DEFICIENCY OF GLUCOSYLSPHINGOSINE: β-GLUCOSIDASE IN GAUCHER DISEASE

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SUMMARY

A deficiency in the activity of glucosylsphingosine: β-glucosidase has been observed in Gaucher's spleen tissue and skin fibroblasts. Preliminary studies indicated the presence of a material similar to glucosylsphingosine in Gaucher's spleen while such a material was not detectable in normal and other pathological control spleen tissue. This is the first report of the natural occurrence of a psychosine-like material in a mammalian tissue.

Gaucher's disease is an autosomal recessive disorder characterized by the accumulation of glucosylceramide in the reticulo-endothelial system. Brady et al. (1,2) demonstrated the deficiency in this disease of glucosylceramide: β-glucosidase employing chemically synthesized glucosyl-1-^{14}C-ceramide as the substrate. This observation was confirmed by Patrick (3) using unlabelled glucocerebroside in studies which also reported that β-glucosidase activity was similarly decreased in Gaucher's spleen tissue when assayed with the non-specific p-nitrophenyl-β-D-glucopyranoside as substrate. Ockerman (4) demonstrated the lack of acid β-glucosidase activity in liver and spleen from patients with Gaucher's disease using 4-methlumbelliferyl-β-D-glucopyranoside as the substrate. Subsequently, this fluorogenic substrate has been employed to detect homozygous and heterozygous carriers of the adult type of Gaucher's disease using leukocytes (5,6) and cultured skin fibroblasts (7,8,9).

In Krabbe's leukodystrophy, a disease characterized by the absence of galactosylceramide: β-galactosidase, Miyatake and Suzuki (10) reported an additional deficiency of galactosylsphingosine (psychosine): β-galactosidase activity. A deficiency in the activity of glucosylsphingosine: β-glucosidase in Gaucher's tissue and its presence in normal and pathological controls has been observed. Preliminary chromatographic and colorimetric evidence is presented for the presence of a material similar to glucosylsphingosine in Gaucher's spleen. This is the first report of the presence of a psychosine-like material in a mammalian tissue.

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METHODS

Glucose-1-^{14}C cerebroside was prepared as previously described (11) and converted to glucose-1-^{14}C sphingosine by alkaline degradation according to the procedure of Taketomi and Yamakawa (12). The alkaline hydrolysate, which was in 90% n-butanol, was repeatedly extracted with water until the aqueous phase was neutral to pH paper. The butanol phase was dried in vacuo in a flash evaporator, the residue was dissolved in 20 volumes of chloroform-methanol (C:M 2:1) and was extracted with 0.2 volumes of 0.1N HCl. The lower chloroform phase was then extracted with 0.4 volumes of methanol: 0.1N aq. HCl (1:1). The combined aqueous phases were made alkaline with sodium hydroxide and extracted with equal volumes of chloroform-methanol-water (86:14:1). The lower chloroform phase was dried in vacuo to obtain a crude product, which was purified by silicic acid column chromatography. Approximately 10 mg of glucosyl-^{14}C sphingosine was applied to a 5 g Unisil (Clarkson Chemical Co., Pennsylvania) column of 1 cm diameter, and eluted with 200 ml of 10% methanol in chloroform followed by 100 ml of 20% methanol in chloroform. The glucosyl-^{14}C sphingosine was recovered in the latter fraction and was further purified by preparative thin-layer chromatography (TLC) on Silica Gel G plates with chloroform-methanol-20% ammonia (14:5:1 v/v) as solvent system. The area corresponding to glucosylsphingosine standard was scraped, packed onto a column and eluted with 100 ml of the developing solvent system. The radioactive glucosylsphingosine obtained in this manner gave a single radioactive spot in three different solvent systems described by Nussbaum and Mandel (13). The final yield of glucosyl-^{14}C-sphingosine was about 35% of the starting material. Glucosyl-1-^{14}C-sphingosine was also synthesized according to the procedure of Shapiro, Rachaman and Sheradsky (14).

The assay employed for the glucosylsphingosine: β-glucosidase activity is similar to the procedure employed by Miyatake and Suzuki for galactosylsphingosine: β-galactosidase (15). The incubation mixture in final volume of 1 ml contained 100 nmoles of glucosyl-1-^{14}C-sphingosine (specific activity 540 cpm/nmole), 0.1 ml of 1M acetate buffer, pH 4.5, and various aliquots of either 20% spleen homogenate prepared in distilled water (2-6 mg protein) or skin fibroblasts grown in culture (0.2 - 1.0 mg protein) as the enzyme source. Control tubes contained boiled enzyme instead of the active enzyme source. The tubes were incubated at 37° for 3 hr in a shaking water bath, and the reaction terminated by the addition of 5 ml chloroform-methanol (2:1). The mixture was made alkaline with 0.1 ml concentrated ammonia after which 0.1 ml of unlabelled glucose solution (1 mg/ml) was added as a carrier. The samples were mixed well, centrifuged and lower phase removed with a Pasteur pipette. The upper phase was washed twice with 2 ml chloroform. One ml of the washed upper phase was transferred to a counting vial and dried in an oven at 110°. The dried residue was dissolved in 1 ml of water, and counted in 15 ml Aquasol (New England Nuclear Corp.) in a Tri-Carb liquid scintillation spectrometer, Packard Model 3380. The sample counts were corrected for counts of the control tubes.

The incubation mixtures for quantitating hexosaminidase activity consisted of 0.5 µmoles of 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucoside, 10 µmoles of citrate-phosphate buffer, pH 4.5, 1.0 mg of hexosaminidase-free bovine serum albumin, 25 µmoles of sodium chloride, and 1-5 µg protein in a total volume of 0.2 ml. The samples were incubated for 30 min at 37° in a shaking water bath. The 4-methylumbelliferone liberated in the reaction was quantitated fluorometrically as previously described (16).

Protein was determined in tissue samples according to the procedure of Itzhaki and Gill (17). Spleen tissue from Gaucher, metachromatic leukodystrophy (MLD) diseases and normal patients were generously provided by Dr. Hugo Moser and Tay-Sachs spleen tissue was provided by Dr. Edwin Kolodny. Niemann-Pick spleen tissues were obtained from Dr. Howard Sloane and Dr. George Rouser. Human skin fibroblasts were kindly supplied by Dr. Aubrey Milunsky.

TABLE 1: Glucosyl sphingosine β-glucosidase and β-N-acetyl hexosaminidase activities in human spleen tissue samples.

Condition	Patient	Age	Sex	Glucosyl sphingosine β:glucosidase nmoles cleaved/mg protein/3 hr.	β-N-acetyl-hexosaminidase nmoles of product formed/mg/ protein/30 min.
Gaucher	H.	60 y.	M	0.20	1931
Infantile Gaucher	S.F.		F	0.03	1492
Gaucher	C.K.		F	0.06	3320
Gaucher	J.C.	43 y.	F	0.11	2688
Infantile Gaucher	J.P.	23 mo.	M	0.0	3388
			average	.08	average 2564
Congenital heart disease	R.D.	6 days	M	1.28	1162
Heart disease	V.S.	73 y.	M	1.30	646
Niemann-Pick	D.D.	9 y.	F	0.58	1432
Niemann-Pick	L.B.	11 y.	F	0.56	1492
			average	0.93	average 1183

Detection of glycosylsphingosine in spleen tissue

The following extraction procedure was employed in order to detect the possible presence of glucosylsphingosine in the human spleen tissue.

Lyophilized material from 0.5 g wet tissue

(1) Disperse in 1 ml water in bath type sonicator
(2) Add 10 ml CH_3OH and incubate at 50° for 15 min
(3) Add 20 ml $CHCl_3$ Mix, let stand and filter

chloroform-methanol (2:1) extract

wash consecutively with:
(i) 6 ml 0.1M KCl in .05M NaOH
(ii) 12 ml C:M:0.1M KCl in .05M NaOH (3:48:47)
(iii) 12 ml C:M:.05M NaOH (3:48:47) discarding each upper phase

Lower chloroform phase

(1) Add 10 ml CH_3OH + 1 ml glacial acetic acid and mix
(2) wash consecutively with:
(i) 6 ml 0.1M KCl + 0.5 ml 1N H_2SO_4
(ii) 12 ml C:M:0.1M KCl (3:48:47) + 0.5 ml 1N H_2SO_4
(iii) 12 ml C:M:H_2O (3:48:47) + 0.5 ml 1N H_2SO_4

Pooled aqueous methanol upper phase

(1) Adjust to pH 9.0 with 10 N NaOH
(2) Extract twice with equal volumes of chloroform-methanol-water (86:14:1)

Combined lower chloroform phase -

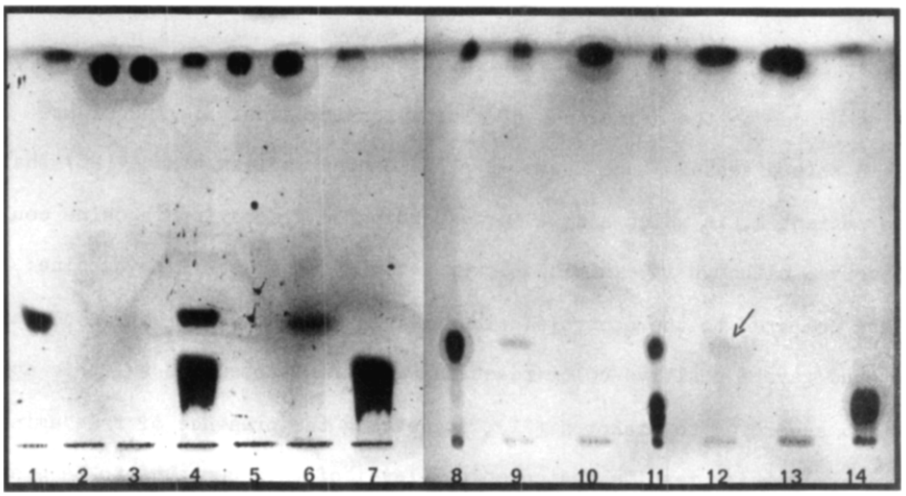
Evaporate to dryness to yield crude glucosylsphingosine type compound. This product in 10% methanol in chloroform was applied to a silicic acid (Unisil) column and the "glucosylsphingosine" fraction was obtained as described earlier for the preparation of glucosyl-l-^{14}C-sphingosine. This material was then subjected to TLC on borate impregnated Silica Gel G plates with C:M:20% ammonia (14:5:1) as the solvent system.

RESULTS

The results of the assays for both glucosylsphingosine: β-glucosidase and hexosaminidase in human spleen tissue samples are shown in Table 1. It is apparent that the activity of glucosylsphingosine: β-glucosidase is markedly diminished in Gaucher's disease samples when compared to that obtained in the normal controls and in another sphingolipidosis, Niemann-Pick disease. The β-N-acetylhexosaminidase activity measured in the Gaucher's spleen were often much higher than the controls suggesting that the acid hydrolase activities of these tissues have not appreciably deteriorated during storage. The deficiency observed in Gaucher's spleen seems specific for the disease since the activity obtained in Niemann-Picks was the same

TABLE 2 Glucosyl sphingosine β-glucosidase and β-N-acetyl hexosaminidase activities in cultured skin fibroblasts

Cell Line	Identity	Sex	nmoles glucosyl sphingosine hydroly./mg prot./3 hr.		nmoles 4-MU-β-N-acetyl-glucosamide hydroly./mg prot./hr.	
446	Normal	F		4.8		3477
498	Normal	M		4.6		5166
295	Normal	M		4.6		4743
497	Normal	M	average	4.8 / 4.7	average	7468 / 5213
502	Gaucher	M		0.94		3213
299	Gaucher	F		0.78		4782
501	Gaucher	F		1.19		4004
119a	Gaucher	F	average	1.21 / 1.03	average	3962 / 3990



TLC on borate impregnated Silica Gel G plate with chloroform-methanol-con. NH_3 (14:5:1 v/v) as the solvent system. The material applied on the plate was extracted from human spleen tissue and purified on silicic acid column as described in the methods section. Lane 1 = Std. glucosyl sphingosine; Lane 2 = Material from atypical Gaucher spleen (ref 20); Lane 3 = Material from normal spleen; Lane 4 = Mixture of std. glucosyl sphingosine and galactosyl sphingosine; Lane 5 = Material from normal spleen; Lane 6 = Material from Gaucher spleen; Lane 7 = Std. galactosyl sphingosine; Lane 8 = Std. glucosyl sphingosine; Lane 9 = Material from Gaucher spleen; Lane 10 = Material from MLD spleen; Lane 11 = Mixture of std. glucosyl sphingosine and galactosyl sphingosine; Lane 12 = Material from Gaucher spleen; Lane 13 = Material from Tay-Sachs spleen; Lane 14 = Std. galactosyl sphingosine. The spots were developed with 50% H_2SO_4-CH_3OH.

as that obtained with controls. Mixing experiments with Gaucher's and normal homogenates failed to indicate either the presence of inhibitors in Gaucher tissue samples or the activation of normals (18,19).

The hydrolysis of glucosylsphingosine is diminished in Gaucher's fibroblasts compared to the normals while the hexosaminidase activity is similar in both Gaucher and normal controls (Table 2). Mixing experiments again failed to demonstrate either the presence of inhibitors in Gaucher cell lines or activation in normal cell lines.

The presence of a material from Gaucher's spleen samples which cochromatographs with glucosylsphingosine on TLC is shown in Fig. 1. A material comigrating with glucosylsphingosine standard could be clearly visualized in Gaucher's tissues. A similar material was absent from normal, metachromatic leukodystrophy and Tay-Sachs controls. This TLC method clearly distinguishes glucosylsphingosine from

galactosylsphingosine. The presence of this material in Gaucher's tissue would be anticipated due to the deficiency of glucosylsphingosine: β-glucosidase observed in this disease. Lane 2 was atypical Gaucher spleen tissue (20) that came from patient H, in which a spot corresponding to glucosylsphingosine could not be observed although it exhibited lower activity of glucosylsphingosine: β - glucosidase compared to the controls. The silicic acid column fraction containing this compound gave a positive color reaction with trinitrobenzene sulfonic acid when assayed according to Siakotos (21), suggesting the presence of free amino group(s). Preliminary results indicated the level of this compound to be elevated in Gaucher's spleen compared to normal non-Gaucher controls.

DISCUSSION

These results demonstrate a deficiency of glucosylsphingosine: β-glucosidase in Gaucher spleen tissue and fibroblasts. This is in addition to the well-documented deficiencies of both glucosylceramide: β-glucosidase and generalized acid β-glucosidase (1-9). This finding is analogous to that reported by Miyatake and Suzuki in Krabbe's leukodystrophy (10) in which they observed the deficiencies of both galactosyl sphingosine: β -galactosidase and galactosylceramide: β - galactosidase. It is not yet known whether the same enzyme protein acts as a glucosylsphingosine: β-glucosidase, glucosylceramide: β-glucosidase and acid β-glucosidase. The suggested presence of a glucosylsphingosine-like material in Gaucher spleen in concurrence with a decrease in the hydrolysis of this compound suggests that this glycolipid may be a naturally occurring compound in mammalian tissue. It is premature to conclude that this material is identical to glucosylsphingosine until complete chemical characterization is provided. The enzymatic deacylation of glucosylceramide has not been demonstrated nor is there experimental evidence suggesting glucosylsphingosine as a glucosylceramide precursor. The in vitro synthesis of glucosylsphingosine from UDP-glucose and sphingosine has been reported (21).

It is tempting to speculate that in Niemann-Pick disease there is a decreased ability to hydrolyze sphingosylphosphorylcholine. This disease is characterized

by the accumulation of sphingomyelin and a deficiency of ceramide phosphoryl-choline:cholinephosphorylhydrolase (22).

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