Biochem. J. (1993) 295, 577–580 (Printed in Great Britain)

Protein catabolism in fibroblasts cultured from patients with mucolipidosis II and other lysosomal disorders

Jürgen KOPITZ, Andrea ARNOLD, Thomas MEISSNER and Michael CANTZ*
Institute of Pathochemistry and General Neurochemistry, University of Heidelberg, Im Neuenheimer Feld 220/221, D-69120 Heidelberg, Germany

Protein catabolism in fibroblasts cultured from the skin of normal individuals and of patients with mucolipidosis II (I-cell disease) and several other lysosomal storage diseases was examined by metabolic labelling with [^{3}H]leucine and following the fate of radioactive proteins in pulse–chase experiments. In mucolipidosis II cells, overall protein degradative rates were found to be distinctly lower than in normal control cells. To distinguish lysosomal from non-lysosomal degradation, labelling experiments were carried out in the presence and absence of 10 mM NH_4Cl, an inhibitor of lysosomal function. It was found that mucolipidosis II fibroblasts exhibited a markedly reduced rate of lysosomal protein degradation, whereas the rate of non-lysosomal degradation appeared normal. Serum and amino acid starvation led to a marked increase in lysosomal protein degradation in normal cells, but had only a minimal effect on that in mucolipidosis II fibroblasts. The specific activities of cathepsins B, H and L were profoundly diminished in all mucolipidosis II cell lines tested. Lysosomal protein degradation in a mucolipidosis III cell line was impaired to a similar degree as in mucolipidosis II cells, whereas it was decreased to a lesser extent in fibroblasts from patients with mucopolysaccharidoses I and VI, galactosialidosis and G_{M1}-gangliosidosis. We conclude that fibroblasts from patients with mucolipidosis II and III have a severely compromised capacity for endogenous lysosomal protein degradation that appears to result from multiple cathepsin deficiency. This lysosomal defect is likely to have pathophysiological consequences.

INTRODUCTION

In eukaryotic cells, macromolecules such as proteins, glycoproteins, glycosaminoglycans (mucopolysaccharides) and glycosphingolipids are predominantly degraded in lysosomes. The importance of this catabolic pathway is highlighted by the existence of so-called storage diseases that are caused by the genetic deficiency of lysosomal enzymes or activator proteins (for a review, see Scriver et al., 1989). However, whereas deficiencies of glycosidases, sulphatases or lipases have been established as the metabolic bases of disorders known as mucopolysaccharidoses, sphingolipidoses, oligosaccharidoses and mucolipidoses, our knowledge relating lysosomal proteinase deficiencies to such disease factors is much less advanced. Several reports have indicated an abnormal lysosomal storage of subunit c of mitochondrial ATP synthase in an animal model and in patients with several forms of ceroid lipofuscinosis or Batten's disease (Palmer et al., 1989, 1992; Hall et al., 1991). If this is the result of a defect of a cathepsin, i.e. a lysosomal proteinase, or is induced by some other factor was, however, not resolved.

We reasoned that the role of cathepsins in lysosomal protein catabolism could be studied in cultured fibroblasts derived from patients with mucolipidosis II (I-cell disease), a multiple lysosomal hydrolase deficiency caused by defective synthesis of the mannose 6-phosphate recognition signal on lysosomal enzymes (reviewed by Nolan and Sly, 1989). A deficiency of cathepsin B (EC 3.4.22.1) has been demonstrated in these cells (Hanewinkel et al., 1987), and additional cathepsin deficiencies could be expected. A previous investigation of intracellular protein catabolism in mucolipidosis II fibroblasts had indicated some abnormality that could not, however, be clearly defined (Cockle and Dean, 1984). This could be due to the existence of two intracellular protein degradative pools in cultured fibroblasts, i.e. a cytoplasmic pool and a lysosomal pool, as has been demonstrated in hepatocytes (Seglen et al., 1981). We have therefore employed methods to measure lysosomal and non-lysosomal protein degradation in fibroblasts cultured from normal individuals and from patients with mucolipidoses II and III and several other lysosomal disorders. The results showed markedly impaired lysosomal, but apparently normal non-lysosomal, protein catabolism in cells from patients with mucolipidoses II and III, presumably as a consequence of a multiple deficiency of lysosomal proteinases. In cells from patients with several other lysosomal diseases, a variable degree of impairment was found.

MATERIALS AND METHODS

Materials

L-[4,5-^{3}H]Leucine (150 Ci/mmol) was purchased from Amersham, Braunschweig, Germany. L-Arginine-7-amido-4-methylcoumarin and N-Cbz-L-arginyl-L-arginine-7-amido-4-methylcoumarin (where Cbz is benzoyloxycarbonyl) were obtained from Bachem, Bubendorf, Switzerland. Azocasein was from Sigma, Deisenhofen, Germany. All other reagents were of analytical grade.

Cell culture

Human skin fibroblasts from patients and normal controls were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum, non-essential amino acids and antibiotics, as described previously (Cantz et al., 1972). Patients had been diagnosed on the basis of clinical data and biochemical investigations, including the determination of lysosomal hydrolase activities.

Abbreviation used: Cbz, benzyloxycarbonyl.

* To whom correspondence should be addressed: Institut für Pathochemie und Allgemeine Neurochemie, Im Neuenheimer Feld 220/221, D-W69120 Heidelberg, Germany.

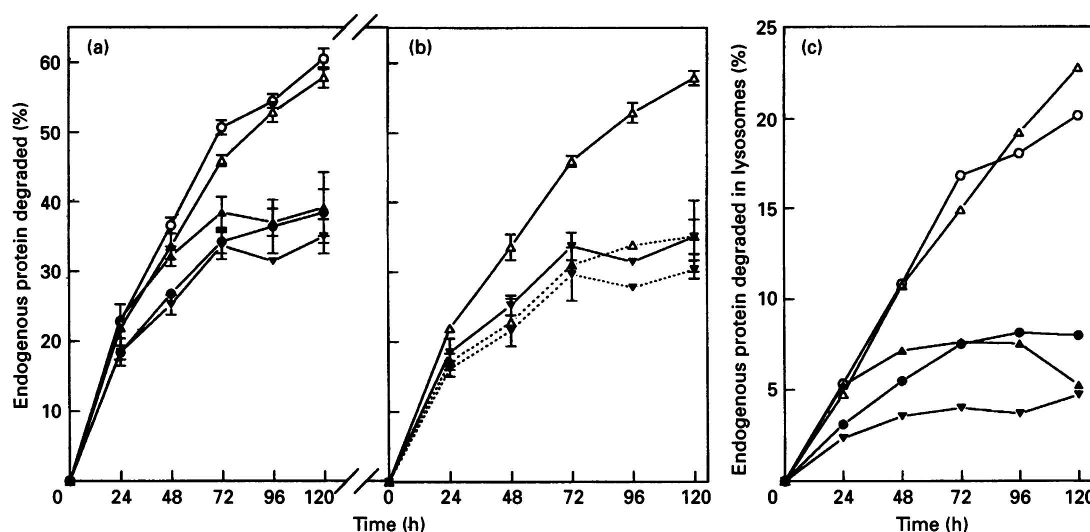

Figure 1 Degradation of endogenous protein in normal and mucolipidosis (ML) II fibroblasts

(**a**) Protein degradation was determined at the indicated time points of the chase after radioactive labelling with [^{3}H]leucine. (**b**) Protein degradation was determined in ML II and normal control cells with chase medium in the presence (broken lines) or absence (solid lines) of 10 mM NH_4Cl. (**c**) The difference between protein degradation in the absence and presence of NH_4Cl was calculated and considered as lysosomal protein degradation. ●, ▲, ▼, ML II fibroblasts; ○, △, normal controls. Error bars represent S.D.s of triplicate cell samples

Enzyme assays

Cathepsins in fibroblast homogenates (1–2 mg of protein/ml) were assayed according to Barrett and Kirschke (1981), with L-arginine-7-amido-4-methylcoumarin as substrate for cathepsin H (EC 3.4.22.16) and *N*-Cbz-L-arginyl-L-arginine-7-amido-4-methylcoumarin as substrate for cathepsin B. One unit of enzyme activity corresponds to 1 μmol of substrate cleaved per min. Cathepsin L (EC 3.4.22.15) activity was determined with azocasein as substrate and the assay was made selective by the inclusion of urea and pepstatin (Barrett and Kirschke, 1981). One unit of activity corresponds to 1 nmol azocasein cleaved per min, assuming a molecular mass of azocasein of 22 kDa and complete hydrolysis into trichloroacetic acid-soluble peptides (Langner et al., 1973). Protein was determined by the method of Bradford (1976).

Protein degradation

The methodology was an adaptation and extension of a procedure developed for HeLa cells (Freshney et al., 1975). Human skin fibroblasts were grown in 24-well tissue culture plates (Falcon). At a near-confluent cell density (about 1×10^4 cells per cm^2), endogenous protein was labelled by including [^{3}H]leucine in the culture medium (0.5 ml of medium per well; 925 KBq/ml) for 72 h. After removal of the radioactive medium, the cell layers were washed three times with 1 ml of medium and chased with another 1 ml of non-radioactive medium. Protein degradation was determined by measurement of the radioactivity released into the medium and of the protein-bound radioactivity. For this purpose, the chase medium of each well was transferred to microcentrifuge tubes and mixed with an equal volume of ice-cold 10% trichloroacetic acid. After centrifugation (13000 *g*; 5 min) the supernatant was counted for radioactivity in a liquid scintillation counter. Radioactivity in cellular protein was determined after washing the cell layer three times with phosphate-buffered saline, fixing the cells with methanol, washing three times with 10% trichloroacetic acid and solubilization with 0.5 M NaOH. The percentage of degraded endogenous protein was calculated in the following manner: {[trichloroacetic acid-soluble radioactivity in medium (d.p.m.)]/[trichloroacetic acid-soluble radioactivity in medium (d.p.m.) + trichloroacetic acid-insoluble radioactivity in cells (d.p.m.)]} × 100.

Lysosomal (ammonia-sensitive) protein degradation was blocked by adding 10 mM NH_4Cl with the chase medium, and lysosomal protein degradation was calculated from the difference in the protein degradation rates in the absence and presence of NH_4Cl. Data for leupeptin-sensitive protein degradation were obtained by adding 2 mM leupeptin to the chase medium instead of NH_4Cl.

RESULTS

After labelling the fibroblast cultures with [^{3}H]leucine for 72 h, radioactivity incorporated into cellular protein ranged from 8×10^6 to 12×10^6 d.p.m./mg in normal controls and from 4×10^6 to 7×10^6 d.p.m./mg in mucolipidosis II fibroblasts. The overall rate of intracellular protein degradation was determined by monitoring the fate of labelled protein in chase experiments. In normal cells, about 60% of endogenous protein had been degraded after 120 h, whereas degradation during the same period was only about 40% of the total in three mucolipidosis II cell lines (Figure 1a).

Incubation of cells in the presence of NH_4Cl was used to block lysosomal protein degradation. Ammonia had a strong effect on protein degradation in normal cells, causing a reduction to about two-thirds the rate in untreated controls, but had a very moderate effect in mucolipidosis II cells (Figure 1b). When the cells were incubated in the presence of the lysosomal cathepsin inhibitor leupeptin, an influence similar to that of ammonia was observed (results not shown).

The difference between protein degradation in the absence and presence of ammonia was taken as a measure of the lysosomal protein degradative capacity. In comparison with normal cells, there was greatly impaired lysosomal protein degradation in all three mucolipidosis II cell lines (Figure 1c).

Table 1 Effect of serum and amino acid deprivation on lysosomal protein degradation in normal and mucolipidosis (ML) II fibroblasts

After [^{3}H]leucine labelling for 72 h, the cells were chased in medium without fetal calf serum and non-essential amino acids, and ammonia-sensitive protein degradation after 48 h of the chase was determined as described. The results were compared with those in incubations containing the regular medium. Results are means ± S.D. of four experiments.

Cell line	Increase in lysosomal protein degradation (fold)
Normal controls	
N15	2.37 ± 0.35
N16	2.46 ± 0.30
ML II	
D.C.	1.10 ± 0.15
R.D.	1.35 ± 0.20
R.G.	1.29 ± 0.17

Table 2 Activities of cathepsins B, H and L in normal and mucolipidosis (ML) II fibroblasts

Cathepsins B, H and L were assayed in homogenates of normal and ML II fibroblasts. The means of the specific activities of the normal controls (cathepsin B, 39.2 munits/mg; cathepsin H, 33.4 munits/mg; cathepsin L, 0.143 units/mg) were set to 100%.

	Activity (% of normal controls)		
Cell line	Cathepsin B	Cathepsin H	Cathepsin L
Normal controls			
Mean ($n = 5$)	100	100	100
Range	89.1–102.5	78.7–115.4	83.5–118.7
ML II			
D.C.	8.3	6.8	6.8
R.D.	6.2	4.0	6.0
R.G.	4.6	3.9	6.4

Table 3 Lysosomal protein degradation in fibroblasts from patients with various lysosomal disorders

The cells were [^{3}H]leucine-labelled and chased for 120 h, and lysosomal protein degradation at the end of chase was determined as described. Results are means ± S.D. of four cell samples.

Cell line	Ammonia-sensitive protein degradation (% of controls)
Controls ($n = 7$)	100 ± 20.9
Mucolipidosis II ($n = 2$)	16.0 ± 13.1
Mucolipidosis III ($n = 1$)	16.1 ± 12.1
Mucopolysaccharidosis I ($n = 1$)	48.1 ± 2.0
Mucopolysaccharidosis II ($n = 2$)	80.7 ± 9.9
Mucopolysaccharidosis IIIA ($n = 2$)	77.3 ± 20.7
Mucopolysaccharidosis VI ($n = 1$)	61.3 ± 0.1
Sialidosis ($n = 2$)	93.9 ± 4.0
Galactosialidosis ($n = 2$)	68.0 ± 18.7
G_{M1}-gangliosidosis ($n = 1$)	62.7 ± 4.0

The lysosomal protein degradative capacity of the fibroblasts was challenged by serum and amino acid deprivation. In normal cells, such starvation more than doubled the amount of protein that was degraded in lysosomes within the first 48 h (Table 1). In mucolipidosis II cells, however, only minor effects on protein degradation were observed under these conditions.

When the specific activities of the proteinases cathepsin B, cathepsin H and cathepsin L were measured in fibroblast homogenates, all three were found to be drastically diminished in the mucolipidosis II cells (Table 2).

It was also of interest to determine the amount of ammonia-sensitive protein degradation in fibroblasts from patients with other lysosomal storage diseases. As shown in Table 3, most of the patients' cell lines exhibited a rate of lysosomal protein degradation that was below that in the normal controls. Not unexpectedly, the degradative impairment was particularly marked in mucolipidosis III, which is a milder variant of mucolipidosis II, but it was also prominent in mucopolysaccharidoses I and VI, galactosialidosis and G_{M1}-gangliosidosis.

DISCUSSION

Studies with cultured hepatocytes and other cell systems had revealed the existence of at least two intracellular protein degradative pools: one that was sensitive to inhibition by ammonia, had turnover rates of several days and was localized in lysosomes, and another that was not influenced by ammonia, turned over more rapidly (within hours) and was confined to the cytoplasm (Knowles and Ballard, 1976; Seglen et al., 1981; Seglen, 1983).

In order to study protein catabolism in fibroblasts cultured from the skin of normal control individuals and of patients with mucolipidosis II and other lysosomal storage diseases, we made use of these findings and developed a simple assay procedure. Cells were grown in microtitre plates in the presence of [^{3}H]leucine, and the degradation of the labelled protein was determined in chase experiments. The contribution of the lysosomal protein degradative pool could then be estimated from the difference of the degradation rates in the absence and presence of ammonia. A comparison of the overall protein degradation of normal control and mucolipidosis II cells showed a marked difference: whereas the half-time of degradation was approx. 72 h in normal cells, it was considerably prolonged in all of the mucolipidosis II fibroblast lines tested. Addition of ammonia markedly slowed protein degradation in normal cells, indicating that about one-third of the total degradation was due to lysosomal proteolysis. As the protein degradative rate of mucolipidosis II cells corresponded to that of normal cells in the presence of ammonia and was barely affected by NH_4Cl treatment, it is clearly the lysosomal, not the cytoplasmic, protein degradation that is severely impaired in mucolipidosis II fibroblasts.

When normal fibroblasts or hepatocytes are exposed to serum and/or amino acid starvation, they respond with increased lysosomal proteolysis. This is a consequence of enhanced bulk autophagy of cytoplasmic proteins mediated via amino acid deficiency (Hendil et al., 1990; Kopitz et al., 1990). Whereas in our present experiments such treatment led to the expected increase in proteolysis in normal controls, there was a very marginal effect in mucolipidosis II fibroblasts, again indicating that proteolytic activities in lysosomes of mucolipidosis II cells cannot meet the demands.

The major proteinases responsible for lysosomal protein catabolism are cathepsins B, D, H and L (Bohley and Seglen, 1992). It has been shown previously that mucolipidosis II fibroblasts, in addition to the known multiple deficiency of lysosomal glycosidases and sulphatases, were also deficient in the proteinase cathepsin B (Hanewinkel et al., 1987). Our results

confirm and extend this finding by showing that mucolipidosis II fibroblasts have very low activities of cathepsins B, H and L. Although the aspartic proteinase cathepsin D was not directly determined in our experiments, it seems of little importance here as the inhibition of cysteine proteinases by leupeptin had no less an effect on protein degradation in normal fibroblasts than did NH_4Cl.

Taken together, the present data demonstrate that fibroblasts from patients with mucolipidosis II have a severely impaired capacity in their lysosomal proteolytic function which is likely to result from the multiple deficiency of cathepsins. Cytoplasmic protein catabolism, on the other hand, seems unaffected.

Earlier investigations with mucolipidosis II fibroblasts had already shown a normal, presumably cytoplasmic, degradation of collagen, but defective lysosomal proteolysis of exogenously added epidermal growth factor (Bienkowski et al., 1990) or low-density lipoprotein (Williams et al., 1982). From a study of the degradation of biosynthetically labelled proteins, Cockle and Dean (1984) concluded that there was an impaired protein degradation in mucolipidosis II fibroblasts that appeared to be non-lysosomal; however, the exact nature could not be satisfactorily explained. The discrepancy with our present findings presumably results from the rather short labelling periods (1 and 16 h versus our 72 h), as it is mainly the slow-turnover proteins that are degraded in lysosomes.

The ammonia-sensitive, i.e. lysosomal, protein catabolism in fibroblasts from patients with other lysosomal storage diseases was also determined. In mucolipidosis III, a milder form of mucolipidosis II, protein degradation was again markedly decreased. In cells from patients with various forms of mucopolysaccharidoses (types I, II, IIIA and VI) and with sialidosis, galactosialidosis and G_{M1}-gangliosidosis, there was a mild to moderate impairment of proteolysis, probably resulting from a secondary disturbance of lysosomal protein degradation by accumulated storage material.

Our results imply that impaired lysosomal protein degradation in mucolipidoses II and III, and perhaps in other lysosomal diseases, is of pathogenic relevance, as proteins are likely to constitute a further class of storage substance and the nature of the accumulated material will have an influence on the clinical symptoms. Moreover, a decreased lysosomal proteolytic capacity is likely to be a limiting factor in the cell's protein turnover as well as its potential to react to amino acid starvation by increasing intracellular proteolysis. The present methodology should also be of use to screen for other possible protein degradative disorders, and experiments with fibroblasts from patients with ceroid lipofuscinoses have been initiated.

We thank Dr. Peter Bohley, Institute of Physiological Chemistry, University of Tübingen, Germany, for helpful discussions, and Ms. Evelyn Becker for word processing of the manuscript.

REFERENCES

Barrett, A. J. and Kirschke, H. (1981) Methods Enzymol. **80C**, 535–561

Bienkowski, R. S., Ripley, C. R., Gitzelmann, R. and Steinmann, B. (1990) Biochem. Biophys. Res. Commun. **168**, 479–484

Bohley, P. and Seglen, P. O. (1992) Experientia **48**, 151–157

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254

Cantz, M., Kresse, H., Barton, R. W. and Neufeld, E. F. (1972) Methods Enzymol. **28**, 884–897

Cockle, S. M. and Dean, R. T. (1984) Clin. Chim. Acta **140**, 257–265

Freshney, R. I., Paul, J. and Kane, I. M. (1975) Br. J. Cancer **31**, 89–99

Hall, N. A., Lake, B. D., Dewji, N. N. and Patrick, A. D. (1991) Biochem. J. **275**, 269–272

Hanewinkel, H., Glössl, J. and Kresse, H. (1987) J. Biol. Chem. **262**, 12351–12355

Hendil, K. B., Lauridsen, A. M. and Seglen, P. O. (1990) Biochem. J. **272**, 577–581

Knowles, S. E. and Ballard, F. J. (1976) Biochem. J. **156**, 609–617

Kopitz, J., Kisen, G., Gordon, P. B., Bohley, P. and Seglen, P. O. (1990) J. Cell Biol. **111**, 941–953

Langner, L., Wakil, A., Zimmermann, M., Ansorge, S., Bohley, P., Kirschke, H. and Wiederanders, B. (1973) Acta Biol. Med. Ger. **31**, 1–18

Nolan, C. M. and Sly, W. S. (1989) in The Metabolic Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), 6th edn., pp. 1589–1602, McGraw-Hill, New York

Palmer, D. N., Martinus, R. D., Cooper, S. M., Midwinter, G. G., Reid, J. C. and Jolly, R. D. (1989) J. Biol. Chem. **264**, 5736–5740

Palmer, D. N., Fearnley, I. M., Walker, J. E., Hall, N. A., Lake, B. D., Wolfe, L. S., Haltia, M., Martinus, R. D. and Jolly, R. D. (1992) Am. J. Med. Genet. **42**, 561–567

Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D. (eds.) (1989) The Metabolic Basis of Inherited Disease, 6th edn., part 11, pp. 1565–1839, McGraw-Hill, New York

Seglen, P. O. (1983) Methods Enzymol. **96**, 737–764

Seglen, P. O., Gordon, P. B., Grinde, B., Solheim, A., Kovacs, A. L. and Poli, A. (1981) Acta Biol. Med. Ger. **40**, 1587–1598

Williams, J. C., Weinstein, D. B., Miller, A. L. and Steinberg, D. (1982) Biochem. J. **202**, 183–190

Received 19 February 1993/27 May 1993; accepted 16 June 1993