MUCOLIPIDOSIS I: INCREASED SIALIC ACID CONTENT AND DEFICIENCY OF AN α-N-ACETYLNEURAMINIDASE IN CULTURED FIBROBLASTS

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Summary:
Extracts of fibroblasts derived from a patient with mucolipidosis I exhibited a fivefold increase in sialic acid content as compared to those of normal cells. About 80% of this sialic acid was linked to other molecules. Using neuraminlactose as a substrate, mucolipidosis I fibroblasts were found to be severely deficient in an "acid" α-N-acetylneuraminidase. Since other lysosomal hydrolase activities were normal, we hypothesize that the basic metabolic lesion in mucolipidosis I lies in a defective degradation of sialic acid-containing compounds due to the genetic deficiency of a neuraminidase.

Mucolipidosis I is a rare congenital disorder characterized by Hurler-like facial features, skeletal dyplasia, cherryred macular spots and neurodegeneration with progressive ataxia, myoclони, impaired speech and mental retardation. Death occurs by the end of childhood (1). Histologic and electron microscopic examination of neuronal, mesenchymal and visceral tissues suggested an abnormal storage of glycoproteins and/or glycolipids within the lysosomes of such cells (1,2). Until present, no information was available concerning the biochemical basis of mucolipidosis I. This is in contrast to mucolipidosis II (I-cell disease) and mucolipidosis III, where the lysosomal storage of glycosaminoglycans and glycolipids appears to result from a defect in lysosomal enzyme localization (3) with deficiency in cultured fibroblasts, and grossly increased levels in serum, of numerous lysosomal hydrolases.

As the catabolism of a number of glycoproteins and glycolipids involves the removal of sialic acid residues, we have now studied the sialic acid metabolism in cultured fibroblasts derived from a patient with mucolipidosis I. This report shows

that these fibroblasts contain an increased level of bound sialic acid, and that they are severely deficient in a α-N-acetylneuraminidase, but no other lysosomal hydrolase.

EXPERIMENTAL.

Reagents: N-Acetylneuraminic acid (NANA) was purchased from Serva, Heidelberg. Neuraminlactose (N-acetylneuraminosyl (α,2-3)-D-lactose) was obtained from Calbiochem, Los Angeles, USA. Clostridium perfringens neuraminidase was purchased from Boehringer, Mannheim.

Fibroblast lines: Fibroblasts were cultured from a skin biopsy of patient D.F. with mucolipidosis I. Analysis of the patient's fibroblasts and serum for lysosomal hydrolase activities, e.g. arylsulfatase A, ß-D-galactosidase, ß-N-acetylhexosaminidases A and B, α-D-mannosidase, and ß-D-glucuronidase yielded normal results, thus excluding the diagnosis of mucolipidosis II or III. A full report on this patient will be published elsewhere (4).

The fibroblast lines were maintained in tissue culture essentially described previously (5).

Sialic acid determination in fibroblast homogenates: Fibroblasts from one to two 75 cm^2 Falcon plastic flasks, which had been grown for at least three weeks containing 3-6 mg of protein, were collected by trypsinization (5), washed three times with 10 ml 0.15 M NaCl, and resuspended in 1 ml distiled water. The cells were ruptured by 5 freeze-thaw cycles (dry ice-acetone). Protein concentration of the broken cell suspension was measured using Lowry's method (6) with bovine serum albumin as standard. After centrifugation at 12,000 g and 4°C for 30 min, the supernatant was transferred to another tube to which an equal volume of 0.2 N sulfuric acid was then added. The insoluble residue of the homogenate was washed twice with 3 ml of distilled water, and resuspended in 1 ml of 0.1 N H_2SO_4. After heating at 80 °C for 60 min, the hydrolysates were analyzed for sialic acid content by the thiobarbituric acid method of Warren (7), values being corrected for interfering substances. N-acetylneuraminic acid was employed as a standard. In some experiments, the samples were treated with neuraminidase (Clostridium perfringens) in parallel to the acid hydrolysis; both procedures yielded similar sialic acid values.

Assay for α-N-acetyl neuraminidase activity: Fibroblasts from at least three week old subcultures grown in 75 cm^2 Falcon flasks were collected as described above, and homogenized in 1 ml distilled water with a Teflon-glass homogenizer. The protein concentration of the homogenates was adjusted to 2-3 mg/ml with distilled water. Neuraminidase was assayed essentially by the method of Tulsiani and Carubelli (8) under conditions appropriate for the lysosomal enzyme. All assays were carried out in duplicate. The incubation mixture consisted of 150 µl homogenate, 40 µl 5 mM neuraminlactose, and 10 µl 1 M sodium acetate buffer, pH 4.2; the actual pH in the incubation mixture

Table I: Sialic acid content of fibroblast homogenates.

Fraction	Nanomoles NANA/mg protein	
	Mucolipidosis I[1)]	Normal controls [2)]
Supernatant	13.5	2.5
	14.o	1.7
Insoluble residue	8.o	7.6
	6.9	6.8

[1)]Mean values of duplicate determinations on two different subcultures.

[2)]Means of duplicate determinations on two different cell lines.

was 4.4 In control tubes, either the homogenate or the substrate were replaced by water. After incubation for 12o min at 37 °C, the liberated NANA was determined using Warren's procedure (7). After subtraction of the homogenate and substrate blanks, enzyme activity was expressed as mU/mg protein, one unit of enzyme activity corresponding to one micromole of substrate transformed per min.

Under these conditions, the enzyme activity of normal homogenates was linear with respect to time and protein concentration. The pH optimum was 4.4, with half-maximal activity at pH 3.8 and pH 5.2.

RESULTS.

1. Sialic acid content of fibroblast homogenates.

Homogenates of cultured fibroblasts from patient D.F. with mucolipidosis I, and from normal individuals, were separated into supernatant and insoluble residue fractions by centrifugation, and their sialic acid content determined. As shown in Table I, there was a much higher sialic acid concentration in the supernatant fraction of mucolipidosis I cells

TABLE II : Sialic acid content of fibroblast extracts.

Cell line		Nanomoles NANA/mg protein	
		Total	Free
Mucolipidosis I	D.F.	21.2	4.4
		13.5	
		14.o	
		22.2	
Mucolipidosis II	I.T.	68.4	3.3
	F.D.	87.6	
Mucolipidosis III	D.B.	12.5	
	L.	12.4	
Normal controls	mean	3.3	1.7
(n=7)	range	1.7 - 5.6	o.6 - 2.7

All values are the means of duplicate determinations. In mucolipidosis I, the data obtained on four different subcultures of cell line D.F. are represented. "Free" sialic acid was determined on unhydrolyzed samples.

than in the corresponding fraction from normal fibroblasts. In the insoluble residue fraction, however, no such difference was discernible.

In Table II, a comparison is made between the sialic acid content of the supernatant fraction of mucolipidosis I, mucolipidoses II and III, and normal fibroblasts. In mucolipidosis I, the sialic concentration was, on the average, about five times higher than that found in the controls. Approximately 8o% of this sialic acid appeared to be bound to other molecules, as judged from experiments where the sialic acid was determined prior to hydrolysis. In mucolipidosis II,

Table III: α-N-Acetylneuraminidase activity of fibroblast homogenates.

Cell line			mU/mg protein
Mucolipidosis I	D.F. [1]		0 0.009 0
Mucolipidosis II	I.T.		0
	T.Y.		0.023
Mucolipidosis III	D.B.		0.088
Normal controls (n=7)		mean	0.305
		range	(0.277-0.348)

[1] Mean values of duplicate determinations on three different subcultures.

the sialic acid concentration was even higher (about 2o times normal), whereas it was approximately threefold elevated in mucolipidosis III.

2. Neuraminidase activity.

The abnormally high amount of bound sialic acid in mucolipidosis I fibroblasts could be due to a defective catabolism of sialoglycopeptides and/or gangliosides. Therefore, the sialidase activity of mucolipidosis I fibroblast homogenates was assayed at pH 4.4 using neuraminlactose as substrate. As shown in Table III, the fibroblasts of our mucolipidosis I patient exhibited a profound deficiency in the activity of a α-N-acetylneuraminidase. A similar neuraminidase deficiency was observed in mucolipidosis II, whereas it was less pronounced in mucolipidosis III.

From a mixture of normal and mucolipidosis I fibroblast homogenates, the neuraminidase activity representing the cal-

TABLE IV: Effect of mixing normal and mucolipidosis I fibroblast homogenates on α-N-acetylneuraminidase activity.

Homogenate	mU/mg protein	
	Found	Expected
Mucolipidosis I	0	-
Normal control	0.217	-
Mixture	0.117	0.109

The samples were adjusted to the same protein concentration before assay. The mixture consisted of equal quantities of the two homogenates.

culated average was obtained (Table IV), thus making it unlikely that mucolipidosis I fibroblasts contain neuraminidase inhibitors.

DISCUSSION.

Our findings suggest that the fibroblasts of patient D.F. with mucolipidosis I accumulate excessive amounts of sialic acid containing compounds because of a profound deficiency of an "acid" (lysosomal?) α-N-acetylneuraminidase. A similar catabolic defect has been reported recently (9,1o), and confirmed in the present study, for fibroblasts from patients with mucolipidoses II and III. However, whereas the sialidase deficiency in mucolipidoses II and III represents only one of many lysosomal hydrolase deficiencies in such cells, it is the only enzyme defect found in mucolipidosis I fibroblasts.

In view of the similarly deficient neuraminidase activity in mucolipidosis I and II fibroblasts the distinctly higher

level of bound sialic acid in the latter is somewhat surprising. Possibly, the mucolipidosis II fibroblasts are lacking in yet another sialidase not detectable using the neuraminlactose substrate, leading to the additional accumulation of certain sialic acid-containing compounds. A neuraminidase acting on mono- and polysialogangliosides and fetuin, but not on neuraminlactose has been described in rat heart muscle (11).

Although our studies were confined to the analysis of fibroblasts derived from a single patient, they support the hypothesis that the basic metabolic lesion in mucolipidosis I consists of a deficiency of a lysosomal neuraminidase, thus impairing the catabolism of sialic acid-containing glycopeptides and/or glycolipids. Most likely, the enzymatic defect results from the mutation of a gene coding for the expression of this neuraminidase.

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