A HYPOTHESIS FOR I-CELL DISEASE: DEFECTIVE HYDROLASES THAT DO NOT ENTER LYSOSOMES

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Skin fibroblasts cultured from patients with I-cell disease ("I-cells") are markedly deficient in several lysosomal hydrolases, though these enzymes are found in surrounding medium. This situation is not brought about by increased lysosomal leakage, since I-cells were found as retentive of ingested enzymes (β-glucuronidase and α-L-iduronidase) as cells of other genotypes. On the other hand, hydrolases from I-cell medium were found only one-fifth to one-tenth as effective as their normal counterparts in experiments that involve uptake of the enzymes from the medium: correction of Hurler cells by α-L-iduronidase, correction of β-glucuronidase deficient cells by β-glucuronidase, and direct measurement of uptake of N-acetyl β-glucosaminidase by fibroblasts deficient in that enzyme.

We suggest that the packaging of lysosomal enzymes requires their secretion followed by specific recognition and uptake. The I-cell mutation would interfere with this process by altering the recognition site on the hydrolases.

I(inclusion)-cell disease is a genetic disorder classified as a "mucolipidosis" because of clinical and biochemical similarities to both mucopolysaccharide and lipid storage diseases (1-4). The disease owes its name to the innumerable inclusions that are seen by phase microscopy in fibroblasts ("I-cells") cultured from the patients' skin (2,3,5). These inclusions can be identified by electron microscopy as lysosomes filled with membrane fragments and other debris (6). The lysosomal storage is, in turn, caused by the deficiency of a number of hydrolases: β-galactosidase, α-L-fucosidase and aryl sulfatase A activities are barely detectable in cultured fibroblasts; α-mannosidase, α-L-iduronidase, N-acetyl-β-glucosaminidase and β-glucuronidase activities are 10-30% of normal. Only acid phosphatase and β-glucosidase activities are at a normal level (2,3,6,7).

Several of the enzymes deficient in I-cells occur in excessive quantity in the culture medium in which these fibroblasts have been grown (8), and are

markedly elevated in the patients' plasma, cerebrospinal fluid, and urine (9). Wiesmann et al. (8,9) have proposed as the basis of I-cell disease, some membrane defect that renders the lysosomes unusually leaky.

To verify the leakage hypothesis, we took advantage of the ability of fibroblasts to take up enzymes from the medium. It is assumed that such uptake occurs by pinocytosis, and that the enzyme therefore becomes lodged in secondary lysosomes (10). Experiments described in this paper show I-cells to be at least as retentive of ingested enzyme as normal cells. On the other hand, the hydrolases released by I-cells into the medium differ from their normal counterparts in that they are not taken up as efficiently by other fibroblasts. We suggest, therefore, that the defect of I-cell disease resides not in faulty membranes that allow enzymes to escape, but in defective enzymes that fail to reach their lysosomal destination.

EXPERIMENTAL

Fibroblasts were maintained in culture as previously described, in modified Eagle's Minimal Essential Medium with 10% fetal calf serum (11); the same medium was used for all uptake experiments. However, for the collection of secretions, fibroblasts were grown to confluence in this medium in 1410 cm^2 Bellco roller bottles; the cells were then fed serum-free medium (12; see modification in ref. 11), which was harvested twice a week. Medium from three roller bottles (750 ml) was concentrated to ca. 30 ml in an Amicon ultrafiltration cell (PM-30 membrane), 15 mg of bovine serum albumin was added as carrier, and protein was precipitated by addition of ammonium sulfate (enzyme grade) to 80% saturation. The precipitate was dialyzed against 0.15 M NaCl, containing 0.01 M sodium phosphate, pH 6.0.

Correction of mucopolysaccharide accumulation was measured as previously described (13). Incubation mixtures for the assay of β-glucuronidase contained 0.1 M sodium acetate, pH 5.0, and either 0.04 M p-nitrophenyl β-glucuronide or 0.002 M phenolphthalein β-glucuronide, as indicated; that for N-acetyl β-glucosaminidase, 0.1 M sodium acetate, pH 5.0, and 0.005 M p-nitrophenyl

N-acetyl β-glucosaminide. Incubation was carried out for 1 hr at 37°, in 0.5 ml. α-L-Iduronidase was assayed essentially as described (14), but on one-fourth the scale.

Enzymes to be incubated with fibroblasts were mixed with medium prior to sterilization through Millipore filters. Mixtures routinely contained 5 ml of medium and 1 ml of 0.15 M NaCl - 0.01 M sodium phosphate, pH 6.0, with enzyme, per 100 mm Falcon Petri dish. After incubation for the desired time, cells were rinsed three times with 0.15 M NaCl and detached from the dish by trypsinization. The cell pellet from each Petri dish was washed twice with 5 ml of 0.15 M NaCl, suspended in 0.2 ml of 0.15 M NaCl and subjected to two cycles of freezing and thawing followed by thorough dispersal in a Kontes "Duall" 1 ml homogenizer. The resultant slurry was used for measurement of enzyme activity and protein content (15).

β-Glucuronidase (Type B-10) was purchased from Sigma; α-L-iduronidase (Hurler corrective factor) was a previously described preparation from human urine (13,14); all substrates were purchased from commercial sources, except for phenyl α-L-iduronide, which was a kind gift from Dr. Bernard Weissmann.

RESULTS

1. Retention of exogenously supplied enzymes by I-cells

Cultured fibroblasts have previously been shown to accumulate substantial levels of bovine β-glucuronidase when that enzyme is added to the medium (7); the activity of endogenous β-glucuronidase is relatively low. I-cells, as well as fibroblasts from a normal individual and a patient with β-glucuronidase deficiency, were incubated with medium containing 1 mg (dry powder) of the bovine β-glucuronidase preparation. After 48 hr, accumulation within the cells was equivalent to 0.88, 0.51, and 0.63 μmole of phenolphthalein glucuronide hydrolyzed per hour, per Petri dish of I-cells, normal and β-glucuronidase deficient fibroblasts, respectively.

Medium containing β-glucuronidase was then replaced by medium without the enzyme and the disappearance of the ingested enzyme activity followed (Fig. 1).

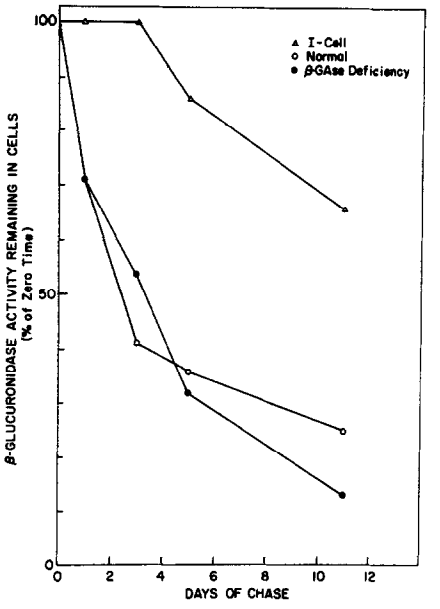

Fig. 1. Decay of β-glucuronidase activity in I-cells, normal and β-glucuronidase deficient fibroblasts, that had been allowed to accumulate bovine liver β-glucuronidase. All values are corrected for endogenous activity. Each point corresponds to the cellular content of one Petri dish (ca. 1 mg protein at the start of the chase); medium was changed in remaining dishes on days 3, 6 and 8.

Contrary to the results predicted by the leakage hypothesis, I-cells were found even more retentive than the other cell lines.

Similar results were obtained with human α-L-iduronidase (13,14). In this case it was impractical to use normal cells because of the high levels of endogenous α-L-iduronidase, and the modest amounts of purified enzyme available; I-cells were therefore compared with iduronidase-deficient cells derived from a Hurler patient. After 48 hr of incubation in the presence of α-L-iduronidase, I-cells and Hurler cells had accumulated respectively, per Petri dish, activity equivalent to 0.86 and 0.62 μmole phenyl iduronide hydrolyzed per 17 hr assay (or 60% and 45% of the activity applied). Medium was changed at that time, and the half-life of the ingested enzyme determined as 9 days, in both Hurler fibroblasts and I-cells.

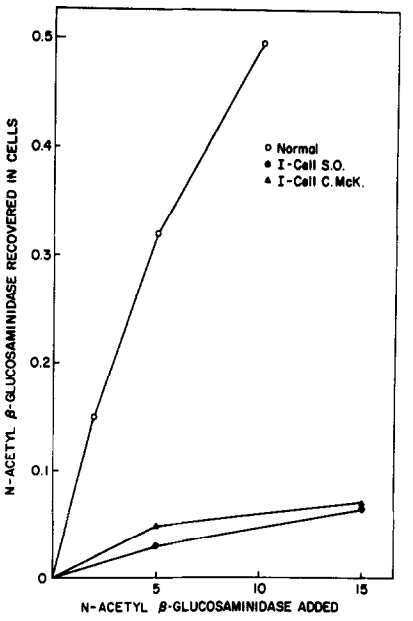

Fig. 2. Uptake of N-acetyl β-glucosaminidase derived from I-cells or normal cells, into fibroblasts from Tay-Sachs (O variant) disease. Values on the abscissa represent activity of enzyme supplied to each Petri dish, in μmoles per hour, whereas those on the ordinate are in μmoles per hour per mg cell protein. All values have been corrected for endogenous activity in the Tay-Sachs (O variant) cells (0.05 μmole per hr per mg protein).

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2. Uptake of enzyme released by I-cells into culture medium

Enzymes in concentrates in which I-cells had been incubated were compared in three experimental situations with enzymes in analogous preparations derived from normal cells, with respect to their ability to enter fibroblasts. The experimental design was restricted by the high endogenous activities in normal fibroblasts; fibroblasts from a mutant deficient in the particular enzyme under study were therefore used as the recipient cells.

The first experiment consisted of direct measurement of the uptake of N-acetyl β-glucosaminidase by cells from a patient with the O variant of Tay-Sachs disease, from which both A and B isozymes of the hexosaminidase are nearly absent (16). Fig. 2 shows that enzyme derived from normal cells was taken up 8 times more effectively than enzyme derived from I-cells. Mixtures

of normal and I-cell enzymes (5 μmoles per hr of each) were taken up additively, thus ruling out diffusible inhibitors or activators as the cause of the difference.

In the following two tests, the uptake of enzymes was followed by indirect means. α-L-Iduronidase deficient (Hurler) and β-glucuronidase deficient fibroblasts accumulate excessive ^{35}S-mucopolysaccharide. The accumulation is reduced to normal (i.e., corrected) by addition of α-L-iduronidase or β-glucuronidase, respectively, to the culture medium (7,13). The correction is associated with entry of the enzyme into the cells; however, the assay for correction is more sensitive, by one order of magnitude, than the direct measurement of enzyme uptake.

Table 1 shows that the α-L-iduronidase from I-cell medium is but one-sixth as effective in correcting Hurler cells as the α-L-iduronidase of normal medium. Likewise, the β-glucuronidase of I-cell medium has less than one-tenth the corrective activity of its normal counterpart.

DISCUSSION

To account for all the evidence presented above, we present as a working hypothesis that packaging of lysosomal enzymes requires intercellular cooperation. Hydrolases synthesized and secreted by a cell are taken up and sequestered into the lysosomes of its neighbors, the uptake requiring specific recognition of the enzyme at the surface of the recipient cells. The recognition site on the hydrolases would have a function analogous to that of β-galactosyl termini in directing plasma proteins into parenchymal liver cells (17,18). In I-cell disease, the mutation would affect this presumed site so that the hydrolases are not recognized, and therefore not taken up by the specific and efficient mechanism. Those defective hydrolases that are stable in the culture medium would accumulate there in measurable excess over the normal.

Our proposal differs from the accepted view of lysosome formation, whereby a primary lysosome filled with hydrolases is formed by budding from the Golgi apparatus or endoplasmic reticulum, and eventually coalesces with a pinocytic vacuole that contains substrates but not enzymes — the GERL concept of Novikoff (10,19). Perhaps the two concepts are not mutually exclusive,

TABLE 1

Relative Effectiveness of Normal and I-Cell Enzymes as Corrective Factors

Enzyme	Corrective activity* / Catalytic activity
α-L-iduronidase	
Normal	46
I-cell	7.5
β-glucuronidase	
Normal	135
I-cell (S.O.)	11
I-cell (C.McK.)	2

*For α-L-iduronidase, the ratio is in Hurler corrective units per μmole phenyl α-L-iduronide hydrolyzed in the 17 hr assay. For β-glucuronidase, the ratio is in corrective units (for the β-glucuronidase deficient cells) per μmole p-nitrophenyl β-glucuronide hydrolyzed in the one hr assay.

since mechanisms for introducing enzymes into lysosomes may differ in different cells or even for different enzymes. The GERL concept has been developed principally from studies of acid phosphatase in parenchymal liver cells, neutrophils, neurons and peritoneal macrophages (20). I-cell disease, however, involves hydrolases other than acid phosphatase, in fibroblasts, histyocytes and lymphocytes; liver, neurons and bone marrow are spared, as shown by enzyme analyses or electron microscopy (3,21).

Recognition sites on lysosomal hydrolases and the complementary sites on cell surfaces should prove a fertile field of study. The subject has obvious relevance to the preparation of purified lysosomal enzymes for potential treatment of lysosome storage disease.

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