Protein degradation in skin fibroblasts from patients with Duchenne muscular dystrophy

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The rates of degradation of [^3H]leucine-labelled proteins have been measured in cultures of skin fibroblasts obtained from normal controls (five subjects) and patients with Duchenne muscular dystrophy (six subjects). Cultures were incubated with [^3H]leucine (10 $\mu\text{Ci/ml}$) for 60 min to label 'short-lived' proteins, and with [^3H]leucine (5 $\mu\text{Ci/ml}$) for 60 h to label 'long-lived' proteins. Optimal wash procedures were devised for removal of [^3H]leucine from the extracellular space and from cell pools before beginning degradation measurements. Re-utilization of [^3H]leucine released from degraded labelled proteins was prevented by supplementing the medium with 4 mM-leucine. Rates of degradation did not depend on the growth state of the cells or on cell age over the range used (passages eight–20). Degradation of long-lived proteins was approximately linear over a 24 h period, at a rate of 1.0% per h. 30% of short-lived protein was degraded within 6 h. No differences were observed between protein degradation in normal fibroblasts and in those from patients with Duchenne muscular dystrophy.

Duchenne muscular dystrophy is a degenerative disorder in which there is a progressive and irrevocable loss of muscle tissue with its replacement by fat and connective tissue. Despite these degenerative changes, muscle regeneration has also been described in Duchenne muscular dystrophy (Cullen & Fulthorpe, 1975) and these paradoxical features of degeneration and regeneration have led to a number of studies of protein synthesis and degradation in dystrophic tissues. Increased autolysis has been described in homogenates of muscle from the dystrophic chick (Iodice *et al.*, 1966) and mouse (129/ReJ-dy/dy; Mayer *et al.*, 1976) and measurements of degradation by tyrosine release in organ baths of dystrophic mouse (C57 BL/6J-dy^{2J}/dy^{2J}) (Libby & Goldberg, 1978) and hamster muscle (Goldspink & Goldspink, 1977; Nakatsu *et al.*, 1978) have indicated increased degradation rates. Using a radioautographic technique, Monkton & Marusyk (1978) claimed that there was increased leucine incorporation in the 129/ReJ-dy/dy mouse *in vivo* but Kitchin & Watts (1973), in a careful study with the same mutant, concluded that there was no increased turnover when differences in pool size were taken into account. Monkton & Marusyk (1979) have applied their technique to samples of biopsied muscle from patients with Duchenne muscular dystrophy and believe that the increased

cytoplasmic protein synthesis is related to muscle regeneration.

Indirect evidence of the importance of protein degradation in muscular dystrophy has come from measurements of proteinase activity in dystrophic muscle and from studies of the effect of anti-proteinases on degenerative changes in dystrophic muscle. Elevated amounts of proteinases have been found in dystrophic mouse (Weinstock *et al.*, 1958; McCaman, 1963; Tappel *et al.*, 1962; Pennington, 1963), chicken (Tappel *et al.*, 1962; Iodice *et al.*, 1966; Peterson *et al.*, 1972) and human (Kar & Pearson, 1978) muscle. In 1976, McGowan *et al.* reported that degenerative changes in dystrophic chick (Connecticut strain) muscle cultures could be prevented by antiproteinases such as leupeptin and pepstatin, and these inhibitors have also been shown to inhibit protein degradation in other systems (Iodice, 1976; Libby & Goldberg, 1978). It has since been claimed that the treatment of dystrophic chickens (Connecticut strain) (Stracher *et al.*, 1978) and mice (129/ReJ-dy/dy) (Schorr *et al.*, 1978) led to a significant improvement, although Enomoto & Bradley (1977) found that antipain, pepstatin and leupeptin had no effect on the lifespan or activity of 129/ReJ-dy/dy mice.

These results indicate that there may be some abnormality of protein turnover in dystrophic muscle but we believe that the studies so far

performed have limitations if protein degradation and the effects of pharmacological agents are to be investigated in Duchenne muscular dystrophy. It is not possible to perform studies of this kind on patients and the nature of a needle biopsy (about 50 mg of tissue, of variable shape with many cut fibres) is not suitable for organ bath experiments. For these reasons we have used cultures of cells from patients with Duchenne muscular dystrophy but rather than rely on subjective assessments of the state of the cultures (McGowan *et al.*, 1976) we have measured protein degradation.

Cell cultures have a number of advantages for studies of protein degradation over studies *in vivo* or measurements made on muscle in organ baths. In these systems there is a delay in the entry and release of labelled materials from the tissue, with consequent problems of re-utilization of released label. It is also difficult to clear the intracellular pools of free-labelled amino acid at the end of the labelling period before beginning the degradation chase. These problems remain in cell-culture methods but are minimized because there is direct and rapid release of label from cells into a very large, excess volume of medium (Poole & Wibbo, 1973). Cell cultures have been used to study changes in protein degradation with culture age (Bradley *et al.*, 1976; Kaftory *et al.*, 1978), viral transformation (Bradley, 1977; Hendil, 1977; Baxter & Stanners, 1978), growth state (Bradley, 1977; Castor, 1977; Hendil, 1977; Robinson, 1977; Baxter & Stanners, 1978) and hormone treatment (Robinson *et al.*, 1976). We have used the intermittent-perfusion technique described by Bradley *et al.* (1976) to measure protein degradation in normal and dystrophic human-skin fibroblasts. Previous studies in this laboratory have shown that the rates of protein synthesis are similar in normal and dystrophic cells (Stephens & Dunn, unpublished work).

Materials and methods

Cell culture

Cultures of skin fibroblasts were established from patients with Duchenne muscular dystrophy and normal controls with standard methods. Biopsies were taken with informed consent from patients and volunteers. All were grown in plastic flasks (80 cm³) with Eagle's minimal essential medium (Paul, 1975), containing 10% newborn calf serum, 10 mM-Hepes* and 50 i.u. of penicillin/ml and 50 µg of streptomycin/ml (growth medium). Cells were sub-cultured weekly. For measurement of protein degradation, cells were plated in six-well (35 mm) culture plates and incubated in a humidified CO₂

incubator [O₂ + CO₂ (95:5, v/v)]. All tissue culture materials were purchased from Gibco-Biocult, Paisley, Scotland, U.K.

Protein degradation

The standard procedure was as follows: cells were plated in six-well plates at a density of approx. 5×10^5 cells per well. [³H]Leucine (sp. activity, 40–60 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) (5 µCi/ml, 1.5 ml/well) was added to one set of cultures for 60 h. At the end of this incubation period, the wells were washed twice with growth medium supplemented with 4 mM-leucine (chase medium), incubated for 2 h at 37°C in the same medium and washed twice again. They were incubated for a further hour during which time a second set of cultures was labelled with [³H]leucine (10 µCi/ml, 1.5 ml/well) to label short-lived proteins. At the end of this period, all cultures were washed twice, incubated for two 15 min periods and again washed with chase medium. Data will be presented to demonstrate that this procedure depletes intracellular pools of free [³H]leucine.

When the incubations and washes were completed, 1 ml of chase medium was added to each well and the plates incubated in a CO₂ incubator. At 0.5, 1.0, 2.0, 4.0, 6.0 and 12.0 h the medium was removed and replaced with fresh medium (1 ml), but at 24 h it was replaced with 5% trichloroacetic acid to extract the remaining labelled leucine in the intracellular pools and the cells dissolved in 1.0 ml of 1.0 M-NaOH. To measure free leucine released into the medium, 100 µl of 25% trichloroacetic acid was added to 500 µl of each medium sample, precipitated proteins were spun down by centrifuging at 2000 g for 10 min and the supernatant was counted. 100 µl samples were counted in 3 ml of scintillation cocktail (Hydroluma; LKB Instruments, Croydon, Surrey, U.K.) with a Rackbeta scintillation-counter (LKB Instruments) and under these conditions corrections for quenching were not necessary. Solubilized cells were neutralized by addition of 100 µl of 1.0 M-HCL. The following samples were counted: at each time point, medium and medium supernatant after trichloroacetic acid precipitation and at 24 h, cell protein and trichloroacetic acid-extractable cell pool. Percentage degradation at each time was calculated as:

$$\frac{\text{Cumulative } [^3\text{H}] \text{leucine released} \times 100}{(\text{Total counts released into the medium} + \text{counts in cell protein and pool at 24 h})}$$

Results and discussion

Depletion of intracellular pools before degradation chase

Estimations of protein degradation in these experiments depend on the measurement of free

* Hepes: 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

[^3H]leucine released into the chase medium, but this can only be used as an accurate estimate of protein degradation if the following conditions are satisfied. First, counts remaining from the medium used to label the cells must be removed. If this is not done efficiently, counting [^3H]leucine from this source will give spuriously high values for leucine release and hence protein degradation during the first 0.5 h of the degradation chase. Secondly, [^3H]leucine in the intracellular pools but not incorporated into protein must be removed; [^3H]leucine released from this source would not be a measure of protein degradation.

Contamination from the labelling medium could be removed quickly as shown by experiments in which monolayer cultures were incubated with the extracellular space markers [^3H]inulin or [^{14}C]polyethylene glycol (mol.wt. 4000). Two washes were sufficient to reduce counts from these markers to background levels (9–15 c.p.m.).

The removal of free [^3H]leucine from the intracellular pools is not so readily achieved. Robertson & Wheatley (1979) have reported that HeLa cells grown in suspension culture have approx. 70% of their free leucine in a pool that exchanges almost instantaneously with the extracellular medium. We were unable to detect such a freely diffusible pool in human-skin fibroblasts because estimates of the extracellular space based on inulin and polyethylene glycol were greater than that possible for leucine. It is possible that this was due to the relatively small number of cells used and to the difficulties of using monolayers rather than suspension cultures for such studies (Wheatley, D. N., personal communication). The acid-soluble pool of these cells could be removed by repeated washings or short incubations. The rate of appearance of [^3H]leucine in medium containing 0, 0.4 or 4.0 mM-leucine and trichloroacetic acid-extractable [^3H]leucine in the cells decreased with time and temperature as shown in Table 1. Washings and two or four 15 min incubations were ineffective at 0°C compared with 27 and 37°C. The latter was clearly the temperature

at which label was most efficiently removed, and as the difference between two and four incubations was small, our standard washing procedure after cell labelling was: two washes, two 15 min incubations at 37°C, followed by two further washes, all with medium containing 4 mM-leucine.

Prevention of re-utilization of released label

Re-utilization of released [^3H]leucine by the cells will give a spuriously low estimate of protein degradation, and experiments in which protein degradation was measured in medium containing 0, 0.4, 4.0 and 8.0 mM-leucine showed that 4.0 mM-leucine was sufficient to prevent re-utilization of released labelled leucine. Results are shown in Fig. 1(a) for short-lived and in Fig. 1(b) for long-lived proteins. It is clear that re-utilization did occur when no leucine was present, but that the addition of 0.4 mM-leucine was sufficient to prevent re-utilization during measurement of short-lived protein degradation. However, the difference in degradation of long-lived proteins at 24 h in medium containing 0.4 and 4.0 mM-leucine was significant ($P = 0.002$ Mann-Whitney rank sum test) and it was decided to supplement normal growth medium with 4.0 mM-leucine for degradation measurements.

Effects of growth state and cell age on protein degradation

Normal cells in culture cease dividing when they reach a certain density and concurrently undergo a variety of metabolic changes; there must be some regulation of the net rate of protein accumulation when cells become density-inhibited, either by inhibition of protein synthesis or by increased protein degradation, or by both means; Balb/3T3 cells for example degrade proteins twice as rapidly when density-inhibited (Neff *et al.*, 1977). It was important to determine if protein degradation in human-skin fibroblasts was dependent on their state of growth.

Cells were plated at densities of 3×10^5 and 1×10^5 cells per well and degradation of proteins

Table 1. *Depletion of intracellular [^3H]leucine pools by washes and incubations in growth medium*

Monolayer cultures were incubated in 1.5 ml of growth medium containing $10 \mu\text{Ci/ml}$ of [^3H]leucine for 60 min. At the end of this period, the cultures were rapidly washed twice with 2 ml of growth medium (a), followed by two (b) or four (c) 15 min incubations. Washes and incubations were carried out at 0, 27 and 37°C. After each procedure 1.0 ml of 5% trichloroacetic acid was added to each well to extract the acid-soluble [^3H]leucine pools. 100 μl samples were counted. Values are means $\pm$ s.d. of counts in the trichloroacetic acid extracts (no. of cultures in parentheses).

Procedure	Counts in trichloroacetic acid cell extracts		
	0°C	27°C	37°C
(a) Two washes	1184 $\pm$ 168 (6)	835 $\pm$ 116 (6)	609 $\pm$ 109 (6)
(b) Two washes + two 15 min incubations	1301 $\pm$ 81 (3)	263 $\pm$ 6 (3)	113 $\pm$ 31 (3)
(c) Two washes + four 15 min incubations	1242 $\pm$ 88 (3)	120 $\pm$ 12 (3)	85 $\pm$ 15 (3)

Fig. 1. Effect of excess leucine in degradation medium on re-utilization of released [^3H]leucine

Cells from patient FANA (normal) were grown in tissue culture plates for 3 days and short- and long-lived proteins were labelled following the standard procedure given in the Materials and methods section. After labelling, cultures were incubated in medium containing 0 (▲), 0.4 (△), 4.0 (●) or 8.0 (□) mM-leucine and the rate of release of [^3H]leucine into the medium was measured. For short-lived proteins, the apparent rate of degradation did not change when the chase medium contained ≥ 0.4 mM-leucine, indicating that 0.4 mM was sufficient to prevent re-utilization. For long-lived proteins, a minimum supplement of 4.0 mM-leucine was required to prevent re-utilization. (a) Short-lived proteins; (b) long-lived proteins. For clarity the 0.5 h points are omitted.

measured 3 days later. Fig. 2 shows that for these cells protein degradation was the same for growing and density-inhibited cultures. Bradley (1977) obtained similar results with the human embryonic-cell strain WI-38, although Castor (1977) demonstrated a small increase in degradation rates (from 1.0 to 1.5%) in confluent cultures of the same cell type. To reduce variation to a minimum, in our experiments cultures were always used when confluent.

Changes in protein degradation have also been reported with increasing cell age in culture (Bradley *et al.*, 1976; Kaftory *et al.*, 1978) but these changes were found only in very late passage, phase III, senescent cells. To avoid differences due to cell age, we used cultures between passages eight and 20, and found no correlation of protein degradation with cell age over this range.

Protein degradation in normal and dystrophic cells

Details of the sources of cells and numbers of experiments performed are given in Table 2 and a

summary of results is given in Table 3. In Table 3, only values for 2, 6 and 24 h are given although degradation at the intermediate time points of 0.5, 1, 4 and 12 h was also measured. Rates of degradation of short-lived and long-lived proteins in these experiments were as shown in Fig. 2. The degradation of short-lived proteins was very rapid over the first 6 h with about 30% of labelled protein degraded in this period. Over the rest of the chase period, the rate of release of [^3H]leucine was greatly reduced with only a further 16% of short-lived proteins degraded over 18 h. Degradation of long-lived proteins in these cells was almost linear over the whole 24 h period, with about 1.0% of total long-lived proteins degraded per hour. These results indicate that a fraction of proteins labelled during the short-term labelling period were degraded at rates similar to those for long-lived proteins.

It is difficult to compare these data directly with those of other studies because 'short-lived' and 'long-lived' proteins are defined by the labelling procedure and the subsequent washing and

Fig. 2. *Effect of cell growth state on protein degradation* Cells from patient DONN (Duchenne muscular dystrophy) were plated such that after 3 days in culture they were confluent (approx. 6×10^4 cells per culture, Δ) or still actively growing (approx. 3×10^5 cells per culture, $\circ$). Degradation of short-lived and long-lived proteins was measured by using the standard procedure. The results showed that there were no significant differences in protein degradation between growing and stationary-phase cells. Each point is the mean of six cultures.

incubation schedules, and the terms ‘short-labelled’ and ‘long-labelled’ might be more appropriate. In our experiments long-lived proteins are those that retain label after a 60h labelling period followed by a 4h chase in cold medium and short-lived proteins are those labelled for 1h followed by a 0.5h chase in cold medium. Others have labelled short-lived proteins for as short as 25 min (Bradley *et al.*, 1976) and long-lived proteins for six generations followed by a 20min cold chase (Baxter & Stanners, 1978). Nevertheless, our results are in agreement with other studies, e.g., Robinson *et al.* (1976) obtained a degradation rate of 1.3% per h for long-lived proteins and 24% of short-lived proteins were degraded by 6h. Our results indicate that protein degradation in dystrophic skin fibroblasts does not differ from that in normal fibroblasts. The results shown in Table 3 cannot be compared by Student’s *t* test or analysis of variance because the variances of the two groups are significantly different. An analysis by using the non-parametric Mann–Whitney test to compare pooled results for normal and dystrophic experiments (13 experiments in each group) showed no significant differences between the two groups at any time ($P > 0.1$).

Ideally these experiments should be performed with muscle cells, but we have used skin fibroblasts

Table 2. *Summary of number of patients and experiments*

	No. of patients	Age range (years, months)	Short-lived proteins		Long-lived proteins	
			No. of experiments	No. of cultures	No. of experiments	No. of cultures
Normal controls	5	0, 8–40, 2	14	63	13	61
Duchenne muscular dystrophy	6	1, 11–8, 4	11	40	13	51

Table 3. *Summary of results on degradation of short- and long-lived proteins in normal and dystrophic cells* Cells were labelled and the degradation of (a) short- and (b) long-lived proteins were measured as described in the Materials and methods section. This Table presents pooled values for normal and dystrophic cultures, giving means $\pm$ s.d. (range in parentheses). A comparison between normal and dystrophic cells at each time point by using the Mann–Whitney non-parametric test showed $P > 0.1$ at all points. There were no differences at intermediate time points.

	No. of cultures	% Degradation		
		2h	6h	24h
(a)				
Normal controls	63	17.76 $\pm$ 2.99 (11.11–26.55)	27.96 $\pm$ 3.69 (16.90–37.65)	47.18 $\pm$ 4.80 (38.31–58.46)
Duchenne muscular dystrophy	40	17.78 $\pm$ 3.26 (11.48–24.59)	27.73 $\pm$ 4.44 (18.84–36.91)	45.00 $\pm$ 5.69 (35.85–50.44)
(b)				
Normal controls	61	2.19 $\pm$ 0.66 (1.30–4.61)	6.19 $\pm$ 1.59 (3.38–10.11)	21.53 $\pm$ 3.56 (15.06–35.89)
Duchenne muscular dystrophy	51	1.92 $\pm$ 0.31 (1.47–2.68)	5.37 $\pm$ 0.95 (4.15–6.93)	19.58 $\pm$ 1.97 (16.46–23.95)

because these can be grown in large numbers, they are a relatively homogeneous population and have been shown to be abnormal in Duchenne muscular dystrophy (Jones & Witkowski, 1979). These negative results obtained with skin fibroblasts do not conflict with the published results in which increased protein degradation has been demonstrated in dystrophic muscle (Goldspink & Goldspink, 1977; Libby & Goldberg, 1978; Nakatsu *et al.*, 1978). However, having established that this technique is sufficiently reproducible, we now feel confident in applying the method to the small numbers of dystrophic muscle-cell cultures that are available to us, and these experiments are in progress.

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