PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 187, 267–272 (1988)

The Differential Effects of Calcium Starvation on Duchenne Muscular Dystrophy Fibroblasts[1] (42662)

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Abstract. The effects of nutrient deprivation on normal and Duchenne muscular dystrophy fibroblasts were examined. The requirements for Ca^{2+} and fetal bovine serum were assessed by their effects on the cells' ability to support viral replication, and by ability of the cells to divide in the presence of low levels of these nutrients. When grown in Ca^{2+}-deficient media, Duchenne fibroblasts supported viral replication at a rate 2- to 2.5-fold greater than did normal fibroblasts. At normal Ca^{2+} levels, Duchenne fibroblasts supported viral replication at levels slightly lower than their normal counterparts. After 48 hr in medium containing 0.2 m*M* Ca^{2+}, the growth of normal cells was arrested, while Duchenne fibroblasts were relatively unaffected. When grown in medium containing either 0.2 or 2.0% serum, the growth of normal cells was arrested within 48 hr, with cell death occurring within 72 hr. Duchenne fibroblasts continued to divide at these serum levels for 72 hr, reaching higher cell densities than normal cells. These results suggest that a defect related to Ca^{2+} metabolism may be part of the Duchenne phenotype, which could be used to identify Duchenne muscular dystrophy cells.

Duchenne muscular dystrophy is an X-linked disease which results in severe muscle wasting and early death in affected males (1). Although the primary defect responsible for this disease has not been identified, several cellular and biochemical abnormalities have been associated with the dystrophic phenotype, and have been reviewed recently (2).

Much evidence suggests the presence of an abnormality in the sarcolemma of DMD muscle cells. Mokri and Engel (3) described the presence of lesions in the sarcolemma observable by electron microscopy. These lesions were associated with areas of increased permeability to horseradish peroxidase. In addition, evidence of Ca^{2+} overloading in dystrophic muscle has been presented (4). It is generally believed that an increase in membrane permeability allows for an abnormal influx of Ca^{2+} into the muscle cell. The resulting increase in cytosolic Ca^{2+} sets in motion a series of events, including a Ca^{2+}-induced mobilization of Ca^{2+} from intracellular stores, and the activation of a Ca^{2+}-dependent protease, which probably contributes to the destruction of the affected muscle (5–7).

It has been demonstrated that abnormalities associated with DMD are expressed in cells other than muscle, including fibroblasts and erythrocytes (8, 9). Fingerman *et al.* described the use of herpes simplex virus thymidine kinase (HSV-TK) to probe the metabolism of DMD fibroblasts (10). Their results pointed to a defect in Ca^{2+} metabolism, which permitted the correct identification of DMD fibroblasts after exposure to low extracellular Ca^{2+} concentrations.

This study was intended to continue to explore the metabolic differences between dystrophic and normal fibroblasts. Metabolic changes were induced by nutrient deprivation, and monitored using a viral plaque assay and measurments of cell growth. The results presented confirm those of Fingerman *et al.* (10), and suggest than an abnormality in Ca^{2+} metabolism is present in dystrophic cells which could contribute to the disease process.

Materials and Methods. *Cells.* The majority of the fibroblasts used in this study were obtained from Eileen Fingerman, formerly with Dr. A. B. Pardee, Dana Farber Cancer Institute (Boston, MA). The normal fibroblasts have the following designations:

[1] This project was supported in part by Grant BRSG SO7 RR 05773-12 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health.

0037-9727/88 $1.50

JF027, GM2987, GM498, GM097B, GM-5756, GM5388, and FS2. Dystrophic fibroblasts included MD3781, MD4978, CM008, MD3604, PN007, and HB017 (isolated from a female carrier of the disease). A human fetal foreskin fibroblast HFF was obtained from Dr. R. Mirkovic, formerly of Texas Tech University Health Sciences Center.

Cells were maintained in Eagle's minimal essential medium (EMEM) (GIBCO, Grand Island, NY) supplemented with 0.25% gentamicin sulfate (Sigma Chemical Co., St. Louis, MO), 25 m*M* Hepes (Research Organics, Cleveland, OH), and 10% fetal bovine serum (FBS, HyClone, Logan, UT). Cells were grown at 37°C in 5% CO_2 and 100% humidity. Calcium-free EMEM (GIBCO) supplemented with the indicated amount of $CaCl_2$ (Malinkrodt, St. Louis, MO) was used for all experiments dealing with the effects of Ca^{2+} on cell metabolism. Fetal bovine serum was present at a concentration of 10% unless otherwise indicated. Prior to use, the FBS was dialyzed in cellulose dialysis tubing (Sargent Welch, Dallas, TX) against 4 liters of phosphate-buffered saline (PBS) containing 138 m*M* NaCl, 1.47 m*M* KH_2PO_4, and 8.1 m*M* Na_2HPO_4) until no Ca^{2+} was detectable using cresolpthalene complexone. For experiments dealing with the effect of serum on cell growth. EMEM containing 1.8 m*M* $CaCl_2$ was supplemented with the indicated concentrations of FBS.

Viral plaque reduction assay. Normal and DMD fibroblasts were grown in 96-well tissue culture clusters (Costar, Cambridge, MA) until confluent. Initial plating density was 1×10^4 cells per well. The media were aspirated from the wells, and the cells were washed twice with 200 μl of PBS. Fresh medium containing the indicated amount of $CaCl_2$ was added, and the cells were incubated for 24 hr. At this time, the media were removed and replaced with 50 μl of Ca-free media containing 20–40 plaque-forming units of vesicular stomatitis virus. Virus was allowed to adsorb for 1 hr. The media were removed, and the cells were overlaid with 0.5% methyl cellulose (Fisher, Fair Lawn, NJ) in EMEM containing the appropriate amount of $CaCl_2$. The cells were incubated until viral plaques were evident upon microscopic examination. At this time, the cells were stained with 1% crystal violet in 80% methanol, and the plaques were counted. To assess the effect of a Ca^{2+} antagonist on viral replication, verapamil (Sigma) was added to the media at a concentration of 2×10^{-7} *M*, 24 hr prior to challenge with virus.

Cell growth assay. Cells were plated in 24-well tissue culture clusters (Becton–Dickinson, Oxnard, CA) at 1×10^3 cells per well and allowed to attach overnight. The media were removed, the cells were washed twice with 500 μl of PBS, and medium containing the indicated amount of $CaCl_2$ or FBS was added. At the indicated times, the cells were removed from the wells by trypsinization, diluted to 10 ml in PBS, and counted in a Coulter Model ZBI counter.

Statistical analyses. The data are expressed as means ± SEM. All comparisons between control and experimental groups were performed using a grouped Student *t* test. All *P* values ≤ 0.05 were considered significant.

Results. *Viral replication in calcium-deficient medium.* These studies utilized a total of 13 fibroblast cell strains isolated from normal and dystrophic patients and affected fetuses. Viral replication in normal and DMD fibroblasts was compared in regular (1.8 m*M* Ca^{2+}) and Ca^{2+} free medium. When grown in medium containing normal levels of Ca^{2+}, normal cells consistently supported viral replication at a greater level than did the dystrophic cells, as demonstrated by viral plaque numbers (Table I). The values in Table I represent the pooled results obtained from at least three experiments with each of the cell strains. When deprived of Ca^{2+} for 24 hr, plaque numbers in normal cells decreased by an average of 65%, compared to a 25% reduction in dystrophic cells. Verapamil, a Ca^{2+} channel blocker, caused a further reduction in viral plaque numbers, but failed to exacerbate the differential effect of Ca^{2+} starvation on viral plaque numbers (data not shown).

Cell growth in calcium-deficient medium. Growth of two normal (HFF and MRC-5) and two DMD (MD3781, and CM008) fibroblast strains was compared in medium containing normal (1.8 m*M*) Ca^{2+} concentration, and Ca^{2+}-deficient (0.2 m*M*) medium (Fig. 1). The cells were seeded at low

TABLE I. VIRAL PLAQUE REDUCTION FOLLOWING Ca^{2+} STARVATION

Cell strain	Calcium concentration 1.8 m*M*	0 m*M*	% Reduction
EG011 (N)[a]	22.5 ± 0.9[b]	11.5 ± 0.9	51
JF027 (N)	27.4 ± 0.7	12.2 ± 0.9	56
GM5388 (N)	26.5 ± 0.8	9.2 ± 0.3	65
GM097B (N)	29.4 ± 0.5	6.8 ± 0.8	77
GM5756 (N)	32.6 ± 1.5	6.1 ± 0.4	81
GM498 (N)	31.1 ± 0.9	10.6 ± 0.6	66
PN007 (DMD)[c]	21.7 ± 0.7	17.4 ± 0.8	20
MD4978 (DMD)	23.6 ± 0.8	15.2 ± 0.5	36
MD3781 (DMD)	22.9 ± 0.7	20.6 ± 0.8	10
CM008 (DMD)	21.2 ± 0.5	12.3 ± 1.7	12
HB017 (DMD)[d]	21.7 ± 0.3	15.6 ± 0.5	28

[a] N, normal.

[b] Number of viral plaques ± SEM; $P \leq 0.001$ for all cell strains except MD3781, for which $P \leq 0.03$.

[c] DMD, Duchenne muscular dystrophy.

[d] DMD carrier.

densities to ensure that contact inhibition would not be a factor in controlling the rate of cell division. The growth of the normal cells was partially arrested after 48 hr in Ca^{2+}-deficient media. Some variability was seen in the sensitivity of the different cell strains to Ca^{2+} deprivation; however, the growth of the normal cells was consistently suppressed. In all DMD cell stains examined, growth levels in Ca^{2+}-deficient media were equal to or better than that seen in medium containing normal Ca^{2+} concentrations. In addition, dystrophic cells reached higher cell densities than did the normal cells at low Ca^{2+} levels. These results are representative of those obtained with the other cell strains tested.

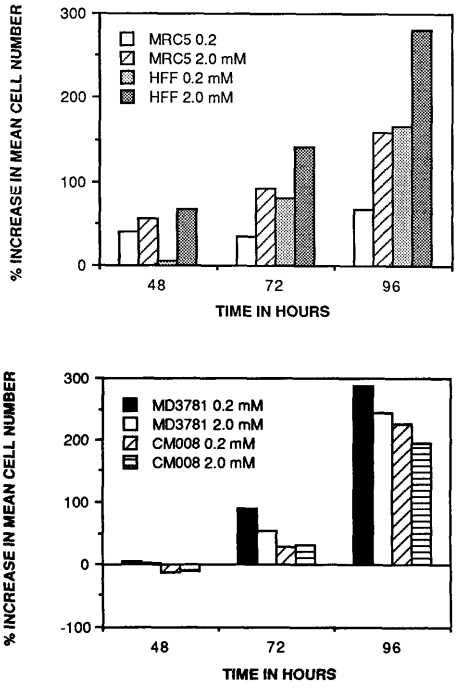

FIG. 1. Growth of normal (HFF, MRC-5) and DMD (MD3781, CM008) fibroblasts in medium containing 2.0 and 0.2 m*M* Ca^{2+}. Graphs illustrate results from a representative experiment. All SEM were ≤1.0.

Serum requirements for cell growth. Fibroblasts normally require 10% FBS for optimal growth *in vitro*. Serum starvation causes a rapid arrest of cell division, followed by cell death. The effect of serum starvation on the growth of normal and DMD fibroblasts is illustrated in Fig. 2. When grown in normal medium (1.8 m*M* Ca^{2+}) containing 10% FBS, normal and dystrophic fibroblasts grew at approximately the same rate over a 96-hr period. By reducing the FBS concentration to 2%, growth of normal cells was arrested within 48 hr, followed by cell death. Dystrophic fibroblasts continued to divide for 72 hr, albeit at a slower rate than seen in medium containing 10% FBS, after which cell death occurred. Similar results were obtained with medium containing 0.2% FBS. Again, normal cells were growth arrested within 48 hr, while dystrophic cells continued to divide for the first 72 hr before dying off. Dystrophic fibroblasts consistently achieved higher cell densities than their normal counterparts at serum concentrations of 2% or less.

Discussion. A role for Ca^{2+} in the pathogenesis of DMD has been suggested (4, 6). However, disturbances in Ca^{2+} homeostasis are believed to be a secondary manifestation of an as yet unidentified genetic abnormality.

Fingerman *et al.* (10) were the first to describe the use of viral replication to probe the metabolism of DMD fibroblasts *in vitro*. Using [^{125}I] iododeoxycytidine incorporation by herpes simplex thymidine kinase in infected cells, they found that Ca^{2+} deprivation had a stimulatory effect on the incorporation of label in infected DMD cells while suppressing incorporation of label in normal cells. The authors concluded that this differ-

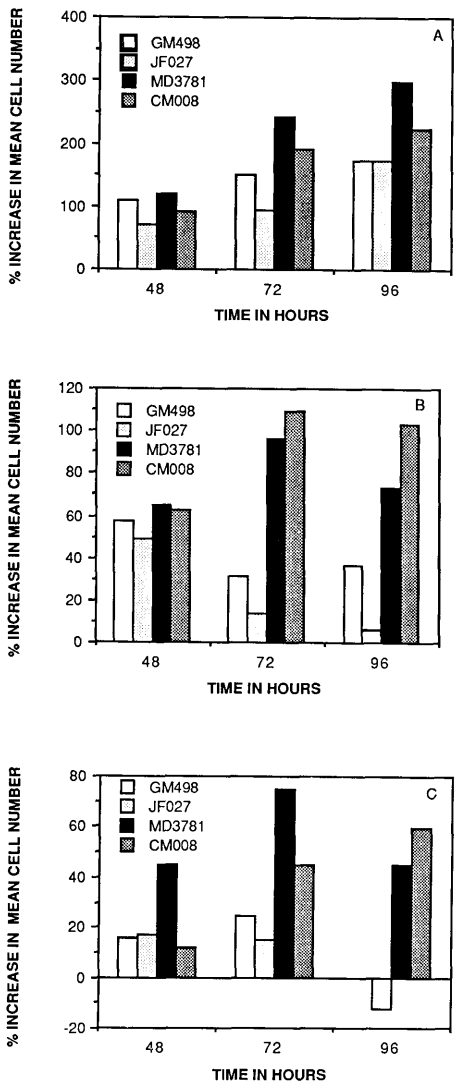

FIG. 2. Growth of normal (GM498, JF027) and DMD (MD3781, CM008) fibroblasts in medium containing 10% (A), 2% (B), an 0.2% (C) fetal bovine serum. Graphs illustrate results from a representative experiment. All SEM were ≤ 1.0.

ential effect was due to abnormally high intracellular Ca^{2+} concentrations in the DMD cells. This hypothesis was further supported by results obtained with Ca^{2+} ionophores and antagonists. It must be noted, however, that increases in cytosolic Ca^{2+} has not been described in DMD fibroblasts. On the contrary, Statham and Dubowitz (11) were unable to detect any differences in the uptake and overall concentration of $^{45}Ca^{2+}$ between normal and dystrophic cells. In affected muscle, increases in intracellular Ca^{2+} are seen, as evidenced by Ca^{2+} precipitates and Ca^{2+}-positive fibers in histological preparations of dystrophic muscle (4, 12).

The present study utilized a plaque assay with vesicular stomatitis virus (VSV) as a probe to identify differences in calcium requirements between normal and dystrophic cells. This RNA virus is capable of infecting a wide variety of cells, and forms well-defined plaques within 36 hr after infection. The results obtained with VSV demonstrate the sensitivity of this assay for monitoring cell metabolism.

The results presented in this study support the hypothesis of Fingerman *et al.* (10). It should be emphasized that the cells used in this study were supplied by these investigators. The precise site of isolation of the cell strains tested was not readily available. Most of the cells were obtained originally from NIGMS Human Genetic Mutant Cell Repository (Camden, NJ), while others were isolated independently from affected individuals. While biochemical differences exist between fibroblasts isolated from different locations, the manifestation of the dystrophic phenotype appears to be a general one which is expressed by many different cell types, regardless of origin (3, 8, 10).

It was found that DMD fibroblasts were significantly more refractory to the effects of Ca^{2+} and serum deprivation than were normal cells. It is interesting to note that at normal Ca^{2+} concentrations (1.8 m*M*), DMD cells consistently supported less viral replication than normal cells. This could be an indication of Ca^{2+}-induced stress in the DMD cells is due to higher intracellular Ca^{2+} concentrations. In addition, the growth of DMD fibroblasts appeared to be stimulated by low extracellular Ca^{2+} levels. Whether these results were due to an increase in membrane permeability to Ca^{2+} or to other factors remains to be determined. Abnormalities in the ability of the sarcoplasmic reticulum to sequester Ca^{2+} have been demonstrated in dystrophic muscle cells (13, 14). This observation could indicate that the abnormality is one of insufficient buffering of cytosolic

Ca^{2+}. In nonmuscle cells, Ca^{2+} is sequestered by the mitochondria and endoplasmic reticulum, as well as by being bound by cytosolic proteins (15). These organelles would be likely candidates for further study.

It is reasonable to assume that if the decrease in the requirement for extracellular Ca^{2+} is due to a defect in the plasma membrane, then requirements for other nutrients might be decreased as well. This was shown to be the case with serum requirements as well. Normal fibroblasts required 10% serum for optimal growth *in vitro*. When the serum concentration was lowered, cell growth was rapidly arrested. Dystrophic fibroblasts displayed a significant decrease in their requirement for serum, as evidenced by their ability to divide at serum concentrations as low as 0.2%. This effect, as well as the decrease in the requirement for Ca^{2+}, parallels those seen in transformed cells. However, growth of both normal and DMD fibroblasts remained contact inhibited.

Growth rates of dystrophic cells have been examined by other investigators with conflicting results. Leichti-Gallati *et al.* (16) reported a significant increase in the doubling times of DMD fibroblasts when compared with those of normal cells, while Wertz and Roses (17) found no significant differences in doubling times. These discrepancies have yet to be resolved. Contact inhibition plays a significant role in the division of cultured fibroblasts. Initial cell densities could be an explanation for the differences in growth rates. For the experiments presented in this study, cells were plated at a low density to ensure that contact inhibition played a minimal role in suppressing cell division over the course of the experiments. In spite of this, however, growth rates varied among the cell strains from experiment to experiment but the final results were consistent.

It appears that the manipulation of extracellular Ca^{2+} levels can be used to identify DMD fibroblasts by a variety of methods. While this approach could form the basis of a prenatal diagnostic test for DMD, it may not be necessary because of recent advances in the isolation of the gene believed to be responsible for this disease. The gene has recently been identified and cloned (18). It is hoped that this will allow the production of a DNA probe to identify the DMD abnormality unambiguously. Identification of the gene product cannot be far behind, and a rational therapy for treatment of DMD may be possible.

Note added in proof. It is worthy to note that two recent reports have described the identification and subcellular localization of the product of the DMD gene locus. Hoffman *et al.* has identified a 400-kDa protein product called Dystrophin which is absent in the muscles of DMD-affected individuals (19). This protein appears to be a constituent of the triads of skeletal muscle which are responsible for the regulation of Ca^{2+} mobilization in the muscle cell (20). It is postulated that the absence of dystrophin in DMD-affected muscle results in a continuous leakage of Ca^{2+} from intracellular stores, which sets in motion a series of events resulting in the destructon of the muscle cell.

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Received September 2, 1987. P.S.E.B.M. 1988, Vol. 187.
Accepted November 2, 1987.