Compartmental distribution of β -hexosaminidase isoenzymes in I-cell fibroblasts

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A characteristic of the human lysosomal disorder I-cell disease is an abnormal excretion of most lysosomal hydrolases, including β -N-acetyl-D-glucosaminidase (EC 3.2.1.30; β -hexosaminidase) by cultured skin fibroblasts. Treatment of I-cell cultures with cycloheximide or tunicamycin demonstrated that (1) I-cell fibroblasts rapidly excrete all newly synthesized β -hexosaminidase, (2) two qualitatively distinct pools of β -hexosaminidase isoenzymes exist inside I-cell fibroblasts, one of which is a rapid-turnover excretory pool, and (3) the induction of an abnormal glycosylation of β -hexosaminidase by tunicamycin in normal or I-cell fibroblast cultures does not affect subsequent excretion of the enzyme.

One of the main biochemical features of I-cell disease (mucopolidosis II) is an abnormal electrophoretic migration of both the intracellular (Lie *et al.*, 1973; Champion & Shows, 1977) and excreted (Vladutiu & Rattazzi, 1975) forms of several lysosomal hydrolases derived from cultures of skin fibroblasts. More recently it has been shown that high-molecular-weight precursor forms of hydrolases are excreted from both normal and I-cell fibroblasts (Hasilik & Neufeld, 1980a); however, the I-cell-derived enzymes are almost completely deficient in phosphate groups (Bach *et al.*, 1979; Hasilik & Neufeld, 1980b).

It is now well established that a mannose 6-phosphate recognition marker on lysosomal hydrolases is responsible for their receptor-mediated uptake by fibroblasts (Kaplan *et al.*, 1977; Sando & Neufeld, 1977; Ullrich *et al.*, 1978; Natowicz *et al.*, 1979). The absence of phosphorylation in I-cell disease may or may not be directly related to the basic defect in this disorder. Although it is generally believed that an abnormality in glycosylation may be the underlying problem in I-cell disease (Sly, 1980), aberrant glycosylation may be secondary to the primary defect (Vladutiu & Rattazzi, 1975). The preferential excretion of hydrolases in I-cell cultures may be due to an abnormality in the vesicle-mediated pathway by which hydrolases migrate to lysosomes, i.e. a defect which promotes an alternative excretory pathway which is present but is minor in normal fibroblasts (Vladutiu & Rattazzi, 1975). This theory was in part supported by the observation that I-cell-type neuraminidase-sensitive

β -hexosaminidase is present in certain normal body fluids (Vladutiu, 1976; Vladutiu & Rattazzi, 1978). However, it is also possible that abnormal glycosylation could shunt the enzymes into this secondary excretory pathway (Vladutiu & Rattazzi, 1975).

The purpose of the present study was to assess whether two distinct pools of β -hexosaminidase existed in I-cell fibroblasts, one intended for excretion and one for packaging in lysosomes. We also wanted to determine if aberrant glycosylation of hydrolases could affect their excretion by normal and I-cell fibroblasts.

Protein-synthesis inhibition by cycloheximide was used to determine if distinct pools of β -hexosaminidase existed and to determine directly whether nearly all newly synthesized β -hexosaminidase is excreted in I-cell cultures (Vladutiu & Rattazzi, 1979a; Hasilik & Neufeld, 1980a).

Tunicamycin, an inhibitor of the formation of N-acetylglucosamine-lipid intermediates used in the biosynthesis of glycoproteins (Takatsuki *et al.*, 1975; Tkacz & Lampen, 1975), was used to assess the role of glycosylation in hydrolase excretion. Concanavalin A binding of hydrolases was used to quantify the effect of tunicamycin on glycosylation. Lectin binding, as a measure of glycosylation, has been used in several studies on hydrolases (Rousson *et al.*, 1979; van Elsen & Leroy, 1979; Kress & Miller, 1979).

Preliminary reports of this work have been presented in abstract form (Vladutiu & Rattazzi, 1979b,c).

Experimental

Tunicamycin was a gift from Dr. G. Tamura, Department of Agricultural Chemistry, University of Tokyo, Tokyo, Japan. Stock solutions were prepared in weak-alkaline water (NaOH) and adjusted to pH 7.4 with 0.1 M-HCl before use (G. Tamura, personal communication). Cycloheximide was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Concanavalin A was obtained from Miles Laboratories, Elkhart, IN, U.S.A.

I-cell fibroblasts (from patient L. T.) were obtained from Dr. George Thomas (Johns Hopkins University, Baltimore, MD, U.S.A.). Normal fibroblast cultures were obtained from our own stocks, and culture GM-41 was from the Genetic Mutant Cell Repository (Camden, NJ, U.S.A.). Cultures were maintained in 25 cm² plastic Petri dishes (Corning Glass Co., Corning, NY, U.S.A.) with Ham's F-10 medium + 15% foetal calf serum + kanamycin (20 µg/ml) (Grand Island Biologicals, Grand Island, NY, U.S.A.). The collection of fibroblast secretions, the preparation of cell extracts, enzyme assay methods and electrophoretic procedures have been described (Vladutiu & Rattazzi, 1975). The fluorogenic substrate 4-methylumbelliferyl *N*-acetylglucosaminide (Pierce Chemical Co., Rockford, IL, U.S.A.) was used for the assay of β -hexosaminidase and for the detection of the isoenzymes after electrophoresis (Vladutiu & Rattazzi, 1975). One enzyme unit was defined as the activity that liberated 1 nmol of 4-methylumbelliferone/h at 37°C. The intra- and extra-cellular β -hexosaminidase activity was expressed as units/µg of cell-extract protein. Lactate dehydrogenase activity in cell culture fluids was determined by the method of Wroblewski & La Due (1955). Electrophoresis of cell extracts and culture fluids was carried out on cellulose acetate gel (Cellologel; Kalex, Manhasset, NY, U.S.A.) in 36 mM-sodium barbital/citric acid buffer, pH 6, as described by Rattazzi *et al.* (1975). The treatment of β -hexosaminidase preparations with *Clostridium perfringens* neuraminidase has been described (Vladutiu & Rattazzi, 1975). Protein concentrations were measured by using the Coomassie Blue dye-binding method (Bradford, 1976).

Monolayers of normal and I-cell fibroblasts were incubated at 37°C in serum-free medium containing 0.25% human serum albumin with a range of concentrations (0.15–8.0 µg/ml) of either tunicamycin or cycloheximide for 2–72 h. A concentration of 4 µg of either drug/ml was found to be the lowest concentration which caused the maximum apparent inhibitory effect on the excretion of β -hexosaminidase in I-cell cultures.

To determine whether tunicamycin treatment affected the glycosylation of excreted enzyme, β -hexosaminidase (550 units/ml) from normal or

I-cell cultures treated with a low dose of tunicamycin (0.5 µg/ml) for 24 h was titrated with concanavalin A (25–400 µg/ml) in 0.018 M-sodium phosphate/1 M-NaCl/0.1 M-MgCl₂ buffer, pH 6.5, at 27°C for 20 h (Rattazzi *et al.*, 1980). Samples were centrifuged at 41 500 *g* for 5 min and the unbound β -hexosaminidase activity in the supernatant was assayed.

The binding affinity of concanavalin A was also used in a unique combination with electrophoresis (Vladutiu & Rattazzi, 1980) to assess whether any of the specific isoenzymes from tunicamycin-treated cultures were deficient in mannosyl residues.

β -Hexosaminidase from tunicamycin-treated (0.5, 1.0 and 4 µg/ml) I-cell culture fluids was quantified by radial immunodiffusion in agarose gel (Ouchterlony, 1958) in order to determine if tunicamycin treatment inactivated β -hexosaminidase, seemingly inhibiting the excretion of active enzyme, while actually allowing the excretion of immunologically active but catalytically inactive enzyme. The assay was performed in 0.5% agarose containing 3% poly(ethylene glycol) and 0.075% (v/v) bovine anti- β -hexosaminidase serum (Bartholomew & Rattazzi, 1974) in 0.1 M-sodium phosphate/0.15 M-NaCl, pH 7.4. Human liver extract (50–500 units of β -hexosaminidase/ml) was used as a standard. The plates were incubated for 5 days at 27°C, washed for 48 h in 0.9% NaCl containing 0.1% Triton X-100, and air-dried. Cellulose acetate gel, soaked in the 4-methylumbelliferyl substrate, was applied to the dried gel surface at 37°C for 3 h, producing a replica of the enzymically active immune precipitation rings. The actual measurement of fluorescence was performed as previously described (Rattazzi *et al.*, 1980). β -Hexosaminidase is stabilized indefinitely by reaction with its specific antibody. It may even be kept in the dried state at room temperature for prolonged periods of time.

Results and discussion

Inhibition of excretion by cycloheximide and tunicamycin

Cycloheximide-treated normal cells maintained a constant intracellular concentration of β -hexosaminidase during 8 h (37.5 units/µg in untreated and 37 units/µg in treated cells), whereas the extracellular activity was decreased to 60% of the corresponding activity in untreated cultures (from 0.042 to 0.026 unit/µg). By 72 h, both the intracellular and extracellular activities were decreased to approx. 60% of control (from 38.1 to 24.5 units/µg in cell extracts and 4.3 to 2.8 units/µg in culture fluids; Fig. 1). These findings are consistent with our earlier report of a half-life of 2.6 days for β -hexosaminidase in normal fibroblast cultures maintained in serum-free medium (Vladutiu & Rattazzi,

Fig. 1. Cycloheximide treatment of normal and I-cell fibroblast extracts (○, △) and culture fluids (●, ▲) during 72 h incubation

●, ○, Normal cultures; ▲, △, I-cell cultures. Results are means of duplicate assays on three to nine culture dishes. For details see the Experimental section.

Fig. 2. Effect of a high dose (4 μ g/ml) of tunicamycin on β -hexosaminidase activity in normal and I-cell fibroblast extracts (△, □) and culture fluids (▲, ■) during 72 h incubation

■, □, Normal cultures; ▲, △, I-cell cultures. Means of duplicate assays on two culture dishes. For details see the Experimental section.

1979a) and also suggest that at any given time the majority of intracellular β -hexosaminidase is already processed and packaged within the cell, presumably in lysosomes.

I-cell fibroblast cultures treated with cycloheximide (4 μ g/ml) for 8 h lost 40% of their intracellular β -hexosaminidase activity (4.3 to 2.8 units/ μ g) and 85% of their extracellular activity (from 0.15 to 0.02 unit/ μ g). These results demonstrated that an accelerated turnover of intracellular and excreted enzyme exists in I-cell fibroblasts as compared with normal cells. By 72 h, the I-cell intracellular activity was 50% of the control (from 5.97 to 3.0 units/ μ g), and the extracellular activity was decreased to less than 8% of that of untreated cultures (from 1.8 to 0.13 unit/ μ g; Fig. 1). The effect of cycloheximide on total protein in normal fibroblasts was negligible, and it decreased I-cell fibroblast protein by 11% in 72 h.

Treatment of normal and I-cell cultures with 4 μ g of tunicamycin/ml did not affect intracellular β -hexosaminidase activity significantly during 8 h incubation at 37°C, whereas after a 4–6 h lag excreted enzyme activity in both cultures was substantially decreased (from 0.169 to 0.081 unit/ μ g in I-cell culture fluids and 0.012 to 0.006 unit/ μ g in normal fluids). By 24 h, I-cell cultures treated with 0.5, 1.0 and 4 μ g of tunicamycin/ml excreted 54, 35 and 26%, respectively, of the activity in untreated cultures, and this activity remained low (4.4 units/

μ g) compared with the controls (19.2 units/ μ g) for 72 h (Fig. 2). I-cell intracellular activity was decreased by 48 h to 65% of that of untreated cultures (from 5.68 to 3.7 units/ μ g) with 4 μ g of tunicamycin/ml. Total protein in I-cell extracts was not significantly influenced by tunicamycin treatment during the incubation period.

The low doses of tunicamycin (0.5 and 1.0 μ g/ml), which significantly decreased β -hexosaminidase excretion, had negligible effects on the intracellular lactate dehydrogenase concentration during a 72 h period, but higher doses were toxic by 72 h.

Although tunicamycin affected excretion of β -hexosaminidase by I-cell fibroblasts, it did not promote the intracellular accumulation of excretory enzyme even when the concentration of tunicamycin was lowered to 0.15 μ g/ml.

The intracellular β -hexosaminidase activity in normal cultures treated with 4 μ g of tunicamycin/ml was decreased to 30% of that of untreated controls (from 31 to 9.4 units/ μ g) by 72 h, and the extracellular activity was decreased to approx. 75% of that of untreated controls (from 3.6 to 2.6 units/ μ g; Fig. 2).

Since the effect of tunicamycin on the excretion of β -hexosaminidase in I-cell cultures was quantitatively similar to that of cycloheximide, this was interpreted to mean that tunicamycin decreased enzyme excretion mainly by inhibiting enzyme synthesis (Hickman *et al.*, 1977); however, an enhanced degradation of newly synthesized enzyme protein (Olden *et al.*, 1978) could not be excluded. Excretion of β -hexosaminidase was more pro-

foundly affected in I-cell cultures than in normal cultures by tunicamycin and cycloheximide. These findings prove earlier suggestions that I-cell fibroblasts synthesize 5-fold more enzyme than do normal fibroblasts daily and excrete more than 95% of it (Vladutiu & Rattazzi, 1979a; Hasilik & Neufeld, 1980a).

Immunological measurement of excreted enzyme

The ratio of immunological to catalytic activity of β -hexosaminidase in culture fluids from I-cell fibroblasts treated with high doses (4 μ g/ml) of tunicamycin for 72h was only 22% greater than in untreated cultures, suggesting that a relatively small portion of the catalytic activity of excreted enzyme may have been inactivated as a result of tunicamycin treatment. Lower doses (0.5–1.0 μ g/ml) of tunicamycin showed only a negligible catalytic inactivation of immunologically active enzyme protein. Thus the apparent decrease in enzyme excretion was not due to decreased catalytic activity of β -hexosaminidase.

Electrophoretic evidence for a distinct excretory pool

Fig. 3 (lanes 1–3) shows diagrammatically that, in the extracts of I-cells treated with either tunicamycin or cycloheximide, two distinct isoenzyme bands of hexosaminidase A and B (bands 2 and 4) disappeared. The loss of these isoenzymes and the coincidental drastic decrease in excreted enzyme strongly suggest that inside I-cell fibroblasts isoenzymes 2 and 4 represent rapid-turnover forms (precursor pool?) of hexosaminidase A and B which are destined for excretion, whereas isoenzymes 1 and 3 are retained intracellularly. The fact that tunicamycin and cycloheximide both effected a loss of isoenzymes 2 and 4 further suggested that tunicamycin inhibited protein synthesis or that an increased rate of enzyme degradation occurred in the presence of tunicamycin.

Isoenzymes 2 and 4 are electrophoretically identical with the isoenzymes that result from

Fig. 3. Composite electrophoretic patterns of β -hexosaminidase (Hex A and Hex B) in extracts (ext.) of I-cell and normal fibroblasts treated for 24 h with tunicamycin (TM) or cycloheximide (CH) (both 4 mg/ml). For details see the Experimental section.

neuraminidase treatment of I-cell-excreted enzyme (Fig. 4, lane 2), suggesting that the I-cell-excreted isoenzymes are sialylated forms of intracellular isoenzymes 2 and 4. However, the cellular site of sialylation is not known, nor have the sialylated forms been demonstrated intracellularly.

A corresponding effect of cycloheximide and tunicamycin on hexosaminidase A and B isoenzymes from normal fibroblast extracts was not apparent (Fig. 3, lane 4), suggesting that if an electrophoretically distinct excretory pool of intracellular β -hexosaminidase does exist in normal fibroblasts it constitutes a very small portion of the total intracellular enzyme.

Fig. 4 shows diagrammatically the effects of tunicamycin and cycloheximide on the residual excreted β -hexosaminidase in normal and I-cell cultures. Lanes 1 and 2 represent the usual pattern of I-cell-excreted β -hexosaminidase before and after neuraminidase treatment, respectively (Vladutiu & Rattazzi, 1978). Tunicamycin had no effect on I-cell hexosaminidase A, whereas hexosaminidase B had a slower mobility, and a new band of activity appeared near the origin (lane 3), as found in the corresponding I-cell extract (Fig. 3, lane 3). Normal excreted enzyme was only affected by the appearance of a similar faint band of activity near the origin (Fig. 4, lane 8). Unlike tunicamycin, cycloheximide treatment did not result in the production of new isoenzymes in I-cell or normal culture fluids (Fig. 4, lanes 5–7).

Evidence of abnormal glycosylation

The results of a concanavalin A titration of β -hexosaminidase derived from the low-dose tunicamycin-treated normal and I-cell fibroblast culture fluids are presented in Fig. 5. The small amount of enzyme excreted from both tunicamycin-treated

Fig. 4. Composite electrophoretic patterns of β -hexosaminidase (Hex) in normal culture fluid (norm. med.) and I-cell culture fluid (ICD med.) in cultures incubated with or without tunicamycin (TM) or cycloheximide (CH) for 24 h.

For details see the Experimental section. Culture fluids in lanes 2, 4, 6 and 7 were also treated before electrophoresis with neuraminidase (N). Arrows denote similar mobility of neuraminidase-treated hexosaminidase B isoenzymes.

Fig. 5. Concanavalin A titration of β -hexosaminidase in concentrated 24 h culture fluids from tunicamycin ($0.5 \mu\text{g/ml}$)-treated (●, ▲) and untreated (○, △) normal and I-cell cultures

●, ○, Normal cultures; ▲, △, I-cell cultures. For details see the Experimental section.

normal and I-cell fibroblasts lost approx. 40% of its concanavalin A-binding capacity. These data strongly suggest that, even though the glycosylation of excreted β -hexosaminidase from both cultures had been considerably affected by tunicamycin treatment, the abnormally glycosylated enzyme was still excreted by the cells.

All the β -hexosaminidase isoenzymes derived from tunicamycin-treated cultures (Figs. 3 and 4) were precipitated by concanavalin A in cellulose acetate gel after electrophoresis, suggesting that mannosyl residues were still present on the isoenzymes. This further suggested that the effect of tunicamycin on glycosylation, indicated by the overall 40% decrease in concanavalin-A reactivity (Fig. 5), did not involve one or more of the isoenzymes preferentially.

In conclusion, our data indicate that I-cell fibroblasts rapidly excrete all newly synthesized β -hexosaminidase, as evidenced by the rapid effect of cycloheximide on excretion.

Our data also demonstrate an intracellular distribution of β -hexosaminidase isoenzymes into a non-excretory pool and a rapid-turnover excretory pool. These pools of isoenzymes may or may not exist in distinct compartments at the subcellular level

in I-cell fibroblasts. Isoenzymes 1 and 3 (Fig. 3, lane 1) may be in lysosomes, since Miller *et al.* (1980a) reported the localization of β -hexosaminidase in a lysosome-rich fraction in I-cell fibroblasts. However, contrary to the chromatographic similarities that Miller *et al.* (1980a) found between normal and I-cell intracellular enzyme, there is considerable evidence showing qualitative differences in the electrophoretic properties (Lie *et al.*, 1973; Vladutiu & Rattazzi, 1975) and subcellular processing (Hasilik & Neufeld, 1980a).

Since I-cell intracellular isoenzyme bands 2 and 4 are most profoundly affected by cycloheximide, the excreted high-molecular-weight precursor forms observed in pulse-chase experiments by Hasilik & Neufeld (1980a) are probably derived from these isoenzyme forms. More direct characterization of the isoenzymes is needed to substantiate this contention.

Although tunicamycin inhibited the synthesis of excretory enzyme, the alteration in glycosylation of β -hexosaminidase induced by tunicamycin did not prevent excretion of the enzyme in normal or I-cell cultures. This finding supports reports on the effect of tunicamycin on lysosomal hydrolases in normal fibroblasts (von Figura *et al.*, 1979) and in I-cell fibroblasts (Miller *et al.*, 1980b), in which it was concluded that glycosylation of hydrolases was not required for excretion.

Sly & Stahl (1978) have proposed a 'phosphomannosyl-enzyme segregation hypothesis' suggesting that a phosphomannosyl recognition marker on hydrolases directs high-mannose-type glycoprotein enzymes to lysosomes. They further suggested that, in I-cell disease, absence of the recognition marker disrupts the segregation process and leads to excretion of nearly all newly synthesized enzymes, including those that have been processed to a complex-type glycoprotein in an unusual pathway associated with the Golgi region. The suggestion of an unusual pathway is similar to our preferential-excretion hypothesis (Vladutiu & Rattazzi, 1975). Neither hypothesis, however, explains how I-cell fibroblasts specifically retain the abnormal β -hexosaminidase isoenzymes 1 and 3, which may reside in secondary lysosomes (Miller *et al.*, 1980a).

We reported that all four of the typical I-cell intracellular β -hexosaminidase isoenzymes are excreted by both normal and I-cell fibroblasts treated with the Na^+/K^+ ionophore monensin (Vladutiu & Rattazzi, 1980). Because monensin blocks transport of glycoproteins out of the Golgi region (Tartakoff & Vassalli, 1977, 1978; Uchida *et al.*, 1979), we suggested that this excretion may arise from pre-Golgi-region vesicles. Since the four excreted isoenzymes comprise two distinct pools of enzyme in untreated I-cell fibroblasts, information on the

diversion to the unusual pathway operative in I-cell disease may be obtained by comparative structural studies of the carbohydrate side chains in the isoenzymes comprising the two pools.

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