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Studies on Ganglioside GM3 and Sialidase Activity in Human Fetal Liver

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Abstract: Ganglioside GM3 and sialidase activities in human fetal liver have been investigated. Gangliosides were extracted from fetal livers by the Folch-Suzuki method and analyzed by high-performance thin layer chromatography (HPTLC). GM3 increased, but lactosylceramide (LacCer) decreased predominantly over the developmental stages. Sialidase in human fetal liver was mainly localized in the lysosomal fraction and its activity was high in the earlier stages of development. The optimum pH for this enzyme was 4.3~4.4. Sialidase was more active with the ganglioside mixture than with GM3, sialyllactose or fetuin. Fetal liver sialidase was still active (20% activity) in the presence of 25% methanol. These results suggested that the changes of the ganglioside GM3 and sialidase activity may be involved in the regulation of cell growth in human fetal liver during development.
Key words: gangliosides, human fetal liver, sialidase.

Gangliosides are a structurally heterogeneous group of animal glycosphingolipids containing one or more glycosidically bound sialic acids (Ledeen and Yu, 1982; Hakomori, 1981). Because of their localization on the cell surface they have been implicated in a variety of membrane-related phenomena; cell surface recognition, growth regulation, immune modulation, and their expression in tumors as oncofetal antigens (Sjoberg *et al.*, 1992). They influence cell proliferation by interacting with membrane-bound proteins such as enzymes and receptors (Bremmer *et al.*, 1986). Rapidly dividing KB, 3T3, or NIL-8 fibroblast cells alter GM3 expression and sialyltransferase I activity according to the cell cycle, at the G_1/G_o phase. Exogenous gangliosides are incorporated into the plasma membrane, leading to an inhibition of cell growth by extending the G_1/G_o phase of the cell cycle. The modulation of EGF-receptor autophosphorylation by ganglioside GM3 is the best-characterized effect of exogenous gangliosides (Rosner *et al.*, 1990). The sialic acid residue of GM3 is metabolically active in growing fibroblasts, and there are several different sialidases that might account for the turnover of GM3 in these cells (Usuki *et al.*, 1988).

Sialidase (neuraminidase, EC 3.2.1.18) catalyzes the removal of sialic acid in α-ketosidic linkage in glycoconjugates. It has been detected in various mammalian tissues and subcellular fractions. The majority of this cellular activity is associated with the lysosomal fraction, but evidences for other membranes and soluble forms exist (Michalski *et al.*, 1982). The cell-dependent degradation of GM3 ganglioside to lactosylceramide (LacCer) by sialidase may relieve the GM3 inhibition of EGF-receptor tyrosine kinase autophosphorylation, thus allowing cell proliferation to continue (Melkerson-Watson and Sweeley, 1991). The purpose of this work was to investigate the gangliosides and sialidase activity in human fetal liver, which has not yet been undertaken.

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Materials and Methods

Materials

Bovine brain ganglioside mixture, monosialoganglioside GM3, lactosylceramide, sialyllactose, fetuin, and *Clostridium perfringens* sialidase (type IV) were purchased from Sigma Chemical Co. (St. Louis, USA). High performance thin-layer chromatographic plates (HPTLC silicagel) were purchased from Merck Co. (Darmstadt, Germany). All other chemicals were from local sources and of the best grade available. Normal human fetal liver obtained during autopsy after death was kindly provided by Dr. H. S. Lee (Department of Anatomy, Ewha Womans University).

Isolation of gangliosides from human fetal liver

The ganglioside mixture was extracted from each liver sample and purified from the total lipid extract according to the Folch *et al.* (1957) and Suzuki (1965)

method with minor modifications. Each sample was homogenized in a motor-driven teflon pestle homogenizer for 1 min with 0.5 vol of distilled water, and the homogenate was mixed with 10 vol of chloroform/methanol (2 : 1). After the homogenate was stirred for 1 h and filtered, the residue was reextracted with 10 vol of chloroform/methanol (1 : 2). Then the chloroform/methanol ratio of the extracts was adjusted to 2:1 by adding chloroform, and the gangliosides were recovered from the crude lipid extracts through separation into the upper phase by the partitioning procedure. The upper aqueous phases were evaporated, dialyzed, and lyophilized. The extract was dissolved in a mixture of chloroform/methanol (1 : 1).

High Performance Thin Layer Chromatography

Gangliosides were analyzed by HPTLC on precoated Silica Gel 60 plates which were activated at 100℃ for 1 h. Gangliosides were developed in chloroform/methanol/0.2% $CaCl_2$ (55 : 45 : 10) and visualized by spraying with orcinol/H_2SO_4 reagents (Svennerholm, 1956). Bovine brain ganglioside mixture and GM3 were used as the standards. The plates were scanned by Ultroscan XL densitomer.

Sialidase (neuraminidase) treatment

Enzymatic treatment with sialidase from *Clostridium perfringens* (EC 3.2.1.18) was carried out by the method of Ando and Yu (1977). The sample was dried with a stream of nitrogen and dissolved in 50 mM sodium acetate buffer (pH 5.0). Then 0.1 unit of sialidase solution was added and the reaction mixture was incubated for 17 h at 37℃. The reaction was terminated by the addition of 2 ml of chloroform/methanol (2 : 1) and evaporated under nitrogen gas.

Subcellular fractionation

The subcellular fractionation of human fetal liver was carried out by the Michalski *et al.* method (1982) with minor modification. The scissored liver was suspended in 3 vol of 0.15 M KCl solution and homogenized. The homogenate was centrifuged for 15 min at 1,500 $\times g$. The pellet was resuspended in 2 vol of homogenation buffer and recentrifuged under the same condition. The mitochondria were removed by centrifugation at 25,000$\times g$ for 3 min and the lysosomal fraction was obtained by centrifuging for 10 min at 63,000$\times g$. The resulting supernatant as well as the fluffy pink layer of the pellet (microsome) was collected and centrifuged at 105,000$\times g$ for 60 min to give the microsomal and cytosolic fractions. After each centrifugation step, the pellets were washed with the homogenation buffer and centrifuged again as above.

Preparation of the lysosomal membrane

The lysosomal fraction was suspended in 10 vol of a hypotonic solution of 5 mM acetate buffer, pH 5.5, and stirred for 30 min at 0℃ to lyse the lysosomes. The suspension was then centrifuged at 3,000$\times g$ for 15 min and the milky supernatant was discarded. The pellet was resuspended in a 10 vol of 1 M NaCl, stirred for 1 h at 0℃ and centrifuged at 105,000$\times g$ for 30 min to obtain a pellet (lysosomal membranes). In solubilization experiments, the membranes were suspended in 5 vol of 20 mM Tris/HCl solution containing 1% Triton X-100, adjusted to pH 6.0, stirred for 1 h, subjected twice to a 20 s sonication in a cooling bath and subsequently centrifuged at 105,000$\times g$ for 30 min.

Sialidase assays

Incubation mixtures for sialidase activity contained 0.05 to 0.15 mg protein from the subcellular fraction and 0.1 M sodium acetate buffer at a final concentration of 75 mM, pH 4.36, in a final volume of 0.2 ml. 11~12 nmol of substrates as bound sialic acid were added in aqueous solution and 0.2 mg of bovine serum albumin was added as a stabilizer. Incubations were carried out routinely at 37℃ and 47℃ for 2 h and stopped by freezing or adding the periodate reagent for the periodic acid/thiobarbituric acid assay. The released sialic acid was determined by Aminoff's method (1961) by measuring absorbance at 532 and 549 nm, and a mathmatical procedure by Warren (1959) was used to calculate the sialic acid concentration. One unit of sialidase is defined as the amount of enzyme required to release 1 nmol sialic acid/h. Specific activity refers to units of enzyme per mg of protein. Proteins were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standards.

Results and Discussion

Separation of human fetal liver gangliosides

Fig. 1 shows the representative HPTLC profile of gangliosides in the human fetal liver aged from 17 to 26 weeks. By densitometric analysis (data is not shown), band 1 was high in younger subjects and progressively decreased in older subjects, whereas GM3 increased from 20% to 80% with development. GM3 is the major ganglioside in human adult liver, where it constitutes more than 90% of the total lipid-bound sialic acid (Riboni *et al.*, 1992). Increases of sialytransferase I and GM3 expression was observed in growth inhibited cells treated by butyrate, but GM3 dramatically decreased in the cells lacking growth control (Machwe *et al.*, 1978). Malkerson and Sweeley (1991) have also reported that exogenous GM3 acts as a growth

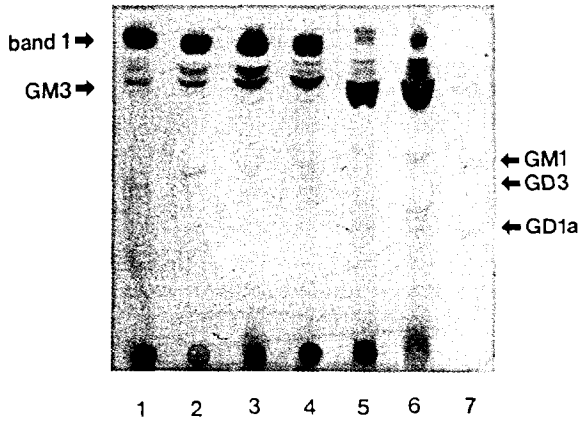

Fig. 1. HPTLC analysis of gangliosides from the human fetal liver in 17, 19, 20, 22, 24 and 26 weeks, lane 1, 2, 3, 4, 5 and 6, respectively. Lane 7 is standard bovine brain ganglioside mixture.

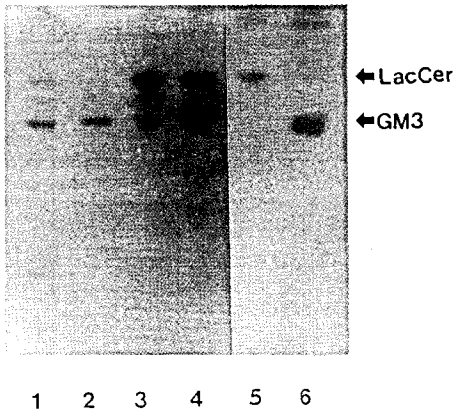

Fig. 2. HPTLC for identification of band 1 in 24 week-fetal liver by sialidase treatment. Lane 1, 3, 4, lactosylceramide derived from GM3 of fetal liver after sialidase treatment; lane 2, GM3 of 24-week fetal liver; lane 5, lactosylceramide derived from standard GM3 after sialidase treatment; lane 6, standard GM3.

inhibitor and lactosylceramide allows cell proliferation to continue. Our results suggest that lactosylceramide and GM3 can control the growth of human fetal liver.

Identification of band 1 by sialidase treatment

GM3 from fetal liver was not degraded by physical shock and GM3 was extracted to the aqeuous phase by the repeated Folch partitioning procedure. When GM3 from a 24 week-fetal liver was treated with Clostridium perfringens sialidase, band 1 was identified as a lactosylceramide as shown in Fig. 2. These data indicate that lactosylceramide in the early stage of fetal liver may allow cell proliferation to continue and that GM3 in the late stage acts as a growth inhibitor. We also found that *Clostridium perfringens* sialidase acted on the fetal liver GM3 and had broad substrate specificity.

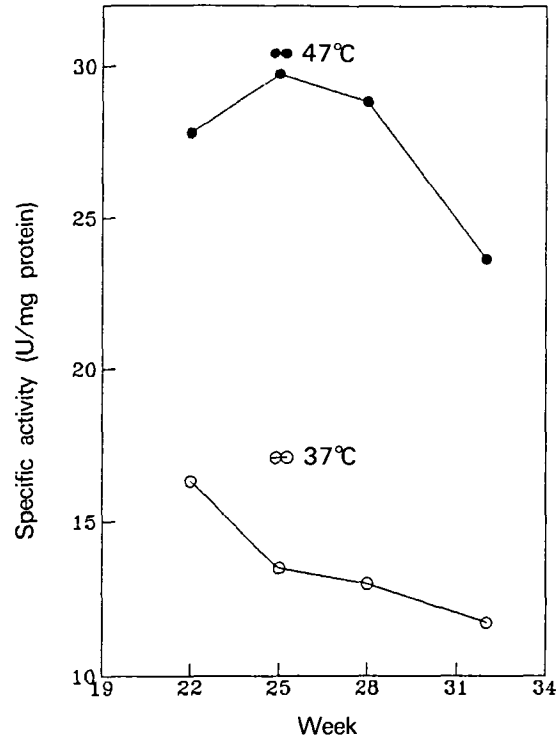

Fig. 3. Developmental changes of the human fetal liver sialidase activity. The lysosomal sialidase was assayed against ganglioside mixture at pH 4.36. Each point of the curve is the mean specific acititiy derived from three independent experiments each performed in duplicate.

Subcellular fractionation of human fetal liver and measurement of sialidase activity

In order to examine the above results on alteration of the ganglioside pattern during human fetal liver development, sialidases from the liver of 22, 25, 28, and 32 week fetuses were prepared by subcellular fractionation. Sialidase in human fetal liver was localized predominantly in lysosomal fraction and higher activity was detected in the early stages of development (Fig. 3). GM3 sialidase activities of human fibroblast cells were higher in growing cells than in growth inhibited cells (Usuki *et al.*, 1988) and cell growth was inhibited by treatment with sialidase inhibitor (Usuki *et al.*, 1988a). Our results support the claim that GM3 sialidase participates in the control of cell growth. Substrate specificities of fetal sialidase were measured. The activity of the fetal sialidase was more active with ganglioside mixture than with GM3, sialyllactose, or fetuin at 37℃ (Fig. 4, Table 1). It has been reported that human adult liver sialidase was preferentially active with bovine submandibular gland mucus glycoprotein, GM3, and ganglioside mixture (Michalski *et al.*, 1982).

Optimum pH, temperature, and thermostability of sialidase activity

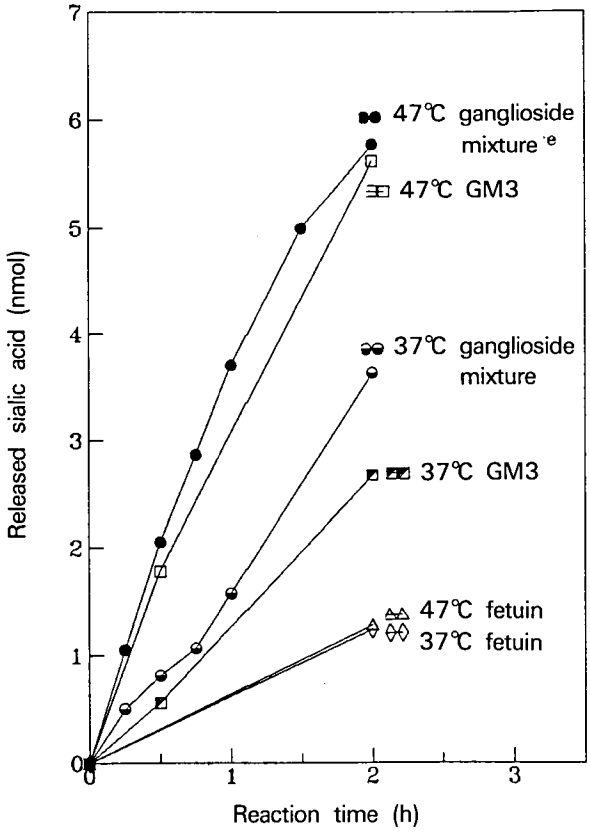

Fig. 4. Substrate specificities of the 22 week-liver sialidase at 37 ℃ and 47℃. The lysosomal sialidase was assayed at pH 4.36.

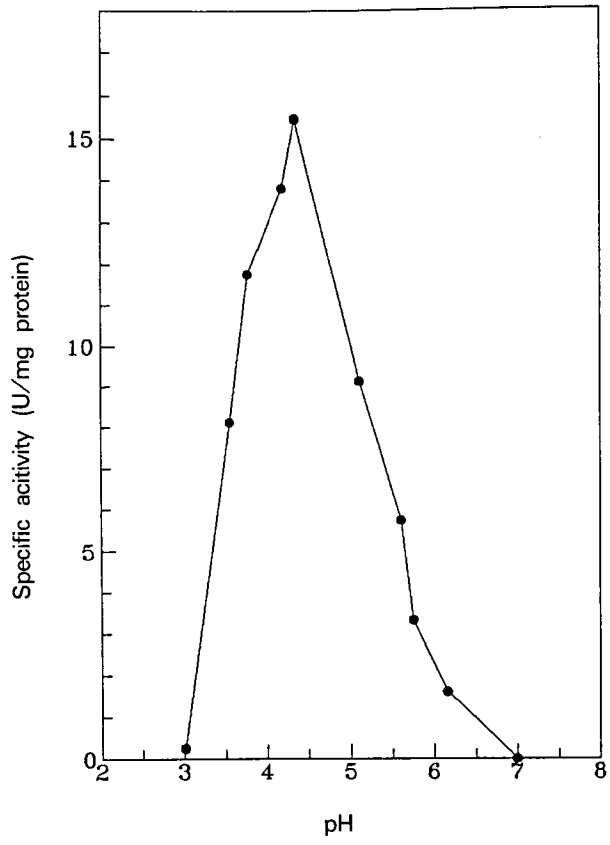

Fig. 5. pH-dependent activities of 22 week-liver sialidase at 37℃. The lysosomal sialidase was assayed against ganglioside mixture.

Table 1. Substrate-dependent sialidase activities in 25 week-fetal liver

Substrates	Specific activity (U/mg protein)
	37℃
Gangliosides mixture	13.6± 1.1
GM3	12.6± 0.8
Sialyllactose	6.9± 0.7
Fetuin	5.6± 0.4

Lysosomal sialidase was assayed at pH 4.36. sp.ac. means specific activity. The data are presented as the mean sp. ac.± s.d.

The optimum pH of the fetal sialidase activity was 4.3~4.4 when determined with a ganglioside mixture (Fig. 5). This low pH optimum is typical for other liver sialidases, especially for those of a lysosomal origin (Spartro and Alhadeff, 1984; Inoute and Shigeta, 1991).

The temperature optimum curve depicted in Fig. 6 indicates that heating to 47℃ enables membrane-bound sialidase to be exposed to the substrate and activate the membrane's sialidase.

Thermostability studies were performed on the fetal sialidase after preincubation without substrate at 40℃ for 30 min and 40 min, and at 47℃ for 50 min (Table 2). No loss or small loss of activity (0-10.3%) was found at 40℃ for 30 min, and 40 min, respectively, whereas approximately 40% loss of activity occurred at 47℃ for 50 min. When compared to human adult liver lysosomal sialidase (Michalski *et al.*, 1982), preincubation of the adult enzyme for 30 min at 40℃ resulted in a 50% decrease of the enzyme activity and enzyme activity was completely destroyed by a 30 min preincubation at 50℃. Thus we found that the fetal sialidase was more thermostable than that of the adult.

The activities of the fetal membrane sialidase and the Triton solubilized sialidase were compared at 37℃ and 47℃. As shown in Table 3, the lysosomal membrane's sialidase was activated 2-fold and Triton insoluble sialidase was activated 1.9-fold, while Triton soluble sialidase was activated 1.2-fold when the incubation temperature was increased from 37℃ to 47℃. This difference could be interpretated as follows; the lysosomal membrane-bound sialidase and Triton insoluble sialidase were exposed more to the substrates at 47℃ and the activity increased about 2-fold.

Effect of methanol on sialidase activity

In order to determine whether the enzyme functions during the separation process, we examined factors af-

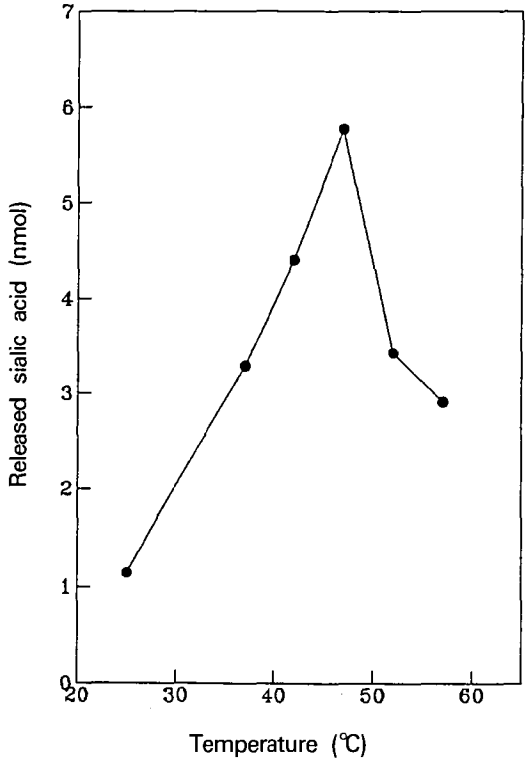

Fig. 6. Temperature-dependent activities of the 22 week-liver sialidase. The lysosomal sialidase was assayed against ganglioside mixture at pH 4.36.

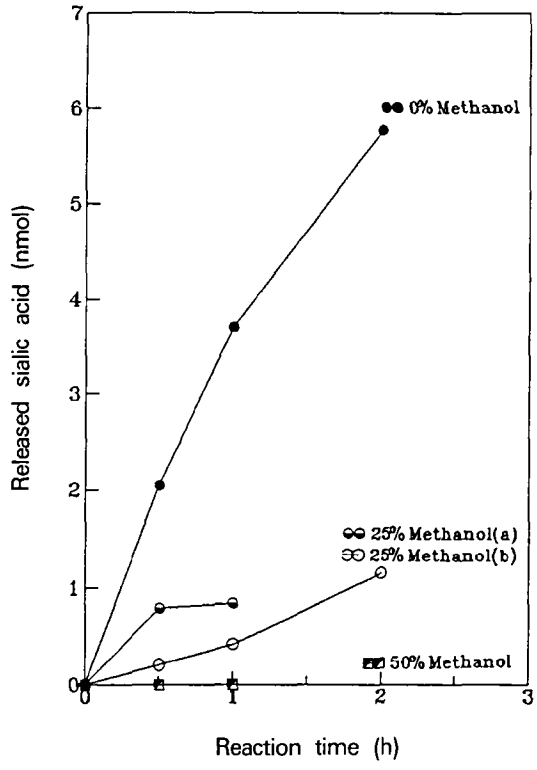

Fig. 7. Effect of methanol on the 22 week-liver sialidase activity. The lysosomal sialidase was assayed against ganglioside mixture at 47℃. a : pH of the incubation mixture was not adjusted; b : pH was adjusted to 4.36.

Table 2. Thermostabilities of 25 weeks-liver sialidase

Preincubation	Specific activity (U/mg protein)
	37℃
0 min	13.6+1.1
40℃ 30 min	13.8+1.3
40℃ 40 min	12.2+1.0
47℃ 50 min	8.1+0.7

Lysosomal sialidase was assayed against ganglioside mixture at pH 4.36.

Table 3. Comparison of sialidase activities in 25 week-liver at 37℃ and 47℃

Enzyme condition	Specific activity (U/mg protein)	
	37℃	47℃
lysosome	13.5+1.0	29.8+1.9
lysosomal membrane	14.7+1.1	30.6+2.5
Triton X-100 soluble	14.9+1.6	17.9+2.0
Triton X-100 insoluble	6.0+0.8	11.3+1.0

fecting the enzyme activity. Inoute *et al.* (1991) reported that 50% ethanol activates the hydrolysis of ganglioside GM3 catalyzed by Clostridium perfringens sialidase by 15 times. We have studied the effects of methanol, which has been used in ganglioside extraction from liver (Fig. 7). No enzyme activity was observed in 50% methanol, but 20% of total sialidase activity remained when the enzyme mixture contained 25% methanol. Therefore, it is possible that the sialidase may still be active in the organic environment during the extraction process of gangliosides,and hydrolyze GM3 to LacCer and sialic acid.

Aknowledgement

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