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ABNORMAL PROTEIN METABOLISM IN SKIN FIBROBLASTS IN VITRO FROM PATIENTS WITH DUCHENNE MUSCULAR DYSTROPHY

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SUMMARY : Rates of protein turnover have been measured on a statistical basis in Duchenne muscular dystrophy and normal skin fibroblast populations in vitro. At comparable numbers of cumulative population doublings, protein synthesis was significantly reduced by about 24 % in DMD fibroblasts as compared to normal fibroblasts ($p < 0.01$, $N=12$). Degradation of short lived proteins was significantly enhanced by about 60 % ($p < 0.05$, $N=18$), and the degradation of long lived proteins was significantly increased by about 28 % ($p < 0.05$, $N=18$) in DMD fibroblast populations in vitro. The enhanced degradation of long lived proteins in DMD fibroblasts can be reduced to basal levels of degradation by the use of the protease inhibitors leupeptin and Ep475 ($p < 0.05$, $N=9$).

Duchenne muscular dystrophy (DMD) is a X-chromosome linked recessive disorder, characterised by a progressive muscle degeneration in the primary organs of manifestation, the skeletal and cardiac muscles (1). The primary genetic defect of this disorder has not been defined, however, the dystrophy of skeletal and cardiac muscles is regarded to be the result of an abnormal protein metabolism (1-4).

Numerous DMD specific cellular and biochemical abnormalities have been demonstrated in erythrocyte-, lymphocyte-, nerve-, and fibroblast cells of DMD patients (1,5,6). DMD skin fibroblasts in vitro express a variety of syndrome specific cellbiological (7-13) and biochemical abnormalities (14-19), e.g. alterations in protein synthesis (12,15-17), in collagen metabolism (15,18), and

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alterations in the degradation of individual proteins (19).Some studies (20,21) describe nonsignificant changes in protein degradation in DMD fibroblast in vitro.

In the present study, protein synthesis and degradation of short and long lived proteins of DMD and normal fibroblast populations in vitro was analysed on a statistical basis. We wil provide evidence,that DMD fibroblasts in vitro express significant altera- in protein synthesis and degradation,that are comparable to the abnormalities in protein metabolism found in DMD in vivo (1-4). Defects in the differential degradation of individual proteins in DMD fibroblasts in vitro have been described by us previously (19).

MATERIAL AND METHODS

Cell cultures : Skin fibroblast populations of 6 male patients with advanced to very advanced manifestations of the DMD syndrome, age 6 to 15 years,and of 6 age-matched normal male probands were established as described elsewhere (18,19).DMD and normal fibroblast cell lines were routinely cultured in Dulbecco's Modification of Eagle's Medium (DMEM,Gibco-Biocult,Karlsruhe,FRG),supplemented with 10 % fetal calf serum (fcs,Seromed,München,FRG,lot No. 107 and 139;Böhringer,Mannheim,FRG,lot No.180),and standard amounts of antibiotics (18).Cells were grown in plastic tissue culture plates (55 cm^2,Falcon,Becton Dickinson,Heidelberg,FRG) in a humidified atmosphere of 95% air and 5% CO_2.Growth rates and cumulative population doublings (CPD) were determined at each transfer by counting the cells in a hemocytometer.For biochemical analyses,cells were seeded into multiwell tissue culture plates (2 cm^2,Falcon,Becton Dickinson,Heidelberg,FRG) at a density of 10^5 cells per well.All biochemical investigations were undertaken with cell populations,6 days after seeding into wells at complete confluency (app. 1.5 x 10^5 cells per well).

Protein synthesis: Cells at complete confluency were labelled for 24 hrs with[3H]-leucine,1 µCi (5 Ci/mmole,New England Nuclear,Dreieich,FRG) in 1 ml of DMEM+10% fcs.After labelling,radioactive medium was removed,and cell monolayers were washed 5 times with phosphate buffered saline (PBS,4°C).1 ml of 5 % trichloroacetic acid (TCA,4°C) was added to each well for 30 min (4°C).TCA-soluble fraction was removed and cell monolayers were rinsed with PBS,solubilised in 1 ml of 0.1 % sodium dodecyl-sulfate-0.1 N NaOH (0.1 % SDS-0.1 N NaOH) and counted in a liquid scintilation system (22). Counts per minute (cpm) were corrected to the cell number (cpm/ 10^5 cells),determined from parallel cultures.

Protein degradation : Cells at complete confluency were labelled for 1 hr with 10 µCi (short lived proteins) or 48 hrs with 1 µCi [3H]-leucine (long lived proteins) in 1 ml of DMEM+10% fcs.After labelling,radioactive medium was removed and cell monolayers were washed 5 times with unlabelled chase medium,which is identical to labelling medium except that [3H]-leucine has been replaced by 2

mM unlabelled l-leucine.After washing, 1 ml of chase medium was added to each well either for 20 min (short lived proteins) or 4 hrs (long lived proteins), in order to flush free radioactive leucine from the cells (prechase period)(22).Prechase medium was removed and fresh chase medium was added to each well to minimize reincorporation of labelled leucine into proteins (22),either for 4 hrs (short lived proteins) or 24 hrs (long lived proteins).Radioactivity soluble in 10% TCA (4°C) was assayed in aliquots of the chase medium.At the termination of each experiment,cell monolayers were rinsed with PBS (4°C) and 1 ml of 5% TCA was added to each well for 30 min (4°C).TCA-soluble fraction was removed and monolayers were counted in a liquid scintilation system.Rates of protein degradation were calculated according to the formula : % Protein degradation=Acid soluble cpm in the medium x 100/cpm in the protein at the end of the experiment + acid soluble cpm in the medium.

The effect of protease inhibitors on the degradation of long lived proteins in DMD and normal fibroblast cell lines was measured as described above in the presence and absence of leupeptin (Sigma Chemicals,München,FRG) and Ep475 (Ep475 was kindly provided by Dr.A.L.Goldberg,Harvard Medical School,Boston,USA).Protease inhibitors were added to the prechase and chase medium at a concentration of 25 µM.

Statistics : For statistical analysis, differences in protein synthesis and degradation between DMD and normal fibroblasts were compared by subsampling in a single factor study with complete randomized design (23). The effects of protease inhibitors were tested for statistical significance by a generalized block design combined with the Dunnett-test (23,24).

RESULTS

Rates of protein synthesis analysed in DMD and normal skin fibroblast populations *in vitro* at cellbiologically well characterised states (CPD 15 to 20),were significantly decreased in DMD fibroblasts by about 24 % as compared to normal fibroblasts ($p < 0.01$, N=12)(Table 1).No differences in the cell size and protein content (25) between DMD and normal fibroblasts analysed could be observed (19).Therefore,rates of protein synthesis were expressed on a per cell basis (table 1).Similar results of a reduced protein synthesis in DMD fibroblasts *in vitro* were obtained using other radioactive tracers,such as[^{14}C]-amino acid mixture (data not shown) or[^{35}S]-methionine (19).

Rates of degradation of short and long lived proteins were measured under various medium and serum conditions,i.e. DMEM minus serum, DMEM + 10% fcs lot No.107, and DMEM + 10% fcs lot No.180,

Table 1 . Protein synthesis in normal and DMD fibroblast populations at comparable numbers of cumulative population doublings in vitro

Cell line	CPD	Incorporation of 3H-leucine (cpm / 10^5 cells)	Mean ± SD (% Change)
HH-4	15.6	3349 ± 194	
HH-6	18.9	3748 ± 87	3839 ± 402
HH-10	15.8	3901 ± 162	
HH-11	18.6	4368 ± 180	
DMD-1	18.2	2781 ± 177	
DMD-3	18.1	3210 ± 122	2903 ± 227**
DMD-4	17.4	2704 ± 115	(- 24%)
DMD-6	18.0	2917 ± 134	

** $p<0.01$, $N=12$

Rates of protein synthesis were measured in normal (HH) and DMD fibroblast populations in vitro, as described in Methods. Each number represents the mean ± SD of triplicate cultures of each cell line. For statistical analysis, data were compared by subsampling in a single factor study with complete randomized design (23).

in 6 DMD and 6 normal fibroblast cell lines at CPD numbers 20 to 22.Depending upon the medium condition,degradation of short lived proteins in DMD fibroblasts in vitro is significantly increased betweem 55 % and 62 % as compared to normal fibroblasts in vitro ($p< 0.05$,$N=18$)(Table 2). Depending upon the medium condition, rates of degradation of long lived proteins were significantly enhanced in DMD fibroblasts between 27 % and 32 % as compared to normal fibroblasts ($p<0.05$,$N=18$)(Table 2). Similar data of an elevated protein degradation in DMD fibroblasts could be demonstrated in kinetic experiments for short lived proteins (0 to 4 hrs) and long lived proteins (0 to 24 hrs) with 3 DMD and 3 normal fibroblast cell lines (Table 3). The abnormally enhanced degradation of short and long lived proteins in DMD fibroblast populations could further be demonstrated at CPD numbers 25 and 30 (data not shown).

Rates of degradation of long lived proteins could be reduced significantly in both,DMD and normal fibroblast populations by treatments with protease inhibitors. Degradation of long lived proteins

Table 2 . Degradation of short and long lived proteins in normal and DMD fibroblast populations in vitro under various medium conditions

Medium condition	% Protein degradation		
	Short lived proteins		
	Normal	DMD	% Change
DMEM minus serum	20.8 ± 1.5	33.7 ± 2.3*	+ 62*
DMEM+10% fcs 107	16.9 ± 1.3	26.2 ± 4.2*	+ 55*
DMEM+10% fcs 180	16.2 ± 2.9	26.0 ± 3.8*	+ 60*
	Long lived proteins		
	Normal	DMD	% Change
DMEM minus serum	24.1 ± 1.1	30.5 ± 2.2*	+ 27*
DMEM+10% fcs 107	17.1 ± 1.1	21.9 ± 0.8*	+ 28*
DMEM+10% fcs 180	16.0 ± 1.7	21.1 ± 0.2*	+ 32*

*$p < 0.05$, N=18

Rates of degradation of short and long lived proteins of 6 normal and 6 DMD fibroblast cell lines were measured under various medium and serum conditions as described in Methods. Numbers shown, represent the mean ± SD of the 6 normal (HH-4,HH-5,HH-6, HH-10,HH-11,HH-12) and the 6 DMD (DMD-1,DMD-2,DMD-3,DMD-4,DMD-5, DMD-6) fibroblast cell lines at the CPD numbers 20 to 22 in vitro, triplicate cultures of each cell line. For statistical significance, numbers were compared by subsampling in a single-factor study with complete randomized design (23).

Table 3 . Kinetics of the degradation of short and long lived proteins in normal and DMD fibroblasts in vitro

	Short lived proteins (% degraded at)		
	1 hrs	2 hrs	4 hrs
Normal fibroblasts	7.3±0.7	12.7±1.1	16.6±1.1
DMD fibroblasts	10.4±0.9*	20.5±1.7**	26.2±1.7**
	Long lived proteins (% degraded at)		
	4 hrs	12 hrs	24 hrs
Normal fibroblasts	3.9±0.5	11.3±0.5	20.7±0.7
DMD fibroblasts	5.4±0.3**	15.5±0.6**	26.5±1.2**

* $p < 0.05$, ** $p < 0.01$, N=9

Kinetic experiments of the degradation of short and long lived proteins were performed as describe in Methods.Normal skin fibroblast cell lines (HH-4,CPD 19.7,HH-6,CPD 20.5,HH-11,CPD 20.1) and DMD skin fibroblast cell lines (DMD-1,CPD 20.8,DMD-3,CPD 19.1, DMD-4,CPD 20.3),triplicate cultures of each cell line were studied.For statistical analysis,data were compared by subsampling in a single factor study with complete randomized design (23).

Table 4 . Effects of protease inhibitors on the degradation of long lived proteins in normal and DMD fibroblasts in vitro

	% Protein degradation		
	Untreated	+Leupeptin	+ Ep475
Normal fibroblasts (% Change)	13.3 ± 0.6	11.3 ± 0.6* (- 15*)	10.5 ± 0.7* (- 21*)
DMD fibroblasts (% Change)	15.5 ± 0.4	12.7 ± 0.5* (- 18*)	11.8 ± 0.6* (- 24*)

*$p < 0.05, N=9$

Rates of degradation o long lived proteins of 3 normal (HH-4, CPD 20.2, HH-6,CPD 23.4,HH-12,CPD 20.7) and 3 DMD (DMD-1,CPD 21.4, DMD-2,CPD 21.9,DMD-3,CPD 23.3) fibroblast cell lines were measured in the absence and presence of protease inhibitors, leupeptin (25 μM) and Ep475 (25 μM) as described in Methods.Numbers shown represent the mean ± SD of the 3 normal and 3 DMD fibroblast cell lines, triplicate cultures of each cell line.For statistical analysis, data were compared by a generalized block design,combined with the Dunnett-test (23,24).The cell lines are the blocking factor (3x3= 9 blocks).Pooled SD for normal fibroblasts : 0.215 .Pooled SD for DMD fibroblasts : 0.28 .

in DMD fibroblasts was reduced significantly by about 18 % (leupeptin,$p < 0.05, N=9$) amd 24 % (Ep475, $p < 0.05, N=9$), as compared to 15 % (leupeptin, $p < 0.05, N=9$) and 21 % (Ep475, $p < 0.05, N=9$) in normal fibroblasts (Table 4).

DISCUSSION

The data reported herein,demonstrate on a statistical basis, that skin fibroblasts in vitro from patients with DMD display significant alterations in protein turnover. The observed decrease in protein synthesis is in agreement with other findings (12,15-19). The concomitant and significant increase in the degradation of short and long lived proteins in DMD fibroblasts vitro is a novel finding,that is supported by recent dat of an enhanced differential degradation of individual [35S]-methionine polypeptides in DMD fibroblasts in vitro,demonstrated by 2-D-gel electrophoresis (19). However,our findings are in disagreement with data of McElligott and Dice (20) and Statham et al (21),who describe differences of non-significant nature in protein degra-

dation between DMD and normal fibroblasts in vitro. The differences in the experimental results could either be caused by the experimental procedures applied or by disparities in the biological materials used by McElligott and Dice (20) and Statham et al (21). In additional experiments,applying the experimental procedures described by McElligott and Dice (20),i.e. omission of the prechase period and repeated sampling of medium aliquots from the same cultures,that could disturb the physiologically balanced conditions of the fibroblast populations analysed,differences of non-significant nature in protein degradation between DMD and normal fibroblasts have been obtained (data not shown).

The results of an abnormally elevated degradation of short and long lived proteins in DMD fibroblasts in vitro are in agreement with recent data,demonstrating a significant differential increase in the rate of degradation of individual [35S]-methionine polypeptides,e.g. tropomyosin proteins,by 2-D-gel electrophoresis (19). In addition, Dunn et al (26) have demonstrated significant quantitative changes in the protein composition of DMD fibroblasts in vitro,applying one dimensional gel electrophoresis techniques. Proteins degraded only few hours after synthesis are generally described as short lived proteins (27). In various cell systems, short lived proteins reflect 3% to 5% of total protein (28-31). Short lived proteins in fibroblasts include several enzymes (32), a fraction of newly synthesised collagen (33),and proteins with abnormal configurations (27).In previous studies (18),we described an 80% increase in the degradation of collagen in DMD fibroblasts in vitro due to an abnormal hyperhydroxylation of proline residues. It remains to be further analysed,whether other noncollagen proteins,e.g. myosin,carry analogous quantitative abnormal modifications of amino acids,e.g. methylation of arginine and histidine (27),that could lead to an abnormally enhanced and preferential

degradation of these proteins in DMD fibroblasts. Proteins degraded with half-lifes of 24 hrs or more,representing 90% to 95% of total cellular protein are generally described as long lived proteins,that are preferentially degraded via the lysosomal degradation pathway (27).In the present study,the abnormally enhanced degradation of long lived proteins in DMD fibroblasts in vitro has been shown to be sensitive to protease inhibitors,like leupeptin and Ep475,which are known to reduce lysosmal protein degradation (34).Thus,the abnormally enhanced degradation of long lived proteins in DMD fibroblasts is most likely caused by a defect in the regulation of lysosomal protein degradation.Clinical long term studies with the protease inhibitors E-64 and E-64-C (Ep475) on DMD patients are in progress (33).Preliminary data show an improvement of muscle function and a decrease in 3-methyl-histidine excretion in DMD patients (36).

The statistically significant abnormalities in protein turnover demonstrated in this study,together with the cellbiological and biochemical alterations reported (7-19),make it very likely,that the deletions in the short arm of the X-chromosome (Xp21) of DMD patients (37),are also present and expressed in DMD fibroblasts in vitro.Therefore,DMD fibroblasts,which are ontogenetically closely related to muscle cells,represent helpful tools for studies into the underlying primary and/or secondary genetic defects of the DMD syndrome.

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