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I-CELL DISEASE: DEFICIENCY OF EXTRACELLULAR HYDROLASE PHOSPHORYLATION

Gideon Bach*, Ruth Bargal* and Michael Cantz+

* Department of Human Genetics, Hadassah-Hebrew University Medical Center, Jerusalem, Israel

+ Institute of Physiological Chemistry, Münster, Germany

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SUMMARY

The content of ^{32}P-phosphorylated residues of purified extracellular N-acetyl-β-hexosaminidase obtained from the fibroblasts of I-cell disease patients was compared to that of control cells hydrolase. The analyses indicated a 60-fold decrease of the radioactivity per unit enzyme activity in the hydrolase synthesized by the patient's fibroblasts compared to the normal enzyme. Electrofocusing demonstrated again a marked decrease in the ^{32}P-content of the I-cell hydrolase while the control enzyme showed the presence of radioactivity in both isozymes, namely hexosaminidase A and hexosaminidase B. Most of the radioactivity could be removed from the hydrolase following incubation with alkaline phosphatase, thus indicating its phosphoester linkage.

Since phosphorylated sugar residues on lysosomal enzymes function as recognition marker for their transport to the lysosomal compartment and for their specific uptake by fibroblasts, the deficiency of phosphorylated residues on the I-cell hydrolase explains the low intracellular and high extracellular lysosomal enzyme levels observed in this disease.

I-cell disease (ICD; mucolipidosis II), a genetic lysosomal storage disorder (1-3), is biochemically characterized by the intracellular deficiency in connective tissue cells, of most of the lysosomal hydrolases and a concomitant increase in their extracellular concentration (2, 4, 5). This intracellular deficiency leads to storage within the lysosomes of various macromolecules. Based on findings with cultured fibroblasts, Hickman and Neufeld (6) proposed that lysosomal hydrolases must bear a specific site (recognition marker) for their receptor-mediated sequestration into the lysosomes. This marker also enables extracellular lysosomal hydrolases to interact specifically with the fibroblast membrane and then be taken-

Abbreviations: I-cell disease - ICD
N-acetyl-β-hexosaminidase-hexosaminidase

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up in high yields by a selective pinocytosis process, while enzymes lacking this site will hardly be cleared from the extracellular medium into the cells. According to these findings it was concluded that the mutation in ICD, causing the abnormal distribution of lysosomal hydrolases in connective tissues, is due to the formation of hydrolases with an absent or defective recognition marker. Recent findings (7-10) indicated the presence of phosphorylated sugars in the recognition marker of hydrolases produced by normal cultured skin fibroblasts.

In our studies to elucidate the basic metabolic defect in ICD, we compared the content of ^{32}P-phosphorylated residues of extracellular N-acetyl--β-hexosaminidase (hexosaminidase) - as a lysosomal enzyme model - of ICD fibroblasts to that of control fibroblasts. In this paper we present evidence of a deficiency in the phosphate content of the hydrolase produced by ICD cells.

While this work was in progress, a preliminary report by Hasilik et al. (11) indicated similar findings of a deficient phosphorylation of lysosomal hydrolases in ICD fibroblasts.

METHODS

Cell cultures - Skin fibroblasts obtained from three ICD patients and two normal controls were propagated and maintained as previously described (12), in 25 cm^2 plastic tissue culture flasks (Falcon). Cultures with density of 2-3 x 10^6 cells per flask were used in all experiments. Experiments were repeated two-three times and the data presented is the average of these determinations.

Radioisotope labeling - Cultures were preincubated for 24 hours with a serum-free medium (Waymouth, MAB 87/3, GIBCO, USA) prior to the radioactivity labeling. The labeling was then carried out with 7.5mCi per flask of carrier-free [^{32}P] orthophosphoric acid (New England Nuclear, USA), dissolved in 4 ml of the Waymouth medium. The cells were incubated for 72 hours prior to harvest.

Hexosaminidase purification - Hexosaminidase activity was determined with 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Koch Light, England) as substrate as previously described (13). One unit of enzyme activity is defined as the amount of enzyme that hydrolases 1 nmol of substrate per hour under the conditions specified. The radioactive medium from two flasks each of the ICD and control fibroblasts was collected and dialyzed for 48 hours against eight changes of 3 liters of 0.04 M sodium phosphate buffer, pH 6.0. The extracellular hexosaminidase was purified by chromatography on concanavalin A and 2-acetamido-N (ε-aminocaproyl)-2-deoxy-β-D-glucopyranosyl-amine sepharose

according to Geiger and Arnon (14). The enzyme was then dialyzed against water and lyophilized. By this procedure the hexosaminidase was purified approximately 700 times. Although the minute quantities of purified enzyme did not enable characterization regarding its purity, previous experience with the placenta preparation indicated a high degree of purity.

Alkaline phosphatase treatment - Purified ^{32}P-labeled hexosaminidase was incubated with E. coli alkaline phosphatase (type III, Sigma Chemicals, USA) as described by Sando and Neufeld (7). Principally, the incubation was carried out in dialysis bags to remove released inorganic phosphate which is inhibitory to the phosphatase. The content of the dialysis bag was then collected and the remaining radioactivity and hexosaminidase activity was determined.

Electrofocusing - For this experiment ^{32}P-labeled hexosaminidase was purified from the medium of ten flasks each of the two genotypes. Acrylamide disc gel electrofocusing of the purified ^{32}P-labeled hexosaminidase was carried out according to the method of Wrigley (15) as previously described (16). The pH range of ampholytes used was 3.5-10. Samples were run in duplicate. At the end of the run the gels were removed by needle irrigation and 2 mm pieces were cut along the gel. Hexosaminidase was determined in the pieces from one gel while the pieces from the second gel were counted for ^{32}P-radioactivity. In both determinations, 0.5 ml water was added to each piece and the gel was ground with a glass rod, and left at 37°C for two hours prior to analysis.

RESULTS

The purified hexosaminidase from both control and ICD fibroblast media had a specific activity of about 7.0-7.5 x 10^3 units/mg protein which is somewhat lower than found in the placental preparations (14). This is obviously due to the instability (even upon freezing) of the medium hexosaminidase while the placental enzyme at that stage of purity was much more stable. The ratio of radioactivity to enzyme activity of purified extracellular hexosaminidase from ICD and control fibroblasts is compared in Table 1. These data indicate a 50-60 fold decrease in the phosphorylation of the hexosaminidase produced by the ICD cells compared to the enzyme obtained from the normal controls.

The decrease in the phosphate content of the ICD hexosaminidase is further indicated by the electrofocusing of the hexosaminidase isozymes. The distribution of the isoenzyme activities and radioactivity is demonstrated in Figure 1 supporting the previous finding, namely that the ICD hexosaminidase (isozyme A and B) contains significantly less, if any, radioactivity per unit enzyme activity when compared to the control enzyme, in which both hexosaminidases A and B were phosphorylated.

Table 1. ^{32}P-content of purified extracellular hexosaminidase

Enzyme source (cell line)		Units of hexosaminidase	Counts/min	Counts/min/units hexosaminidase
Control	Experiment No.1	35	1130	32
	Experiment No.2	18	400	22
ICD	Experiment No.1	987	540	0.5
	Experiment No.2	604	300	0.45

Incubation with bacterial alkaline phosphatase caused the release of approximately 75% of the labeled phosphate from both normal and ICD preparations, proving a phospho-ester linkage in the hydrolase. The remaining phosphate is not susceptible to the enzyme, possibly because the high instability of the hexosaminidase at this stage of purification causes it to denature and became insoluble.

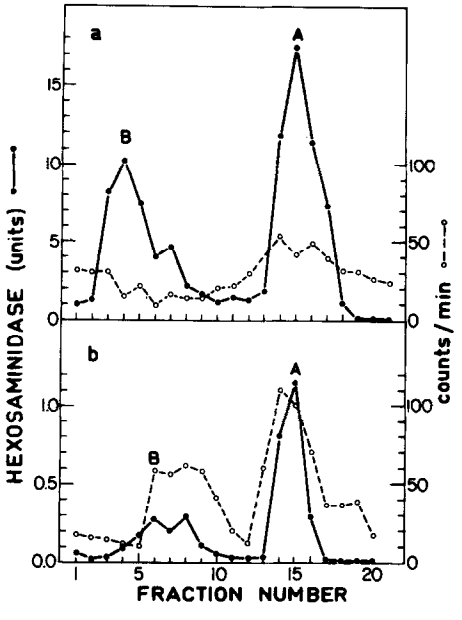

Fig. 1. - Isoelectric focusing of extracellular ^{32}P-labeled hexosaminidase obtained from ICD fibroblast - a and control cells - b. 946 units (570 counts/min) of purified hexosaminidase from ICD and 12 units (270 counts/min) from control were applied to each gel. By this technique the hexosaminidase is separated to its main two isozymes. A - hexosaminidase A; B - hexosaminidase B. ●——● units hexosaminidase/fraction; o-------o radioactivity, counts/min/fraction.

Table 2. Incubation of purified ^{32}P-labeled hexosaminidase with alkaline phosphatase

Enzyme source (cell line)	Alkaline phosphatase	Remaining radioactivity (counts/min)
ICD	+	65
ICD	-	209
Control	+	71
Control	-	231
Control*	+	219

250 counts/min from Control and ICD were incubated with the phosphatase.

* This incubation was carried out in 0.1 M phosphate buffer pH 7.0 instead of the Tris-HCl buffer. The phosphate ions are inhibitory to the phosphatase.

DISCUSSION

This report indicates that lysosomal hydrolases synthesized by ICD fibroblasts are deficient in phosphorylated residues. Since phosphohexose moieties on lysosomal enzymes are thought to be involved in their receptor-mediated endocytosis and transport into the lysosomes (7-10), this deficiency may explain the abnormal distribution of connective tissue hydrolases in ICD. It can therefore be hypothesized that in the absence of the phosphorylated marker, the hydrolases can neither be transported from their biosynthetic site to the lysosomal compartment, nor can they be recaptured from the extracellular environment. Consequently, there is an intracellular deficiency and an extracellular accumulation of these enzymes.

The small quantities of labeled hexosaminidase obtained from the fibroblast culture media did not enable a full characterization of its purity. However, a high degree of purification may be assumed from our previous experience with placental hexosaminidase subjected to the same purification steps (14), as well as from the focusing experiment showing most of the ^{32}P-label of the normal preparation to be associated with hexosaminidase activity.

Our present data do not reveal whether the defect in the ICD hydrolases involves only a deficiency of phosphorylation or a defect in a larger residue of which the phosphate is only a part. The isolation of larger quantities of enzyme is necessary to answer this question by direct chemical analysis.

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