Ganglioside GM3 sialidase activity in fibroblasts of normal individuals and of patients with sialidosis and mucopolipidosis IV

Subcellular distribution and some properties

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Sensitive assays for the determination of the ganglioside sialidase activity of fibroblast homogenates were established using ganglioside GM3, ³H-labelled in the sphingosine moiety, as a substrate. Ganglioside GM3 sialidase activity was greatly stimulated by the presence of the non-ionic detergent Triton X-100 and was further enhanced by salts such as NaCl; the optimal pH was 4.5. The subcellular localization of this activity was determined by fractionation using free-flow electrophoresis and found to be exclusively associated with the marker for the plasma membrane, but not with that for lysosomes. This Triton-stimulated ganglioside sialidase activity was selectively inhibited by preincubating intact cells in the presence of millimolar concentrations of Cu²⁺, suggesting that the activity resides on the external surface of the plasma membrane. In normal fibroblast homogenates, ganglioside GM3 sialidase was also greatly stimulated by sodium cholate. In contrast to the Triton X-100-activated reaction, however, it was not diminished by prior incubation of intact cells in the presence of Cu²⁺. Only after cell lysis was Cu²⁺ inhibitory. The cholate-stimulated ganglioside sialidase activity thus paralleled the behaviour of the lysosomal 4-methylumbelliferyl- α -D-N-acetylneuraminic acid (4-MU-NeuAc) sialidase. In fibroblasts from sialidosis patients, the cholate-stimulated ganglioside GM3 sialidase activity, but not that of the Triton-activated enzyme, was profoundly diminished. In fibroblasts from patients with mucopolipidosis IV (ML IV), both the Triton X-100- and the cholate-stimulated ganglioside GM3 sialidase activities were in the range of normal controls. The Triton-activated enzyme was associated with the plasma membrane in the same manner as in normal cells. Our findings suggest that, in human fibroblasts, there exist two sialidases that degrade ganglioside GM3: one on the external surface of the plasma membrane, and another that is localized in lysosomes and seems identical with the activity that acts on sialyloligosaccharides and 4-MU-NeuAc. As neither activity was found to be deficient in ML IV fibroblasts, our results argue against the hypothesis of a primary involvement of a ganglioside GM3 sialidase in the pathogenesis of ML IV.

INTRODUCTION

In cultured human fibroblasts there are at least two genetically distinct sialidase activities: one that is active towards oligosaccharides, glycoproteins and the synthetic substrate 4-methylumbelliferyl- α -D-N-acetylneuraminic acid (4-MU-NeuAc) and which is genetically deficient in the lysosomal storage disorder sialidosis, and a different activity that cleaves terminal sialic acid residues from gangliosides and is stimulated by detergents such as Triton X-100 (Cantz & Messer, 1979; Caimi *et al.*, 1979; O'Brien & Warner, 1980; Cantz, 1982). A partial or profound deficiency of the ganglioside sialidase has been described in fibroblasts from patients with another lysosomal storage disease known as mucopolipidosis IV (ML IV) (Bach *et al.*, 1979; Caimi *et al.*, 1982; Ben-Yoseph *et al.*, 1982). Studies on the subcellular distribution showed the oligosaccharide-specific activity to reside in lysosomes (Cantz *et al.*, 1981; Zeigler & Bach, 1981). The sialidase activity towards ganglioside substrates, on the

other hand, appeared to be associated with the plasma membrane (Zeigler & Bach, 1981). This was unexpected because biochemical and electron-microscopic studies of ML IV fibroblasts and tissues suggested an abnormal accumulation of gangliosides and other complex carbohydrates within the lysosomes (Berman *et al.*, 1974; Bach *et al.*, 1975, 1977; Crandall *et al.*, 1982).

To fully understand the catabolism of gangliosides in cultured human fibroblasts, a detailed knowledge of the properties and topology of the relevant sialidase(s) is essential. Previous investigations on the subcellular distribution of ganglioside sialidase activity were hampered by a lack of specificity and sensitivity of the assay procedure, and it was therefore not clear if a less prominent activity of some other cellular compartment remained undetected. In this study we used sensitive radiometric assays for the determination and characterization of ganglioside GM3 sialidase activity and investigated its subcellular localization employing fractionation

Abbreviations used: ML IV, mucopolipidosis IV; ganglioside GM3, NeuAc(α 2-3)Gal(β 1-4)Glc(β 1-1)-ceramide; 4-MU-NeuAc, 4-methylumbelliferyl- α -D-N-acetylneuraminic acid; sialidase (neuraminidase), N-acetylneuraminyl hydrolase (EC 3.2.1.18)

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by free-flow electrophoresis. Our findings show that human fibroblasts contain two ganglioside GM3 sialidases that can be differentiated by their detergent requirements, their subcellular distribution, their sensitivity to inhibitors and by the study of genetic deficiency diseases.

Parts of this work have been published in preliminary form (Lieser *et al.*, 1986; Lieser & Cantz, 1988).

MATERIALS AND METHODS

Reagents

Ganglioside GM3, purified from human spleen, was kindly provided by Dr. Günter Schwarzmann, Department of Organic Chemistry and Biochemistry Bonn University, F.R.G. It was ^3H -labelled in the ceramide moiety by the borohydride reduction method (Schwarzmann, 1978) and had a specific radioactivity of 305 Ci/mol. 4-MU-NeuAc was purchased from Koch-Light, Colnbrook, U.K. Triton X-100 (pure) and sodium cholate (analytical grade) were obtained from Serva, Heidelberg, Germany; DEAE-Sephacrose CL-6B was from Pharmacia, Freiburg, Germany and Instagel was from Packard. All other reagents were of analytical grade.

Cell culture

Human skin fibroblasts from patients and normal controls were maintained in Eagle's minimum essential medium (Serva, Heidelberg, Germany) supplemented with 10% fetal calf serum (Boehringer, Mannheim, Germany), non-essential amino acids and antibiotics, as described previously (Cantz *et al.*, 1972).

Enzyme assays

Fibroblasts were harvested by trypsinization (Cantz *et al.*, 1972) and homogenized in distilled water by ten strokes of a Teflon-glass homogenizer. Protein was determined according to Peterson (1977).

Sialidase activity towards gangliosides was determined in incubations containing 250 pmol of [^3H]GM3, 5 μmol of sodium acetate buffer, pH 4.5, 3.75 μmol of NaCl, 20 μg of Triton X-100, and 10–20 μg of homogenate protein in a total volume of 50 μl . Ganglioside GM3 sialidase activity was also determined in presence of the detergent sodium cholate by mixing 10 μl of a 1% (w/v) solution with 10 μl of 0.5 M-sodium acetate, pH 4.4, yielding a fluffy precipitate. Homogenate protein (10–60 μg) and 200 pmol of [^3H]GM3 ganglioside were then added to give a final volume of 50 μl .

After incubation for 60 min at 37 °C, the reactions were stopped by the addition of 900 μl of methanol. The product, lactosyl- ^3H ceramide, was separated from undegraded substrate by ion-exchange chromatography on small columns (0.5 cm $\times$ 2 cm) of DEAE-Sephacrose CL-6B equilibrated with methanol. After washing with 3 ml of methanol, lactosyl- ^3H ceramide was eluted with 4 ml of methanol/1 M-sodium acetate (9:1, v/v). The radioactivity was determined by adding 8 ml of Instagel and counting in a Searle Mark III liquid scintillation counter.

Sialidase activity towards the synthetic substrate 4-MU-NeuAc was determined as described earlier (Harzer *et al.*, 1986).

Subcellular fractionation by free-flow electrophoresis

Fibroblasts were grown in 600 cm² culture dishes (Nunc, Wiesbaden, Germany). After 30–40 days of subculture, the cells of 2–3 dishes were collected by

scrapping in phosphate-buffered saline (137 mM-NaCl/2.7 mM - KCl / 5.4 mM - Na₂HPO₄ / 1.5 mM - KH₂PO₄, pH 7.2 (without Ca²⁺ and Mg²⁺) and centrifugation for 10 min at 500 g. A mitochondrial-lysosomal fraction was prepared by differential centrifugation, and subcellular particles were fractionated by electrophoresis in a free-flow apparatus, as described previously (Harms *et al.*, 1980). In the fractions, protein concentration, as well as the marker enzymes β -N-acetylhexosaminidase, alkaline phosphodiesterase I, succinate-INT [2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrafolium chloride] reductase and galactosyltransferase, were determined according to previously published procedures (Harms *et al.*, 1980).

Inhibition studies with intact cells

Cells were trypsinized, washed twice with 0.9% (w/v) NaCl and resuspended in 0.25 M-sucrose/0.01 M-triethanolamine/HCl buffer, pH 7.4. To the suspension, CuSO₄ solution was added to a final concentration of 0.02 M and the mixture was incubated at 37 °C for 20 min. The cells were then collected by centrifugation, washed three times with 0.9% NaCl, homogenized and assayed for sialidase activity.

RESULTS

Optimized conditions for the determination of sialidase activity towards ganglioside GM3 were established in homogenates of normal fibroblasts. The activity was greatly stimulated by the presence of the non-ionic detergent Triton X-100. This stimulation was however not only dependent on the Triton X-100 concentration but was also influenced by the concentration of the homogenate protein; higher concentrations of the latter requiring higher concentrations of detergent. Beyond a certain detergent-to-protein ratio, however, enzyme activity decreased, presumably due to inactivation (Fig. 1). Because of these considerations, the homogenate protein concentration in subsequent experiments was generally

Fig. 1. Dependence of ganglioside GM3 sialidase activity on the detergent and homogenate protein concentrations

Homogenates with final protein concentrations of 0.04 mg/ml (○), 0.1 mg/ml (▲), 0.2 mg/ml (□), 0.4 mg/ml (●), and 0.6 mg/ml (■) were incubated in the presence of substrate and the Triton X-100 concentrations indicated, as described in the Materials and methods section.

Fig. 2. Subcellular distribution of the Triton X-100-stimulated ganglioside sialidase activity

A lysosomal-mitochondrial fraction obtained by differential centrifugation and containing 1.3–2.1 mg of protein was fractionated by free-flow electrophoresis as described under Materials and methods. (a) Normal control fibroblast line, (b) ML IV fibroblast line. $\square$, Ganglioside GM3 sialidase; $\bullet$, alkaline phosphodiesterase I; $\blacktriangle$, β -N-acetylhexosaminidase; $\triangle$, INT reductase; $\blacksquare$, galactosyltransferase; $\circ$, protein.

adjusted to 0.4 mg/ml, and the concentration of Triton X-100 was fixed at 0.04% (w/v) for maximal activation. Under these conditions, the activity was linear with time for incubation periods up to 90 min (results not shown). The specific activity was stimulated by salts such as NaCl, KCl, NaH_2PO_4 , and Na_2SO_4 , 75 mM-NaCl having a maximal effect of about three times the control value. Variation of the proton concentration in the reaction mixture, while maintaining an optimal ionic strength of 110 mM by the use of NaCl, yielded a maximum at pH 4.5, with half-maximal activities at pHs around 4.0 and 5.0.

The subcellular distribution of the Triton X-100-stimulated ganglioside sialidase was studied by subjecting a lysosomal-mitochondrial fraction from fibroblasts of normal individuals and ML IV patients to fractionation by free-flow electrophoresis. Fig. 2 shows a representative experiment. In both the normal and the ML IV cells, the Triton-stimulated ganglioside GM3 sialidase activity co-distributed with the plasma membrane marker alkaline phosphodiesterase I and was clearly distinct from the distribution of the lysosomal marker β -N-acetylhexosaminidase and the markers for mitochondria (INT reductase) and Golgi (galactosyl transferase). The small amounts of ganglioside sialidase activity present in the lysosomal fractions essentially paralleled that of the alkaline phosphodiesterase I, indicating that it represented a contamination with plasma membrane. This interpretation was confirmed when the ratio of sialidase-to-phosphodiesterase activities in pooled and concentrated lysosomal fractions was determined and found to be identical with that in fractions containing the bulk of the plasma membrane. The recovery of the ganglioside GM3 sialidase activity in the fractions was about 90%, ruling out any significant inactivation during fractionation.

The observed plasma membrane localization of the Triton-stimulated ganglioside GM3 sialidase led us to probe its sensitivity to exogenous agents by incubating suspensions of intact fibroblasts under iso-osmotic conditions prior to homogenization and activity assays. Where-

as treatment with 0.4 and 0.8% (w/v) trypsin at 37 °C for up to 60 min was without effect, there was a drastic decrease in the ganglioside sialidase activity after treatment of the cells with CuSO_4 , as shown in Fig. 3. The ganglioside sialidase was similarly inhibited when the preincubation step was done at 0 °C, when there was no endocytosis (results not shown). On the other hand, such inhibition was not observed in case of the sialidase acting on the water-soluble substrate 4-MU-NeuAc (Fig. 3). As CuSO_4 was strongly inhibitory to 4-MU-NeuAc sialidase in the homogenate (results not shown), this was taken as evidence that 4-MU-NeuAc sialidase was localized intracellularly, most likely in lysosomes.

In normal fibroblast homogenates, ganglioside GM3 sialidase activity was also found to be greatly stimulated by sodium cholate, a maximal effect being reached at a

Fig. 3. Effect of Cu^{2+} -pretreatment of intact fibroblasts on Triton X-100-stimulated ganglioside and 4-MU-NeuAc sialidases.

Experimental details are given in the Materials and methods section. $\square$, $\blacksquare$, 4-MU-NeuAc sialidase incubated with 5 and 40 mM- CuSO_4 , respectively; $\circ$, $\bullet$, ganglioside sialidase incubated with 5 and 40 mM- CuSO_4 , respectively.

Table 1. Sialidase activities of fibroblast homogenates

Assay system ...		Specific activity			
		[³ H]GM3/ Triton X-100 (pmol/min per mg of protein)	[³ H]GM3/ cholate (Cu ²⁺ treated cells) (pmol/min per mg of protein)	4-MU-NeuAc (nmol/min per mg of protein)	
Cell line					
Normal controls		Mean Range	21.6 (<i>n</i> = 10) 14–32	101 (<i>n</i> = 2) 81–121	3.33 (<i>n</i> = 17) 1.62–4.75
ML IV	S		26	93	2.52
	L		30	106	3.53
	F		22	104	3.27
	V		15	92	
	R		28	103	
	G		28		
Sialidosis	F		13	1	0.02
	M		11	2	0.04
	G		11	6	0.14
ML II	D		2	0	0

Fig. 4. Stimulation of ganglioside GM3 sialidase activity by sodium cholate

Homogenates of normal (○), sialidosis (□), copper-treated normal (●), copper-treated sialidosis (■) and heat-inactivated (95 °C, 30 min) normal (▲) fibroblasts were incubated in the presence of varying concentrations of sodium cholate as described in the Materials and methods section.

concentration of 0.2% (w/v) (Fig. 4). Unlike the Triton X-100-activated reaction, however, this activity was not diminished upon prior treatment of the cells with copper; rather, the specific activity was somewhat increased. In homogenates of fibroblasts from sialidosis cells, there was only a little cholate-stimulated ganglioside GM3 sialidase activity; this residual activity was completely abolished by copper treatment (Fig. 4). Mixing normal and sialidosis fibroblast homogenates yielded the expected intermediate activities, ruling out the presence of soluble inhibitors in sialidosis fibroblasts (results not shown). The results therefore suggest that most of the

cholate-stimulated ganglioside sialidase activity is of lysosomal origin, and that preincubation of whole cells with Cu²⁺ leads to inhibition of any non-lysosomal ganglioside sialidase.

A comparison of the various sialidase specific activities in fibroblasts from patients with ML IV, sialidosis, mucopolipidosis II and from normal controls is presented in Table 1. The Triton X-100-stimulated ganglioside GM3 sialidase activities of all six ML IV lines were in the range of normal controls, whereas the sialidosis lines exhibited values of 51–60%, and a ML II line one of 9% [ML II is a disorder of lysosomal enzyme targeting resulting in a multiple hydrolase deficiency (Neufeld & McKusick, 1983)]. When the ML IV cells were assayed under conditions of higher substrate concentration according to Caimi *et al.*, (1982), no enzyme deficiency was observed either (results not shown). The cholate-stimulated ganglioside GM3 sialidase activity was determined after prior copper treatment of intact cells and is shown in the second column of Table 1. Again, all ML IV lines tested exhibited specific activities in the normal range. In the three sialidosis patients and a ML II line, on the other hand, a complete-to-nearly-complete deficiency of this activity was observed. These results were mirrored by the findings towards the synthetic substrate 4-MU-NeuAc (final column, Table 1), showing normal activities in the ML IV lines tested and, as expected, deficient activities in sialidosis and ML II.

DISCUSSION

By employing sensitive methods for the determination of ganglioside sialidase activity in combination with gentle and fast subcellular fractionation by free-flow electrophoresis, we were able to show that human fibroblasts exhibit two different activities towards ganglioside GM3.

The ganglioside GM3 sialidase activity stimulated by the non-ionic detergent Triton X-100 was found to reside exclusively in the plasma membrane as it co-distributed

with the marker enzyme alkaline phosphodiesterase I in the subcellular fractionation experiments. A very small contribution to the lysosomal fraction is ascribed to contamination with plasma membrane, which may be a systematic feature of lysosomes (Draye *et al.*, 1987). As there was good recovery of the ganglioside sialidase activity during fractionation, it is unlikely that any other such activity remained undetected because of inactivation or loss.

This ganglioside sialidase activity was completely inhibited upon incubation of intact cells in the presence of Cu^{2+} , conditions that did not affect lysosomal 4-MU-NeuAc sialidase activity. As the inhibition was also observed at low temperatures where the cell membrane was 'frozen' and there was no endocytosis, it was likely that Cu^{2+} did not get inside the cell and the inhibitor thus reacted with an enzyme that was situated on the external surface of the membrane. Our results therefore confirm and extend other observations of a plasma membrane localization of the Triton-stimulated ganglioside sialidase of human fibroblasts (Zeigler & Bach, 1981; Chigorno *et al.*, 1986; Usuki *et al.*, 1988).

Ganglioside GM3 sialidase activity was also observed in normal control fibroblasts when sodium cholate was used as a detergent. This activity, however, was different from the Triton X-100-stimulated enzyme in that it was not inhibited upon prior incubation of intact cells with CuSO_4 . Only after cell lysis was Cu^{2+} again inhibitory. The cholate-stimulated ganglioside GM3 sialidase activity thus paralleled the behaviour of the 4-MU-NeuAc sialidase activity, a known lysosomal hydrolase (Verheijen *et al.*, 1983, 1987). Moreover, most of the ganglioside sialidase activated by cholate was deficient in sialidosis fibroblasts. Our findings therefore suggest that there is a cholate-stimulated ganglioside GM3 sialidase in human fibroblasts that represents a genuine lysosomal activity and which is a property of the sialidase that is deficient in the sialidosis disorder. This interpretation is supported by the recent observation of an abnormal accumulation of gangliosides with terminal sialic acid in autopsy tissues of a sialidosis patient (Ulrich-Bott *et al.*, 1987), and by the demonstration that lysosomal sialidases purified from human liver or placenta released sialic acid from gangliosides in the presence of detergents (Michalski *et al.*, 1986; Hiraiwa *et al.*, 1988).

It therefore appears likely that in human fibroblasts there exist two sialidases which degrade gangliosides: one that is situated on the external surface of the plasma membrane and which may be involved in intra-membranous sialic acid cleavage, and another enzyme that is localized in the lysosomes and which seems identical with the activity that acts on sialyloligosaccharides. The presence of both lysosomal and plasma membrane ganglioside sialidase activities, the latter acting on endogenous membrane sialolipids as well as exogenous substrates, has been described in herpes simplex virus-transformed hamster fibroblasts (Schengrund *et al.*, 1973, 1976).

Our present results are of relevance also for the discussion of the pathogenesis of the lysosomal storage disease ML IV. Upon subcellular fractionation by free-flow electrophoresis, the Triton-stimulated ganglioside GM3 sialidase activity of ML IV fibroblasts showed essentially the same plasma membrane localization as in normal cells. Although the subcellular distribution of the lysosomal and Golgi markers in the normal and ML IV

cells appeared somewhat different, it is not clear if such differences are disease-related, as there was some variation between experiments, perhaps due to particle aggregation under the conditions of low ionic strength. The specific activities of the Triton X-100-stimulated ganglioside GM3 sialidase, the cholate-activated ganglioside sialidase and the 4-MU-NeuAc sialidase were all in the range of normal controls. We could thus not confirm earlier reports of a partial-to-profound deficiency of ganglioside sialidase activity in ML IV fibroblasts (Bach *et al.*, 1979; Caimi *et al.*, 1982; Ben-Yoseph *et al.*, 1982). As ML IV fibroblasts show an abnormal accumulation of sulphate-labelled mucopolysaccharides (Bach *et al.*, 1977) and such sulphated glycosaminoglycans are now known to be potent inhibitors of the Triton-stimulated ganglioside sialidase of fibroblasts (Baumkötter & Cantz, 1983), we consider it possible that in a homogenate such inhibition was the cause of the abnormally low activities observed in the previous investigations. Under our much more sensitive assay conditions, such inhibitors are presumably sufficiently diluted to result in normal activities, with the probable exception of an ML II cell line that showed a significantly decreased activity. Earlier findings of an altered microdistribution of the ganglioside sialidase in the plasma membrane of ML IV fibroblasts in conjunction with decreased solubilization properties (Zeigler & Bach, 1985) may rather point to a defect in membrane biochemistry. Altogether, our present findings argue strongly against the concept that a deficiency of the ganglioside sialidase of the plasma membrane is the primary genetic defect in ML IV.

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