemizygotes that would be universally applicable. In contrast, a common feature of all DMD hemizygotes thus far examined has been absence or severe reduction in the amount of dystrophin in skeletal muscle (Hoffman et al. 1987*a*). This indicates that a more generally applicable assay for the diagnosis of hemizygote males—and, by extension, of somatic heterozygote female carriers—would involve analysis of the protein rather than of the nucleic acid. Such an analysis could prove particularly valuable (*a*) for those kindreds not segregating a deletion or in whom creatine kinase determinations are uninformative (Hyser et al. 1987) or (*b*) where linkage analysis is not practicable (Goodship et al. 1988). Although in most women it will not be possible to use the G6PD phenotype as a control for lyonization, a statistical probability could be assigned to the resulting diagnosis, as a function of the number of clones studied. Before such an assay could be applied clinically for genetic counseling, it would be necessary to obtain better estimates both of the patch size of lyonization in skeletal muscle and of the relative frequencies of gonadal mosaics (Bakker et al. 1987; Darras and Francke 1987; Wood and McGillivray 1988) and heterozygotes for DMD mutations.

Note added in proof.—Mosaic expression of dystrophin protein was found in immunostained sections of skeletal muscle from three symptomatic carriers of DMD (Arahata et al. 1989).

Acknowledgments

We are most grateful for patient referrals from directors of Muscular Dystrophy Association clinics and for the many DMD carriers who donated blood or muscle specimens. We acknowledge many valuable and pleasurable discussions with our colleagues: Pamela Talalay, Guy M. McKhann, Victor A. McKusick, and Richard T. Johnson. These studies were supported in part by grants from the Muscular Dystrophy Association and by National Institutes of Health grant AR38231. E.P.H. is the Harry Zimmerman Postdoctoral Fellow of the Muscular Dystrophy Association, L.M.K. is an Associate Investigator of the Howard Hughes Medical Institute, D.R.J. is a Postdoctoral Fellow of the Muscular Dystrophy Association.

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