Extent of shock-induced membrane leakage in human and mouse myotubes depends on dystrophin

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SUMMARY

A lack of the cytoskeletal protein dystrophin causes muscle fiber necrosis in Duchenne/Becker muscular dystrophies (DMD/BMD) and in murine X-linked muscular dystrophy (MDX). However, no overt disease symptoms are observed in dystrophin-less cultured myotubes, and the biological function of dystrophin in normal muscle cells is still unknown. In this work, we have extended our studies on a model system, using hyposomatic shock to determine stress resistance of muscle cells. In frozen sections of control human and mouse myotubes, dystrophin was shown to be localized at the cell periphery as in mature muscle fibers. Dystrophin-less DMD and MDX myotubes were more susceptible to hyposomatic shock than controls, as monitored by the uptake of external horseradish peroxidase and release of the soluble enzymes creatine kinase or pyruvate kinase and of radiolabelled proteins. Control experiments indicated that this difference is not due to differences in metabolism or ion fluxes. Treatment with cytochalasin D drastically increased the shock sensitivity of myotubes and abolished the difference between dystrophin-less and control cells. These results lend further support to the suggested stabilizing role of dystrophin in the context of the membrane-cytoskeletal complex.

Key words: dystrophin, Duchenne dystrophy, MDX mouse, myotube, muscle culture, osmotic shock, cytochalasin D, sarcolemma, microfilament system

INTRODUCTION

Dystrophin is a high molecular mass polypeptide present in very small amounts in several types of vertebrate muscle cells and in neurons (Hoffman et al., 1988). There is no known enzymatic activity of dystrophin. In the muscle fiber, dystrophin’s subsarcolemmal localization (Watkins et al., 1987; Salviati et al., 1989) and non-covalent attachment to both transmembrane glycoprotein complexes (Ibraghimov-Beskrovnaya et al., 1992) and cytoskeletal elements (Levine et al., 1990) suggests a role in either the functional modulation of membrane components such as cation channels (Fong et al., 1990; Franco and Lansman, 1990), or in the mechanical stabilization of the plasma membrane (Koenig and Kunkel, 1990; Menke and Jockusch, 1991; Petrof et al., 1993), or both. As deficiencies in the dystrophin gene are the cause of one of the most frequent fatal hereditary diseases, Duchenne muscular dystrophy (DMD), it would be important to understand the functional role of dystrophin in normal muscle cells. Because the pathology of DMD is complicated by secondary effects such as the replacement of muscle by connective tissue, model systems for experimental studies would be most desirable. The MDX mouse (Bulfield et al., 1984) with a nonsense mutation in the dystrophin gene (Sicinski et al., 1989) provides an animal model of the dystrophin-less condition. However, only certain muscles of the MDX mouse, such as the diaphragm (Stedman et al., 1991), show a pathology similar to that of human DMD muscle.

However, in both human and mouse dystrophies, ongoing muscle fiber leakage or deterioration can be demonstrated by elevated serum levels of soluble muscle proteins (Pennington, 1980; Bulfield et al., 1984; Jockusch et al., 1990; Emery, 1993).

Cell cultures of myotubes, of either human DMD or murine MDX origin do not reproduce the pathological changes seen in dystrophic muscle in situ, and we argue that this may be so because dystrophin is only indispensable in muscle cells subjected to mechanical stress. We therefore devised a simple procedure in which isolated muscle fibers or cultured myotubes are subjected to membrane stress by exposure to a hypotonic environment. While hyposomatic conditions are not relevant as such for DMD pathogenesis in situ, there is good evidence (see below) that, due to osmotic pressure exerted by the solutes inside the cell, they can serve as a model for mechanical stress on the cell membrane-cytoskeletal complex. Using this model, we could indeed demonstrate a lower stress resistance in dystrophin-less isolated muscle fibers and cultured myotubes from MDX mice as compared to normal controls (Menke and Jockusch, 1991). This view has found support from subsequent in situ mechanical stress experiments on mature muscle of MDX mice (Petrof et al., 1993).

Here we show that the cell culture model also reveals a lowered stress tolerance for dystrophin-less human muscle cells and give further evidence of the mechanical nature of the stress response as well as of the importance of dystrophin’s interaction with the cytoskeleton.
MATERIALS AND METHODS

Animals and human biopsy material
C57BL/10-mdx mice used for breeding were originally obtained in 1985 from Dr Bücher, Munich. In this paper, MDX represents the disease phenotypes due to either the mdx/y (male) or mdx/mdx (female) genotypes. Human muscle tissue was obtained from biopsies of seven 5- to 12-year-old-DMD patients. The clinical diagnosis of the younger patients was verified by immunostaining of muscle biopsies with anti-dystrophin antibodies (Dys 1+2, from Novocastra, Newcastle upon Tyne, UK). Control samples were from 7 patients (older than 45 years) who had undergone a hip-joint surgery. None of these patients suffered from a muscular disorder. Informed consent was obtained.

Cell culture
Muscles from MDX or C57BL/10 mice were dissected and enzymatically dissociated by gently shaking for 30 minutes in Eagle’s minimal essential medium (MEM) with 2% fetal calf serum (FCS), containing 1.0 mg/ml collagenase, 1.5 mg/ml protease and 2.0 mg/ml DNase. The cells were cultured in growth medium (GM: 20% FCS, 4% chick embryo extract (CEE) and 1% penicillin-streptomycin in Ham’s F12 medium). To induce differentiation of confluent cultures, the medium was changed to fusion medium (5% FCS and 5% horse serum (HS) in MEM).

For human myotube cultures, biopsy samples weighing 200 to 600 mg were enzymatically dissociated with 0.1% collagenase (type II; Sigma, Deisenhofen, Germany) and 0.2% trypsin (Difco, Hamburg, Germany) in calcium- and magnesium-free phosphate buffered saline (CMF-PBS), 25 mM glucose, 25 mM saccharose, 25 µg/ml gentamycin and 0.1% bovine serum albumin (BSA). The growth medium GM1 contained 5% FCS, 5% HS in a 1:1 (v/v) mixture of Ham’s F12 and CMRL 1415 with 2.5 mg/ml glucose, 0.292 mg/ml glutamine and 50 µg/ml gentamycin (Brinkmeier et al., 1993). Afterwards the cells were plated on collagen-coated tissue culture dishes in growth medium (GM), which contained 10% FCS and 2% CEE in Ham’s F-12 medium. After the cells had reached confluency the growth medium was changed to fusion medium (FM) containing 3% FCS and 3% HS in MEM. The cultures were incubated at 37 °C and in a 5% CO2 atmosphere. Number-coded muscle cultures were obtained from the University of Ulm, and, after reseeding, used for the shock experiments. The code was only released after the evaluation of the experiments.

Immunological methods and reagents
To localize dystrophin, indirect immunofluorescence was performed on cryosections of cultured myotubes. For this purpose, cultures were detached by trypsin treatment and harvested by centrifugation at 200 g in conical plastic tubes. The tubes with the pellet were shock frozen in liquid N2, and the frozen pellets recovered after crushing the tubes. The pellets were mounted, and sections were cut with a cryostat (Leitz, Wetzlar, Germany). The air dried sections were pretreated with 3% bovine serum albumin (BSA) in CMF-PBS, washed three times in CMF-PBS, incubated with a monoclonal anti-dystrophin antibody (Dys-2) for 1 hour at 20 °C or overnight at 4 °C and stained with a Texas Red-labelled anti-mouse IgG antibody (Amersham Buchler, Braunschweig, Germany). Detergent permeabilized muscle cultures were stained with anti-titin (T12; Boehringer, Mannheim, Germany). Observation and photography was done with an Axioshot/UV photomicroscope (Zeiss, Oberkochen, Germany). One-dimensional electrophoresis for dystrophin immunoblots was carried out in an SDS-polyacrylamide gel system with 0.15% bisacrylamilde (Ho-Kim et al., 1991). After electroblotting, the nitrocellulose was blocked with 5% BSA for 1 hour at 20 °C or overnight at 4 °C and stained with a Texas Red labelled anti-mouse IgG antibody (Amersham Buchler, Braunschweig, Germany). Phosphatase activity was visualized with a chlor-indolylphosphate/nitroblue tetrazolium substrate.

Other chemicals
Cytochalasin D, triethylammonium chloride and A23187 were purchased from Sigma (Deisenhofen, Germany).

Osmotic stability test
Cultures were washed twice with CMF-PBS and afterwards treated with shock solutions containing 0.01 M phosphate buffer (pH 7.4) and

CONTROL  HUMAN  DMD

WT  MOUSE  MDX

Fig. 1. Cross striation and immunocytochemical localization of dystrophin in cultured myotubes as used for shock experiments. After 9 to 15 days in fusion medium, genetically dystrophic and normal human and mouse myotubes showed cross striation as demonstrated by anti-titin staining (A, control; B, DMD; F, WT mouse; G, MDX). In frozen cross sections (8 µm) of these cultures, a peripheral localization of dystrophin was seen in control myotubes (C, human; H, mouse), whereas dystrophic myotubes showed no staining (D, E, DMD; I, K, MDX). Bar, 20 µm.
variable concentrations of saccharose as the osmotically active substance, to yield osmotic values from 20 to 200 mosmol (280 mosmol is physiological). After exposing the muscle cultures for 30 minutes to the shock medium, creatine kinase (CK) activity of the supernatant shock medium and the remaining enzyme activity of the cells, after lysis by freezing in distilled water, were determined. The CK activity was measured with an assay kit (Merck, Darmstadt, Germany) at 25°C. In other experiments, the release of $[^{35}S]$methionine-labelled proteins was determined. Cultures were labelled for 19 hours in 1 ml medium containing 30 pmol $[^{35}S]$methionine (sp. act. 14.8 TBq/mmol; NEN, Dreieich, Germany). Subsequently, the labelling medium was replaced by chase medium containing 7.5 mM nonradioactive methionine. After washing with CMF-PBS, the cultures were exposed to shock solutions. The radioactivity of the released trichloroacetic acid (5% TCA, 0°C) precipitatable protein in the shock solution and of the cells was determined.

In order to test membrane penetration by external proteins, 0.1 mg/ml horseradish peroxidase (HRP, RZ 3.0; Sigma, Deisenhofen, Germany) was added to the shock solution. Osmolarity was determined with an osmometer (Knauer, Berlin, Germany). After 30 minutes, the cultures were fixed in 4% paraformaldehyde and stained for HRP with diaminobenzidine. The overall staining intensity of treated cultures was determined by scanning with HP ScanJet II (Hewlett Packard, USA) and the QuantiScan program (Microbiol. Systems Ltd, UK).

The results of the shock experiments were tested with Student’s $t$-test for significance. The data were plotted with the program Meßwertanalyse (PD program, J. Altmann).

RESULTS

By immunoblotting, we have verified that human and murine control myotubes contained dystrophin, at a concentration of at least 30% of that in mature muscle (as judged by the amount of protein required to yield similar signal intensities), whereas none was found in the cultures from DMD patients (thus confirming the diagnosis) or from MDX mice (not shown). In the cultures used for shock experiments, more than half of the murine and 5-15% of the human myotubes showed cross-striations. Within each species, the degree of maturation was not visibly dependent on the genotype (Fig. 1A,B,F,G). Dystrophin immunocytochemistry on frozen sections revealed a peripheral localization of dystrophin in virtually all murine and human control myotubes after 14 days in fusion medium. There was no staining in myotubes from DMD patients or MDX mice (Fig. 1C-E, H-K).

Membrane defects of myotubes caused by hypoosmotic shock were demonstrated in two ways: by the entry of an externally added marker enzyme and by the release of cytosolic proteins, monitored either by radiolabel or by enzyme activity.

In a control experiment, the enzyme release technique (Menke and Jockusch, 1991) was used to evaluate the role of some ion channels and of metabolism. Blockade of plasmalemmal K$^+$ channels by triethylammonium ions (TEA), the introduction of non-physiological Ca$^{2+}$ permeability by the Ca$^{2+}$ ionophore, A23187, and the interference with metabolism by azide (10 mM, not shown) or low temperature shifted the stability curves, but did not abolish the difference between dystrophin-less MDX and control mouse myotubes (Fig. 2).

The entry of an enzyme horseradish peroxidase (HRP, 44 kDa), added to the shock medium, was demonstrated histochemically. Whereas at the most a few single myotubes of untreated cultures were HRP-positive, a considerable percentage of myotubes was HRP-positive in shock treated human cultures, both of control and of DMD origin (Fig. 3). When HRP- and shock-treated cultures were cultured overnight and then stained, almost no HRP-positive myotubes were left.

**Fig. 2.** Effect of interfering with ion fluxes and metabolism on the response of MDX and normal mouse myotubes to hypoosmotic shock. Shown is the amount of creatine kinase activity (relative to the total enzyme content of the culture), released into the supernatant after a 20 minute hypoosmotic shock treatment at the osmolarity shown on the abscissa (in mosmol). Upper left, standard conditions, 37°C (std; replotted data from Jockusch and Menke, 1991); upper right, shock treatment on precooled culture, at 4°C; lower left, K$^+$ conductance blocked with 100 µM triethylammonium chloride (TEA); lower right, Ca$^{2+}$ ionophore A23187 added at 4 µM during shock treatment.
Thus, despite their seemingly intact morphology immediately after treatment (Fig. 3), the permeabilized cells were unable to survive. This was not due to toxic effects of the HRP preparation as shock-treated cultures also detached in the absence of HRP. Densitometric scanning of the extinction of cultures stained for HRP activity allowed for a semiquantitative estimation of HRP entry. The results shown in Fig. 4 indicate a dependence on the osmolarity of the shock solution and

Fig. 3. Penetration into human myotubes of an external protein, horseradish peroxidase (HRP). (A,C,E) control; (B,D,F) DMD. During buffer treatment for 30 minutes, medium contained 100 µg/ml HRP. (A,B) Non-shocked (300 mosmol); (C-D) subjected to hypoosmotic shock (C,F, 96 mosmol; E,F, 45 mosmol). After formaldehyde fixation, cultures were washed and stained for HRP. In a control experiment, HRP-positive (membrane damaged) myotubes would not survive a subsequent incubation at cell culture conditions. Bar, 100 µm.
suggest greater cell damage in DMD as compared to control myotubes. Similar results have been obtained with MDX and control murine myotubes (not shown).

In a second set of experiments, the release of $[^{35}\text{S}]$methionine-labelled soluble proteins was studied. The size distribution of polypeptides released from osmotically shocked cells was determined by SDS-gel electrophoresis of the released proteins. A broad spectrum of molecular masses was found, with the upper limit at about $M_r$ 150,000 (not shown). For quantitative measurements, released TCA precipitable $^{35}\text{S}$ counts were determined. Using the release of $^{35}\text{S}$-labelled soluble proteins and enzyme release, dystrophin-less and control myotubes of mouse and human origin were compared. The $^{35}\text{S}$ experiment confirmed our previous enzyme release experiments on mouse myotubes, and both techniques revealed a stability difference between dystrophin-less and control, for myotubes of human origin (Fig. 5), thus supporting the semi-quantitative results of the HRP influx experiment (Fig. 4). Control cultures with fibroblasts alone yielded a relatively small signal in the case of CK and PK release. There was no difference between the stabilities of fibroblasts of dystrophic and control origin (Fig. 5, diamond symbols).

In order to test the significance of the microfilament system, enzyme release experiments were performed on myotubes that were pretreated with cytochalasin D (Fig. 6). There was a dramatic shift, of $>70$ mosmol, in the mid point of the shock effect, towards lower stabilities. In the case of human myotubes, the difference between dystrophin-less and control was totally abolished, and it was reduced below statistical significance in the case of mouse myotubes.

**DISCUSSION**

In the case of cultured myotubes, a prerequisite of the difference in sarcolemmal stability between dystrophin-less cells and controls being due to dystrophin would, of course, be the presence in sufficient amounts and proper subsarcolemmal localization of this protein, comparable to the situation in mature muscle fibers. Here we show that this condition is fulfilled for both murine and human myotubes. We found that human DMD myotubes, like murine MDX myotubes, are more susceptible to hypoosmotic stress than are their dystrophin-containing counterparts. Thus, already at a relatively early stage of muscle development as compared to a fully mature muscle fiber, a stabilizing function of dystrophin can be demonstrated. This view was supported by measurements on a myogenic rat subline, L185, and its subline L8 with a spontaneous mutation in the dystrophin gene (U. Nudel, personal communication). The latter was more sensitive to osmotic stress than the former (Menke, 1994).

The different osmotic stability could reflect the ‘history’ of cells originating from a dystrophic as compared to a normal organism, rather than the absence versus presence of dystrophin. This possibility was addressed by cloning cell strains

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**Fig. 4.** Quantitative estimation of the influx of HRP. After a shock experiment as shown in Fig. 3, the amount of HRP reaction product was estimated by densitometric scanning. Insets show tracings of absorbance using white light (ordinate, arbitrary units) vs length (abscissa, total length 20 mm); the areas (from 3 independent tracings per point) were used to plot the sensitivity curves.

**Fig. 5.** Leakage of soluble proteins from dystrophic and normal myotubes. Leakage of $[^{35}\text{S}]$methionine-labelled proteins and of the muscle specific enzymes creatine kinase (CK) and pyruvate kinase (PK) as a function of the osmotic value of the shock medium. DMD and MDX myogenic cultures showed a greater leakage than dystrophin containing control cultures. Each point is the mean of 16-26 measurements on 7 independent biopsies. m, myotube cultures (some mouse and all human cultures contained fibroblasts); f, cultures with fibroblasts only. All differences between cells of dystrophic and control origin at the 96 mosmol value are significant, with $P=0.0001$. 

from a mdx/+ heterozygous female. Of four lines, three expressed dystrophin (indicating that the wild-type X-chromosome was active, and the mutated X-chromosome was Lyon-inactivated) whereas one did not (indicating that the wild-type X-chromosome was inactivated). Again, stabilities differed according to dystrophin content (Menke and Jockusch, unpublished results). Recently, a low dystrophin content has been demonstrated in fibroblasts (Hugnot et al., 1993). Although this may explain certain abnormalities of dystrophic fibroblasts, no significant stability difference has been found in our assay system.

A difference in osmotic resistance could either have an essentially mechanical basis, or it could be due to differences in compensatory ion fluxes or in metabolism. In the case of MDX muscle cells, the latter possibility appears unlikely as interference with K⁺ channels did not eliminate the difference between MDX and normal cells, nor did the Ca²⁺ ionophore, A23187 (Fig. 2; Jockusch et al., 1993). Cl⁻ conductance is very low in myotubes and does not seem to play a role in the stability of adult muscle fibers, as shown by mutant samples with reduced sarcolemmal Cl⁻ conductance (Menke and Jockusch, 1991). Furthermore, metabolism is of no importance in the difference between dystrophin-less and control muscle cells.

As hypoosmotic treatment induces membrane blebs, both in isolated muscle fibers (Menke and Jockusch, 1991) and in myotubes (unpublished SEM observations), its predominant effect is most likely mechanical stress, causing swelling and local rupturing of membranes. This phenomenon has been studied extensively in non-muscle cells, e.g. erythrocytes (Davidson, 1970; Savitz et al., 1964) and kidney cell lines (Clegg, 1992). It is assumed that cell stability is dependent on both submembranous cytoskeletal proteins like spectrin in erythrocytes (dystrophin and spectrin share structural homologies; Liu et al., 1990; Svoboda et al., 1992), and the microfilament system connected to these. Evidence for the latter is based on the observation that the microfilament disrupting drug cytochalasin D greatly reduces the cell’s resistance to hypoosmotic shock (Clegg, 1992). In our experiments, cytochalasin D treatment not only destabilized the myotubes but also abolished the difference between dystrophin-less and normal. This provides strong evidence that dystrophin is only relevant for membrane stability in conjunction with the microfilament system. For this reason, the absence of dystrophin would not necessarily lead to measurable effects in the lipid bilayer (see Hutter et al., 1991; Hutter, 1992).

Previous work has indicated higher cytosolic Ca²⁺ concentrations in dystrophic than in normal muscle cells (Bodensteiner and Engel, 1978) and has led to the hypothesis that Ca²⁺ may be involved, e.g. by the activation of proteolysis (Duncan, 1978; Turner et al., 1988; Kämper and Rodemann, 1992), in the necrosis of dystrophin-less muscle cells. However, more recent measurements cast doubt on the finding in elevated Ca²⁺ levels, either steady state or transient, in dystrophin-less cultured myotubes (Pressmar et al., 1994; Rivet-Bastide, 1993) and mature muscle fibers (Head, 1993; Gailly et al., 1993).

The alternative hypothesis of membrane leakage (Rowland, 1976) due to mechanical instability (Karpati and Carpenter, 1986) has been put forward prior to the identification of the dystrophin gene. The present work strongly supports this hypothesis: although more complicated mechanisms cannot be rigorously excluded, the most straightforward explanation for the differential effects of osmotic shock would be a role for dystrophin in mechanical stabilization of the cell membrane-cytoskeletal complex. On the basis of in situ studies on the diaphragm, Stedman et al. (1991) arrived at the conclusion that mature MDX muscle fibers within an intact muscle are less resistant to mechanical stress than normal ones. This supports the biological relevance of our hypoosmotic stress model.

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