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duchenne muscular dystrophy 5′-nucleotidase
fibroblasts

Abnormal Growth Kinetics and 5′-Nucleotidase Activities in Cultured Skin Fibroblasts from Patients with Duchenne Muscular Dystrophy

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Summary

The experiments reported herein compare growth kinetics and biochemical properties of cultured skin fibroblasts from patients with Duchenne muscular dystrophy (DMD) and matched normal controls.

On day 7 after plating (6000 cells/cm^2) cell number and DNA per dish are significantly reduced ($P < 0.001$) in the cultures from DMD patients ($n = 14$), compared to those from controls ($n = 10$). Moreover DMD cells contain less lipids and proteins per dish but more per cell than normal fibroblasts (not significant). Variations of media (McCoy's medium instead of Eagle's minimum essential medium) and sera (human cord serum instead of fetal calf serum) resulted in the same differences between DMD and control cells.

Cell kinetic experiments (plating density: 2000 cells/cm^2) show increased doubling times of DMD fibroblasts ($P < 0.001$; $n_{DMD} = 5$; $n_{controls} = 4$) whereas plating efficiency is equal for both DMD and controls.

On day 7 the activity of the membrane bound enzyme 5′-nucleotidase either per mg protein or per μg DNA is significantly elevated in cells from DMD patients ($P < 0.0005$; $n_{DMD} = 8$; $n_{controls} = 9$) independent of cell density.

Thus all findings in cultured DMD fibroblasts: increased doubling time, tendency to more voluminous cells, and elevated 5′-nucleotidase activity per cell suggest, that the DMD cells behave similar to prematurely aging cells. Until now we were not able to check whether any alterations of the plasma membrane are inducing early senescence or, reversely, premature aging is the cause of the postulated membrane alterations.

If these findings were to be confirmed in cultured amniotic cells from DMD fetuses, they could serve as a potential prenatal diagnosis of the disease.

Speculation

As a systemic disease, Duchenne muscular dystrophy is also expressed in cultured skin fibroblasts by a growth delay of so far unknown origin. Since cultured amniotic cells are metabolically similar to fibroblasts, corresponding growth kinetic alterations might be expected in amniotic cells from DMD fetuses. This would provide a suitable method for prenatal diagnosis.

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder, which, therefore, occurs almost only in boys. Muscle weakness and wasting become apparent in the first years of life and are slowly deteriorating until early death in the second or third decade. No effective therapy exists at present.

The degeneration of muscle fibers is most probably based on membrane alterations (23, 24, 33, 38, 42). Biochemical and morphologic abnormalities have been found not only in muscle fibers, but also in red cells (6, 14, 18, 20, 22, 30, 39) and lymphocytes (1, 13, 27, 40). All these findings are supposed to be secondary or tertiary alterations. The underlying metabolic defect is not yet known.

Cultured fibroblasts obtained from explants of human skin biopsies have proved to be very useful for the investigations of numerous inborn errors of metabolism. Studies of cultured fibroblasts from DMD patients have been initiated only very recently. There are reports of altered morphology (43), reduced cell aggregation (17), decreased protein synthesis (5, 29), and decreased lysosomal dipeptidyl aminopeptidase I activity (11).

Herein we report the results of investigations on cultured fibroblasts from patients with DMD: plating efficiency, doubling time, effects of different media and sera, ^{14}C-thymidine incorporation in DNA, and activity of the membrane bound enzyme 5′-nucleotidase (EC 3.1.3.5.).

MATERIALS AND METHODS

MATERIAL

We studied fibroblasts growing from skin biopsies of 14 patients with DMD, aged 4 to 14 yr, and of 10 normal boys matched by age, who were undergoing surgical treatment. The cells were usually cultured in Eagle's minimum essential medium (MEM), supplemented with nonessential amino acids and 10% fetal calf serum (FCS). To test the influence of different growth conditions, McCoy's 5A modified medium (26) was used instead of MEM, and MEM was supplemented with 10% human cord serum instead of FCS.

METHODS

Cultures. Skin biopsies were finely minced with scissors, incubated at 37°C in 95% air and 5% CO_2 in 25-cm^2 flasks (Corning) and fed twice weekly with MEM + 20% FCS. After 3 or 4 wk monolayers of fibroblasts were subcultured and frozen in liquid N_2. Cells were rapidly thawed when desired, grown to confluency in 75-cm^2 flasks (Falcon) and subcultured by trypsinization (0.25% for 5 to 10 min). Cell lines were between passage 5 and 10, and for any given experiment all cell lines were at the same passage number ± 1.

For characterization of fibroblasts on day 7, cells were plated at a density of 6000/cm^2 in 100-mm Petri dishes (Corning). For all other experiments, density was 2000 cells per cm^2. Cultures were prepared in triplicates per strain and per day. Cell countings were done in a hemicytometer and cell diameter was measured in a coulter counter. The medium was renewed twice weekly (8 ml/dish). Plating efficiency was calculated as described by Couzin (8). It represents the ratio of the cell number 24 hr after plating relative to the inoculation value ×100%. Doubling time was calculated from the exponential growth ranges of the curves (34) (third to sixth day).

Biochemical Analyses. For biochemical analyses cultures were

Table 1. *Characterization of fibroblasts from patients with DMD and controls on day 7 after plating (6000 cells/cm²): lipids, proteins, DNA, cell count, cell diameter*

		Controls		DMD		P/*t* test
Cell count/dish	$\bar{x}$	2.31×10^6 C	(10)[1]	1.33×10^6 C	(14)	<
	S.E.	0.13		0.06		0.0005
DNA/dish	$\bar{x}$	25.24 μg	(9)	15.54 μg	(13)	<
	S.E.	1.46		0.93		0.001
Protein/dish	$\bar{x}$	673.11 μg	(9)	551.11 μg	(9)	NS[2]
	S.E.	34.51		30.30		
Total lipid/dish	$\bar{x}$	195.38 μg	(6)	160.54 μg	(6)	NS
	S.E.	9.60		9.57		
DNA/cell	$\bar{x}$	12.30 pg	(11)	13.10 pg	(14)	NS
	S.E.	0.75		0.93		
Protein/cell	$\bar{x}$	291.39 pg	(9)	414.37 pg	(9)	NS
	S.E.	1.54		4.29		
Total lipid/cell	$\bar{x}$	84.58 pg	(7)	120.71 pg	(6)	NS
	S.E.	0.64		1.84		

[1] Number of strains are in parentheses.
[2] Not significant.

Table 2. *Plating efficiency and doubling time of DMD and control fibroblasts*

			Controls	DMD	*P/t* test
Plating efficiency (after 24 hr)	cc[1]	$\bar{x}$	76.50% (6)[2]	78.20% (7)	ns[3]
		S.E.	10.74	6.27	
Doubling time	cc	$\bar{x}$	30.04 hr (4)	54.92 hr (5)	<
		S.E.	1.065	3.75	0.001
Doubling time	DNA	$\bar{x}$	31.61 hr (5)	58.98 hr (8)	<
		S.E.	1.18	5.96	0.001

[1] Cell count.
[2] Number of strains are in parentheses.
[3] Not significant.

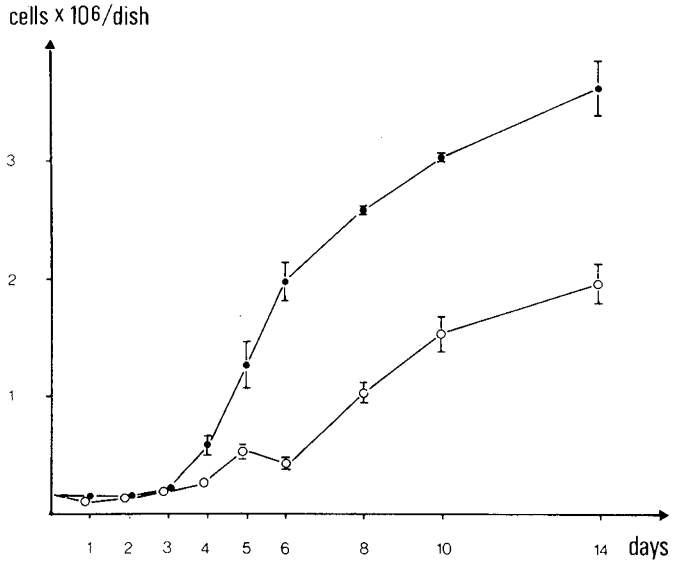

Fig. 1. Cell kinetics per dish; $\bar{x} \pm$ S.E. ●, controls (four strains); ○, DMD (five strains).

washed twice with NaCl and scraped gently into 10 ml of NaCl with a rubber policeman. After centrifugation (1500 × *g*, 5 min, 4°C), the cells were washed once with NaCl and sonified for 30 sec at 30 W in ice. Protein was measured by the method of Lowry *et al.* (21) and DNA was measured by the fluorometric method of Hinegardner (12). For 5′-nucleotidase (EC 3.1.3.5.) we adapted the method of Widnell (41), and for determination of total lipids the method of Zöllner and Kirsch (44) was used.

Thymidine Incorporation. Fibroblasts were incubated during 6 hr in medium with 1 μCi ^{14}C-thymidine per dish (3, 7). After incubation cells were scraped and sonified as described before. During 15 min in ice-water nucleic acids were precipitated with 0.4 n perchloric acid and sedimented by centrifugation (30,000 × *g*, 20 min, 4°C). Sediments were washed once with 0.2 n perchloric acid. DNA was extracted by an incubation in 1 n perchloric acid for 20 min at 70°C. The supernatant was measured directly in a scintillation counter.

RESULTS

CHARACTERIZATION OF NORMAL AND DMD FIBROBLASTS ON DAY 7

As can be seen in Table 1, cell number and DNA per dish are significantly reduced in the cultures from DMD patients, compared to those from controls on day 7 after plating. However, lipids and proteins per dish are reduced in DMD cells, although not significantly. Lipids and proteins per cell are increased in DMD, but also not significantly. DNA per cell is within the same range for DMD patients and controls.

Cell number per dish and DNA per dish were also measured under different growth conditions: McCoy's medium instead of MEM, both with 10% FCS, and in one set of experiments FCS in MEM was replaced by human cord serum. However, these experimental variations resulted in the same differences between the DMD and control fibroblasts.

The mean cell diameter was slightly increased in the DMD strains (23.1 μ) compared to the controls (21.0 μ), but again this difference was not significant.

CELL KINETICS

Six, 12, 18, and 24 hr and 1, 2, 3, 4, 5, 6, 8, 10, 12, and 14 days after plating cell counts and DNA measurements were performed. In DMD fibroblasts we found a significant increase of the doubling time (Table 2, Figs. 1 and 2), and correspondingly a reduced ^{14}C-thymidine incorporation into DNA (measured on days 3, 4, 6, 8, 10, and 13). However plating efficiency is the same (Table 2).

5′-NUCLEOTIDASE

On day 7 after plating 5′-nucleotidase was determined and its activity was expressed per dish, per protein, and per DNA. In DMD fibroblast cultures there is a statistically significant increase of 5′-nucleotidase activity per mg protein and per μg DNA, whereas the total activities per dish are the same for both DMD and normal cells (Fig. 3). The dependence of 5′-nucleotidase

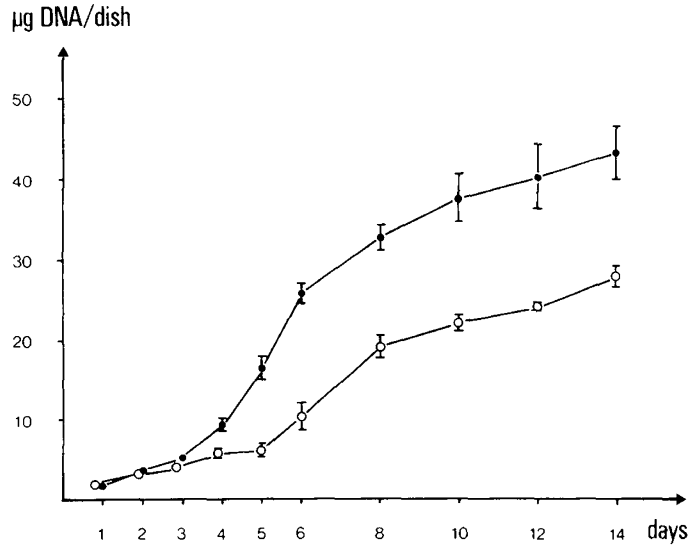

Fig. 2. DNA kinetics per dish; $\bar{x} \pm$ S.E. ●, controls (five strains); ○, DMD (eight strains).

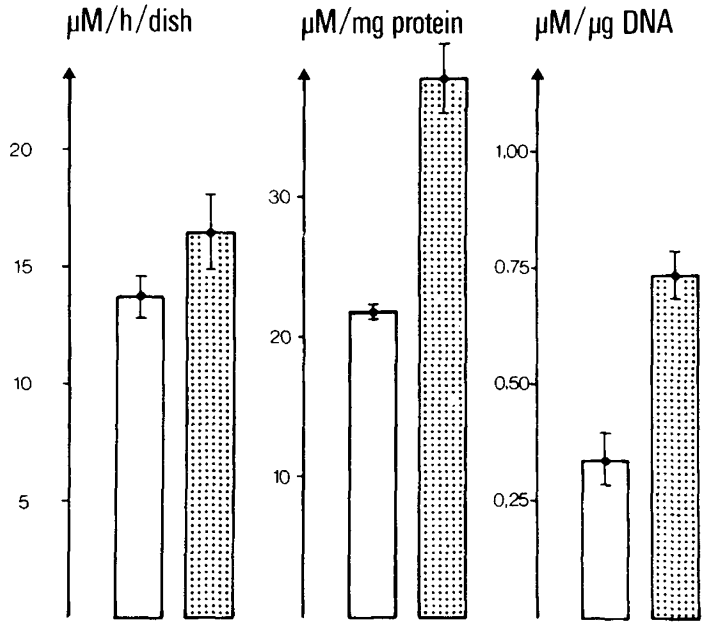

Fig. 3. 5′-Nucleotidase activities on day 7. □, controls (nine strains); ▨, DMD (eight strains); $\bar{x} \pm$ S.E., activity per mg protein: $P < 0.0005$; activity per µg DNA: $P < 0.0005$.

activity on different cell densities was investigated on days 4, 7, 11, and 14. On day 4 cell density of the controls corresponded to the density of DMD cells on day 7, and on day 7 cell density of the controls is similar to DMD cell density on day 11. However, 5′-nucleotidase activity of DMD cell cultures exceeds the activity of the control cultures, independent of the cell density.

DISCUSSION

Compared to normal fibroblasts under identical culture conditions, DMD fibroblasts are significantly reduced in cell number and DNA content per dish on day 7. DMD cells contain less lipids and proteins per dish but more per cell than normal fibroblasts, although not significantly. These findings can be explained as follows: the cell number includes both large and small cells, and the amount of lipids and proteins measured represents the total content of the cell population. If the cell count is reduced in the DMD strains, but the relative proportion of the *larger* cells was increased compared to the controls, the decrease of proteins and lipids *per dish* may not be statistically significant. However, if only a proportion of the DMD cells were enlarged, the average amount of proteins and lipids *per cell* would be increased, but might also fail to be statistically significant. These opposite trends were actually observed (Table 1) and suggest more voluminous cells in the strains from the DMD patients.

This would also be in agreement with the observed tendency toward a slight increase of the mean diameter of DMD cells. There are three possible explanations for the reduced cell number and DNA content per dish observed: (1) the DMD cells may stop proliferation earlier than the control cells, (2) their plating efficiency might be reduced, or (3) their doubling time increased. Kinetic studies on plating efficiency and doubling time show that the observed difference is probably due to a delayed doubling rate of the DMD cells. These findings would be in accordance with the observations of Rodemann (29). He found a decreased number of generations achieved by DMD fibroblasts in culture. In contrast Pena *et al.* (25) did not find any differences in growth kinetics between normal fibroblasts, obtained from the Institute for Medical Research, Camden, NJ, and DMD cells obtained from the Repository for Mutant Human Cell Strains, Montreal, Quebec. A possible explanation for these controversies could be that their DMD and control cell lines were from different sources, so that the conditions for their primary fibroblast cultures are therefore probably not comparable with our matching procedure. Roses *et al.* (31) also found maximal growth rate and maximal cell density to be similar in both DMD and controls, but they do not mention experimental data or culture conditions. Therefore, it is difficult to compare our results with the work of Roses *et al.* (31).

A number of investigators were looking for biochemical, physiologic, and morphologic differences between cultured fibroblasts of DMD patients and controls. Boulé *et al.* (5) and Ionasescu *et al.* (15, 16) studied protein synthesis, Statham (36) protein degradation, Cullen and Parsons (9) and Wyatt and Cox (43) inclusion bodies, Gelman *et al.* (11) lysosomale enzymes, Jones and Witkowski (17) cell adhesiveness, Kohlschütter *et al.* (19) phospholipid composition, and Statham and Dubowitz (35) calcium exchange. However, none of these authors gives any data about growth kinetics.

Since the reduced cell number and DNA amount on day 7 and the increased doubling time in DMD fibroblasts could be the result of an alteration in the plasma membrane, and since many reports suggest a membrane defect in DMD for various cells (2, 6, 13, 18, 20, 23, 29, 30, 39, 41), we studied the activity of the membrane bound enzyme 5′-nucleotidase. We found a significant increase of its specific activity in the DMD cells. This could be the result of an increased amount of plasma membrane per cell, which would be in agreement with the tendency of DMD cultures to produce more voluminous fibroblasts. Scholte and Busch (32) did not observe these differences for the 5′-nucleotidase activities; however, in their study there is no information on culture conditions and the number of subcultures, and the comparison was made only between one control and one DMD cell line. At the moment we cannot explain the discrepancies between the findings of the two laboratories.

Obviously the DMD mutation is also expressed in the cultured fibroblasts from the patients. This interpretation is in agreement with the literature. Rodemann (29) points out the decreased generation number of DMD fibroblasts as a specific behaviour of aging cells. Moreover, there is a strong correlation between cell size and the onset of cell division (28), and it may be justified to define voluminous cells, which are no longer participating in the division cycle, as senescent (10). Finally 5′-nucleotidase activity in different human cells was found to be inversely proportional to their rate of proliferation (37). Thus, all our findings in cultured DMD fibroblasts—increased doubling time, tendency to more voluminous cells, and elevated 5′-nucleotidase activity per cell suggest, that the DMD cells behave similarly to prematurely aging cells. Until now we were not able to check whether any alterations

of the plasma membrane are inducing early senescence or, reversely, whether premature aging is the cause of the postulated membrane alterations.

As amniotic cells are metabolically resembling fibroblasts, we are now investigating cell kinetics and 5′-nucleotidase activities of cultured amniotic cells from DMD carriers, aiming at a possible prenatal diagnosis of DMD.

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