MUCOPOLYSACCHARIDOSIS III A (SANFILIPPO A DISEASE) : DEFICIENCY OF A HEPARIN SULFAMIDASE IN SKIN FIBROBLASTS AND LEUCOCYTES

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SUMMARY : Cultured skin fibroblasts and peripheral leucocytes from patients with Sanfilippo A disease are strikingly deficient in sulfamidase activity (sulfamatase, EC 3.1.6.?), as measured with heparin - N - $^{35}SO_4$. A partial sulfamidase deficiency was found in the cells of the heterozygote carriers. Since Sanfilippo A fibroblasts have normal sulfate ester hydrolase activities towards oligosaccharides prepared from $^{35}SO_4$-labelled heparan sulfate by nitrous acid treatment, the basic defect in Sanfilippo A disease is considered to be the inactivity of a heparin (heparan sulfate) sulfamidase.

Sanfilippo's syndrome or mucopolysaccharidosis III is an autosomal recessive transmitted disorder of heparan sulfate catabolism, characterized by an increased intralysosomal storage and an excessive urinary excretion of that glycosaminoglycan (1). Although the disorder appears as a distinct clinical entity, the existence of two different genotypes has been elaborated. These biochemically defined subtypes were arbitrarily designated Sanfilippo A and Sanfilippo B disease respectively (2).

As in the other mucopolysaccharidoses the disturbed mucopolysaccharide catabolism of fibroblasts, cultured from the skin of Sanfilippo A patients, can be remedied by adding a specific protein to the culture medium. After 850-fold purification from human urine, this protein - the Sanfilippo A corrective factor - released inorganic sulfate from heparan sulfate extracted from Sanfilippo A cells (3). It was therefore proposed that in Sanfilippo A disease a heparan sulfate sulfatase has been rendered inactive by mutation. However, the linkage of the sulfate bond splitted by the enzyme remained unknown. This paper provides evidence that in Sanfilippo A fibroblasts and leucocytes the activity of a heparin sulfamidase (sulfamatase, EC 3.1.6.?) hydrolyzing N-sulfate bonds shows profound deficiency.

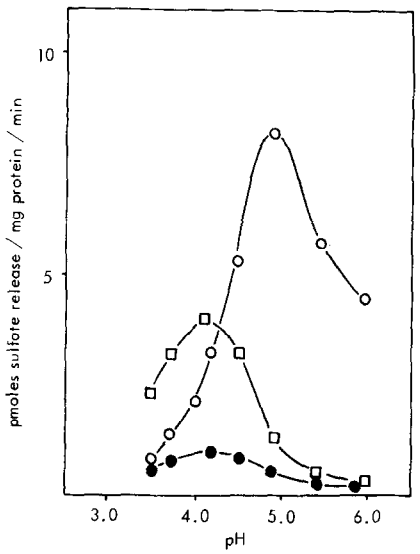

Fig. 1: pH activity curves of heparin sulfamidase from normal (o—o) and Sanfilippo A fibroblasts (●—●), and of heparan sulfate sulfate ester hydrolases from normal fibroblasts (□—□).

Materials and Methods

Materials: Heparin-N-$^{35}SO_4$ (charge 32, specific activity 15 µCi/mg) and $Na_2{}^{35}SO_4$ (carrier free) were purchased from Amersham-Buchler, Biogel P 2 from Bio-Rad, Sephadex G-25 and dextran (molecular weight 250000) from Pharmacia.

Preparation of $^{35}SO_4$-labelled heparan sulfate: Calf aorta tissue segments were incubated in vitro in the presence of $Na_2{}^{35}SO_4$ (0.5 mCi/20 g tissue) as previously described (4). Heparan sulfate was isolated according to l.c. 5, 6. The final heparan sulfate preparation, which was essentially free of other glycosaminoglycans, contained 1.4 µmoles glucosamine, 1.46 µmoles uronic acid , and 1.04 µmoles sulfate (0.63 µmoles glucosamine-N-sulfate) per mg ; the specific radioactivity was 450 000 cpm/mg.

N-sulfate-free heparan sulfate oligosaccharides were prepared by treatment with nitrous acid (7). The degradation products were chromatographed on a Sephadex G-25 column (1.5 x 41 cm), equilibrated with 1.0 N NaCl. The radioactive material appearing in the void volume was pooled and desalted on a Sephadex G-25 column.

Determination of sulfatase activities : Skin fibroblasts were grown in Eagle's minimum

Table I

Sulfatase activity of cultured skin fibroblasts towards $^{35}SO_4$-labelled heparan sulfate

The incubation mixture contained 50 µl cell homogenate, 0.1 M sodium acetate, pH 4.0, 0.02 % sodium azide, and 125 µg heparan sulfate (test I) or 125 µg heparan sulfate obtained after digestion with Sanfilippo A cells (1 mg heparan sulfate, 2 mg cell protein, pH 4.5, 72 hours), dialysis and ethanol precipitation (test II), final volume 110 µl.

	Test I	Test II
	% sulfate release / 0.1 mg cell protein / 8 hours	
Normal	2.5	5.5
Sanfilippo A	0.9	0.3
Sanfilippo B	2.0	7.9

essential medium as previously described (3). The genotypes of different cell lines were determined by cross-correction tests using the same reference cell lines as in Dr. E.F. Neufeld's laboratory (National Institutes of Health, Bethesda, USA).

Fibroblasts grown to confluency in a 75 cm^2 plastic flask were harvested by trypsinization, suspended in 0.5 ml 0.075 N NaCl and subjected to 10 cycles of freezing and thawing.

White blood cells were isolated essentially as described by Moser (8), except that the leucocytes of 10 ml blood were suspended in 0.25 ml 0.15 N NaCl.

The incubation mixture for the determination of heparin sulfamidase activity in fibroblasts and leucocytes (for leucocytes values are given in parenthesis) had the following composition: 50 (50) µl cell homogenate, 0.1 M sodium acetate, pH 5.0, 0.02 % NaN_3, 7 (20) µg heparin-N-$^{35}SO_4$, final volume 65 (150) µl. Boiled enzyme preparations served as controls. Incubation was for 4 (16) hours at 37 °C.

For the determination of heparan sulfate sulfate ester hydrolases (O-sulfate sulfatases) in fibroblasts the incubation mixture contained 50 µl cell homogenate, HNO_2-degraded heparan sulfate (17 nmoles ester sulfate), 0.1 M sodium acetate, pH 4.0, and 0.02 % NaN_3 in a final volume of 65 µl. Incubation was for 7 hours at 37 °C.

At the end of the incubation period an aliquot of the mixture was applied on a paper strip (Schleicher and Schüll, No. 2043a) and subjected to electrophoresis in a barbital - acetate - N-cetylpyridinium chloride (CPC) buffer (0.028 M 5,5-diethylbarbituric acid, sodium salt, 0.028 M sodium acetate, 0.116 M NaCl, and 1 % CPC), pH 8.6 (9). Electrophoresis was carried out at room temperature for 90 min at a gradient of 7.7 volts per cm. The strip was cut in 1-cm segments for counting in a liquid scintillation spectrometer. Undegraded heparin and sulfated oligosaccharides migrated up to 7 cm in the presence of CPC, whereas inorganic sulfate had a mobility of 9 - 10 cm. The sulfamidase activity was calculated on the basis of a heparin-N-sulfate content of 1.46 µmoles/mg. Sulfate was released linearly with time in all assays.

Other methods: Protein was determined by staining with amido black (10). Radioactivity measurements were done as described (3).

Results

$^{35}SO_4$-labelled heparan sulfate from bovine aorta serves as a substrate for heparan sulfate sulfatases when incubated in vitro with fibroblast homogenates. Normal, Sanfilippo A and Sanfilippo B cells liberated inorganic sulfate as judged by the criterion of electrophoresis in CPC (Tab. I), although Sanfilippo A fibroblasts constantly released only about half the amount. After exhaustive digestion of heparan sulfate with Sanfilippo A cell extracts, the partially degraded material was even a better substrate for sulfatases of normal and Sanfilippo B cells, whereas no further sulfate liberation was brought about by Sanfilippo A cells. One of the possible explanations for this observation could be the existence of different heparan sulfate sulfatases.

As possible substrates for N-sulfate and O-sulfate sulfatases heparin-N-$^{35}SO_4$ and O-$^{35}SO_4$-labelled, HNO_2-degraded heparan sulfate were tested. It is shown in Tab. II that fibroblasts of all genotypes tested so far had ester sulfate sulfatase activities towards heparan sulfate. Towards heparin-N-$^{35}SO_4$, however, Sanfilippo A fibroblasts were strikingly inactive with regard to sulfate release (less than 6 % of the mean value of

Table II

Sulfatase activities in cultured skin fibroblasts

Genotype		Heparin-sulfamidase	Heparan sulfate-sulfate ester hydrolases
		pmoles sulfate / mg protein / min.	
Normal:	N.S.	7.75	2.02
Sanfilippo A:	B.B.	0.44	4.36
	T.D.	0.15	3.59
	S.T.	0.57	7.56
	U.Wi.	0.18	5.15
	P.Wo.	0.15	6.90
Sanfilippo B:	A.F.	7.81	3.20
	S.K.	13.2	5.31
	P.S.	8.26	5.11
Hurler:	N.R.	5.65	2.80
Hunter:	McD.	9.28	2.53
	P.E.	12.0	4.64
Maroteaux-Lamy:	R.M.	11.3	4.18
Sanfilippo A Heterozygotes:	H.Wo.	4.81	
	G.Wo.	5.26	

other genotypes). Cells of the obligate heterozygote parents of one Sanfilippo A patient exhibited reduced sulfamidase activities (51 % and 56 % of the mean value).[+]

Beside skin fibroblasts, peripheral leucocytes exhibited measurable sulfamidase activity, whereas serum was inactive. Leucocytes from four Sanfilippo A patients in two families

[+] In control experiments sulfate release was also determined by chromatography on a Biogel P-2 column (1.5 x 29 cm), equilibrated with 0.15 N NaCl. Similar results were obtained.

Table III

Sulfamidase activity in peripheral leucocytes

	Sulfamidase*
V. Family:	
Patient B.V.	0.03
Patient S.V.	0.01
Patient I.V.	0.04
Father H.V.	0.28
Mother M.V.	0.18
W. Family:	
Patient P.W.	0.04
Sister D.W.	0.30
Sister U.W.	0.35
Father H.W.	0.26
Mother G.W.	0.33
Normals $_{+}$ (7) (mean $\pm$ s.d.)	0.54 ± 0.21

* expressed as pmoles sulfate liberated by leucocytes of 10 ml blood per minute

had remarkably diminished sulfamidase activities (less than 10 % of normal controls, Tab. III). In the leucocytes of their heterozygote parents intermediate activities were found.

N-sulfatase and O-sulfatase activities differed in their pH activity curves. Normal fibroblasts released sulfate from N-sulfate bonds optimally near pH 4.9 (Fig. 1). The residual activity of Sanfilippo A fibroblasts was highest at pH 4.2. The enzymatic hydrolysis of sulfate ester bonds had a maximal rate at pH 4.2. It is not clear whether the residual activity in Sanfilippo A cells is partially due to the existence of sulfamidase isoenzymes or due to the action towards some labelled O-sulfate groups, which may

occur in heparin-N-$^{35}SO_4$ in low amounts (about 8 % as determined by HNO_2 degradation).

Discussion

The results described give evidence for a profound sulfamidase deficiency in fibroblasts and leucocytes derived from individuals affected with Sanfilippo A disease. Reduced activities were found in the cells of heterozygote carriers. On the other hand, sulfatase activity towards a heparan sulfate degradation product, which contained exclusively sulfate ester groups, was normal in Sanfilippo A fibroblasts. It seems therefore justified to claim the inactivity of a sulfamidase as the basic defect in Sanfilippo A disease.

Additional evidence would be obtained by the demonstration that the Sanfilippo A corrective factor is identical with a sulfamidase. During three purification steps sulfamidase and corrective activities could not be separated from each other (11).

The glycosaminoglycan which by its faulty degradation is accumulated in Sanfilippo A fibroblasts is heparan sulfate rather than heparin. Since heparin shares typical structural features with heparan sulfate, and both are the only biological macromolecules containing N-sulfate groups, one could assume that the degradative pathway for both substances has common steps. A N-acetyl-α-D-glucosaminidase could be shown to be involved both in the catabolism of heparin and heparan sulfate (12). The same is assumed to hold true for the sulfamidase.

A mammalian heparin sulfamidase has been detected so far only in lymphoid tissues (13). The detection of sulfamidase activity in peripheral leucocytes should be of some clinical significance since it facilitates the genotype-specific diagnosis of Sanfilippo A disease, avoiding the more complicated and time consuming method of fibroblast typing. Further methodological improvements are needed to determine the heterozygote carrier state with certainity, which would be helpful for genetic counseling.

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