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G_{M1} gangliosidosis
gangliosides

β-Galactosidase in Tissue Culture Derived from Human Skin and Bone Marrow: Enzyme Defect in G_{M1} Gangliosidosis

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Extract

β-Galactosidase activities were determined in fibroblast preparations derived from the bone marrow and skin of a patient with G_{M1} gangliosidosis and from 11 control subjects. Two β-galactosidase substrates were employed in these studies: ganglioside G_{M1}, labeled with tritium in the terminal galactose by a new procedure, and *o*-nitrophenyl-β-galactopyranoside. The G_{M1}-β-galactosidase activity in the cells of the patient was reduced 17- to 30-fold compared with the activity in the cells of the control subjects; also, the cells of the patient exhibited an 11- to 30-fold depression in *o*-nitrophenyl-β-galactosidase activity (ONP-β-galactosidase). These results demonstrated that fibroblasts cultured from an affected subject might be employed for diagnostic purposes. The G_{M1}- and *o*-nitrophenyl-β-galactosidase activities of fibroblasts cultured from normal amniotic fluid have also been determined; *o*-nitrophenyl-β-galactosidase activities ranged from 61 to 153 units/10^6 cells and the G_{M1}-β-galactosidase activities varied between 45 and 95 units/10^8 cells.

Speculation

Determination of G_{M1}-β-galactosidase activity in fibroblasts cultured from skin may permit the detection of individuals heterozygous for G_{M1} gangliosidosis. The presence of a β-galactosidase capable of releasing the terminal galactose from ganglioside G_{M1} in fibroblasts derived from amniotic fluid offers a basis for possible intrauterine diagnosis of G_{M1} gangliosidosis.

Introduction

Generalized gangliosidosis (G_{M1} gangliosidosis) is a rare inherited metabolic disorder characterized by the accumulation of large quantities of G_{M1} ganglioside, galactosyl-(1 → 3)-N-acetyl-galactosaminyl-(1 → 4)-[(2 → 3)-N-acetylneuraminyl]-galactosyl-(1 → 4)-glucosyl-(1 → 1)-[2-N-acyl]-sphingosine, in the brain and visceral organs [9, 11, 22]. Clinically, the disorder is manifested by progressive neurological deterioration, accumulation of glycolipid in neurons and reticuloendothelial cells, skeletal and facial features similar to

those seen in Hurler's syndrome, and death by 2 years of age. DERRY *et al.* [3] have proposed that there is a second form of G_{M1} gangliosidosis in which ganglioside G_{M1} (G_{M1}) accumulates only in the brain without osseous defects or visceromegaly.

A deficiency of *o*- and *p*-nitrophenyl-β-galactosidase activity has recently been demonstrated in the liver, spleen, kidney, and brain of four patients with G_{M1} gangliosidosis [2, 12, 15]. In addition, OKADA and O'BRIEN [12] demonstrated depressed levels of G_{M1}-β-galactosidase activity in tissues from two of these patients. Deficiency in the activity of lysosomal β-galactosidase, which normally cleaves the terminal galactose from the oligosaccharide moiety of G_{M1}, probably accounts for the accumulation of G_{M1} in generalized gangliosidosis.

We have determined the β-galactosidase activity with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate in fibroblast cultures derived from the skin and from bone marrow of a patient with G_{M1} gangliosidosis and in cultures from 11 control subjects. An additional method using tritiated G_{M1} (G_{M1}-^{3}H) was also developed to assay the enzyme.

Subjects and Methods

Patient MB (NIH 07-22-86) was 14 months old at the time the biopsies for initiation of cell cultures were obtained; he died at the age of 2 years. The diagnosis of G_{M1} gangliosidosis was established by isolating and quantifying the gangliosides from portions of brain and liver [10, 22]. These tissues contained 1,900 and 108 μg of G_{M1} sialic acid/g wet weight, respectively, an increase of 20–50 times the normal G_{M1} content [3, 19, 20].

Control cells were derived from one normal subject and from patients with diseases other than G_{M1} gangliosidosis. The control subjects ranged in age from 6 months to 26 years of age. Cultures were initiated from bone marrow aspirates and from 3-mm punch skin biopsies [23]. The growth medium used was Eagle's 'minimum essential medium' containing the nonessential amino acids [5], 10% fetal bovine serum and neomycin (50 μg/ml). Usually in 3 weeks sufficient outgrowth of fibroblasts from primary explants occurred to permit subcultivation of the cells at 3- to 6-day intervals. The cells used for subcultivation were removed from the glass surface and dispersed with 0.25% trypsin. As viewed under the light microscope, the cell morphology and growth pattern of the cells from the patient with G_{M1} and those from the control subjects were the same.

Cells used for enzyme assays were grown as monolayer cultures in 32-oz prescription bottles. At the time of harvesting the cells were rinsed with Ca^{2+}, Mg^{2+}-free Dulbecco's saline solution [4] and removed from the glass by treatment with this solution containing: (in percent) trypsin, 0.05; ethylenediaminetetraacetate (EDTA), 0.005; and methylcellulose, 0.2. Cells, 30–60 $\times 10^6$, were then suspended in 40 ml of Dulbecco's saline containing 0.1% methylcellulose, and a 0.5-ml aliquot was removed for counting [28]. The cell suspension was then centrifuged at $600 \times g$; the pellet was resuspended in 10 ml of cold methylcellulose-saline and centrifuged at $600 \times g$. The cell pellet was stored at $-80°$ until processed further.

Other cells were suspended in 1 ml of 0.25 M sucrose containing 2.5% Cutscum (isooctylphenoxypolyoxyethanol) [29] and disrupted by the microtip of a sonifier [30]. The output control selector was set at no. 2 and the output of the microtip was 1.2 amp. The cells were sonified at 0° for two 30-sec periods, interrupted by a 1-min pause. Aliquots of the resulting preparation were assayed immediately or after storage at $-70°$. Repeated freezing and thawing did not affect the activity of the various enzymes assayed.

The incubation mixture for the assay of ONP-β-galactosidase activity contained an aliquot of the fibroblast preparation representing approximately 10^5 cells; 25 μmoles of sodium acetate buffer, pH 5.1; 1.33 μmoles of ONPG; 200 μg of Cutscum; and water in a final volume of 0.2 ml. After incubation for 90 min at 37°, the reaction was terminated by the addition of 0.1 ml of human serum albumin (100 mg/ml) and 0.5 ml of 4% trichloroacetic acid. The clear supernatant solution obtained by low speed centrifugation ($500 \times g$) was pipetted into 0.4 ml of 1 M sodium carbonate. The *o*-nitrophenol concentration was determined by measuring the optical density at 415 mμ. One unit of ONP-β-galactosidase activity was defined as the amount of enzyme required to catalyze the hydrolysis of 1 nmole (nanomole) of ONPG/h under the standard conditions.

G_{M1}, isolated from the brain of patient MB, was tritiated by oxidation with galactose oxidase followed by reduction with sodium borohydride-^{3}H (17). The radioactive G_{M1} was purified by column chromatography on Anasil S [13]. The purified G_{M1}-^{3}H migrated as a single band on thin-layer plates of silica gel G that were developed in either chloroform–methanol–2.5 N ammonium hydroxide (60:35:8) or *n*-propanol–water (7:3) [27]. The labeled ganglioside had the same R_F as authentic G_{M1}. Incubation of the G_{M1}-^{3}H with rat brain β-galactosidase [7] demonstrated that more than 90% of the tritium was in the terminal galactose moiety.

G_{M1}-^{3}H was used as a specific substrate for the determination of G_{M1}-β-galactosidase activity. The incubation mixture contained an aliquot of the fibroblast

Table I. β-Galactosidase activities in fibroblasts cultured from bone marrow and skin biopsies of control subjects and of a patient with G_{M1} gangliosidosis

Patients	Age	Type of biopsy	Diagnosis	β-Galactosidase activity with substrate		Activity of other lysosomal enzymes	
				ONPG[1]	G_{M1}-^{3}H [2]	Acid phosphatase[3]	β-N-acetyl-galactosaminidase[4]
			Control cultures				
CC	8 years	Marrow	Inclusion body encephalitis	67	65	493	33
AS	4 years	Marrow	Niemann-Pick disease	79	84	499	30
MM	2 years	Marrow	Niemann-Pick disease	95		572	37
JT	18 months	Marrow	Undifferentiated lipidosis		56		
VW	15 months	Marrow	Mental retardation	99	91	373	30
JF	6 months	Marrow	Niemann-Pick disease	111		327	30
OS	59 years	Skin	Rheumatic heart disease	86			
PO	22 years	Skin	Niemann-Pick disease	59		395	22
PU	21 years	Skin	Normal volunteer	92	75	401	31
RH	5 years	Skin	Vogt-Spielmeyer disease	63			
PW	18 months	Skin	Niemann-Pick disease	105	81		
VW	15 months	Skin	Mental retardation	79	52	328	25
			Patient's cultures				
MB	18 months	Marrow	G_{M1} gangliosidosis	6	<3	464	43
MB	18 months	Skin	G_{M1} gangliosidosis	3	<3	398	39

[1] Activity expressed as nmoles *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed/10^6 cells/h.
[2] Activity expressed as nmoles galactose-^{3}H released/10^8 cells/h.
[3] Activity expressed as nmoles *p*-nitrophenylphosphate hydrolyzed/10^6 cells/h.
[4] Activity expressed as nmoles *p*-nitrophenyl-β-N-acetyl-galactosaminide hydrolyzed/10^6 cells/h.

preparation representing approximately 10^5 cells; 25 μmoles of sodium acetate buffer, pH 5.1; 2.5 nmoles of G_{M1}-^{3}H (17 mCi/mmole); 1 μmole sodium deoxytaurocholate [29]; 200 μg of Cutscum; and water in a final volume of 0.2 ml.

After incubation for 90 min at 37°, the reaction was terminated by heating for 3 min at 100°. The galactose liberated from G_{M1} was separated from excess G_{M1} and the other reaction product, ganglioside G_{M1} [31], by thin-layer chromatography. Chromatography was performed on thin-layer plates of silica gel G with chloroform–methanol–2.5 N ammonium hydroxide (60:35:8) as solvent. The area corresponding to galactose was scraped and counted in a liquid scintillation counter with a Triton-toluene counting fluid [1]. One unit of G_{M1}-β-galactosidase activity was defined as the amount required to catalyze the release of 1 nmole galactose-^{3}H/h under the standard conditions. The galactosidase activity as measured with ONPG or G_{M1}-^{3}H was linear with respect to time and enzyme concentration over the range examined.

Acid phosphatase and β-N-acetyl-galactosaminidase activities of the tissue culture preparations were also determined [8, 18]. The substrates, *p*-nitrophenylphosphate and *p*-nitrophenyl-β-N-acetylgalactosaminide, were obtained commercially [32].

Results and Discussion

A deficiency of both ONP-β-galactosidase and ganglioside G_{M1}-β-galactosidase activities was found in fibroblast cultures derived from skin and from bone marrow biopsies of patient MB (table I). The ONP-β-galactosidase activity in his cultures was 2.5–10% of the activity in the cultures from control subjects. With G_{M1}-^{3}H

as substrate, cell cultures of patient MB demonstrated no activity by an assay capable of detecting 3 units of activity per 10^8 cells; G_{M1}-β-galactosidase activity was less than 6% of that in the least active control culture. The deficiency of activity against either galactoside was specific for G_{M1} gangliosidosis (table I).

The activities of two other lysosomal hydrolases, acid phosphatase and β-N-acetylgalactosaminidase, were determined to demonstrate the metabolic competency of the cells from patient MB. The activities of these enzymes were not depressed (table I). Aliquots of a single sonically disrupted cell preparation were used for multiple β-galactosidase and 'control' enzyme assays. In most cases the values in table I are the means of values from two or three assays. In three subcultures of skin and two of the bone marrow, cells of patient MB exhibited low levels of ONP- and G_{M1}-β-galactosidase activity.

The results of assays for ONP- and G_{M1}-β-galactosidase activities in control cells with and without admixture of cells from the patient are shown in table II. Cells from the patient did not inhibit ONP-β-galactosidase activity; however, there appeared to be a decrease in the G_{M1}-β-galactosidase activity of the control cells in the presence of cells from patient MB. This depressed activity was eliminated by tripling the concentration of G_{M1}-^{3}H in the incubation mixtures. These experiments indicate that the lower β-galactosidase activities in the mixtures probably resulted from dilution of the labeled substrate by endogenous G_{M1} and not from the presence of an inhibitor in the patient's cells.

Table II. β-Galactosidase activity in mixtures of cells from control subjects and patient MB

Control subjects	Type of cell	Enzyme activity in cells from	
		Control alone	Control+MB[1]
	ONP-β-galactosidase[2]		
PO	Skin	63	59
PU	Skin	81	78
AS	Bone marrow	80	83
	G_{M1}-β-galactosidase[3]		
AS	Bone marrow	80	71
VW	Bone marrow	95	78
VW	Skin	57	46

[1] For each incubation $2–4 \times 10^5$ control cells were used; in the mixtures 5×10^5 cells from patient MB were added.

[2] Activities expressed as nmoles ONPG hydrolyzed/10^6 control cells/h.

[3] Activities expressed as nmoles galactose-^{3}H released/10^8 control cells/h.

The marked difference between the ONP- and G_{M1}-β-galactosidase activities in the cells of the control subjects was probably the result of the different solubility characteristics of the two substrates. ONPG is water soluble and, therefore, probably more available to the enzyme than G_{M1}, which presumably was present in micellar form. Further metabolism of the galactose liberated from G_{M1} would also result in a lower apparent G_{M1}-β-galactosidase activity. The latter possibility is probably of minor importance, however, since more than 90% of the G_{M1} could be recovered following incubation.

We have also cultured a number of cell lines from the fetal cells present in amniotic fluids, obtained by transabdominal amniocentesis or at therapeutic abortion after 12–20 weeks of gestation. ONP-β-galactosidase and G_{M1}-β-galactosidase activities were assayed in four of these lines. The ONP-β-galactosidase activities ranged from 61 to 153 units/10^6 cells; the G_{M1}-β-galactosidase activities varied between 45 and 95 units/10^8 cells [24]. Thus, cultures derived from amniotic fluid may make possible the detection of G_{M1} gangliosidosis in the developing fetus as early as week 22 of gestation.

Sodium borohydride-^{3}H, with a specific radioactivity of 400 mCi/mmole, was used in the preparation of the G_{M1}-^{3}H, and yielded a labeled substrate that permitted the determination of the G_{M1}-β-galactosidase activity in 10^5 fibroblasts. Sodium borohydride-^{3}H is now available with a specific radioactivity of 6 Ci/mmole. If this reagent was employed, a 15-fold increase in the specific radioactivity of G_{M1}-^{3}H could be obtained. Use of this substrate and prolonged incubation of the assay might permit the determination of the G_{M1}-β-galactosidase activity in cultured cells as early as 2 weeks after the amniotic fluid was obtained.

After this work was completed, Pinsky *et al.* [14] reported depressed levels of ONP-β-galactosidase activity in cultured fibroblasts of a patient in which G_{M1} accumulated only in the brain.

'Generalized' and 'cerebral' G_{M1} gangliosidosis may represent two manifestations of homozygosity for the same defective gene. The amounts of visceral mucopolysaccharides and visceral glycolipids are two differences in the chemical pathology of these two forms of the disorder [21]. It seems possible that these two forms of G_{M1} gangliosidosis may actually result from two different mutations at the β-galactosidase locus.

Compared with the normal enzyme, the mutant enzymes might possess different relative affinities for G_{M1} and mucopolysaccharides. Alternatively, one mutation might lead to a more severe deficiency of

enzymatic activity and, therefore, to a more generalized accumulation of G_{M1}. The ready accessibility of this enzymatic activity in cultured fibroblasts may assist in the further assessment of these two possibilities. It is also entirely possible that enzymatic analysis of cultured fibroblasts may permit detection of the heterozygous state in G_{M1} gangliosidosis.

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30. Branson Instruments Inc., Stamford, Conn.
31. The structure of G_{M2} is identical to that of G_{M1} minus the terminal galactose.
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