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Change in Contents of Biologically Active Sphingolipids Modulating Cell Growth and Survival in Hepatoma 27 Compared to Rat Liver

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Abstract—The contents of bioactive sphingolipids (sphingomyelin, ceramide, glucosyl- and lactosylceramides, gangliosides) were studied in rat hepatoma 27 and rat liver. The amounts of sphingomyelin, ceramide, and glucosyl- and lactosylceramides were about twofold and that of gangliosides was about 3.5-fold increased in the tumor compared to normal tissue. Since sphingomyelin promotes angiogenesis, glucosyl- and lactosylceramides stimulate proliferation, gangliosides inhibit apoptosis, but ceramides suppress proliferation and stimulate apoptosis, it is obvious that the balance of these effectors in hepatoma 27 moves with the tumor growth.

Key words: sphingolipids, gangliosides, glucosylceramide, lactosylceramide, tumor, sphingomyelin, proliferation, apoptosis

Sphingolipids are natural biologically active endogenous compounds with a sphingoid base (as a rule, sphingosine D-*erythro*-(2S,3R,4E)-2-amino-4-octadecene-1,3-diol) in the structure which are involved in cell proliferation, differentiation, and apoptosis as secondary messengers or extracellular modulators. Data now available are summarized in a great number of reviews. It has been established that ceramide (N-acylsphingosine) inhibits cell proliferation and stimulates differentiation and apoptosis (reviews [1-3] and the literature cited therein), whereas products of ceramide metabolism (glucosyl- and lactosylceramides) stimulate proliferation and inhibit apoptosis (reviews [2, 4] and the literature cited therein). Sphingomyelin was recently shown to promote tumor growth, metastasis, and angiogenesis [5]. Obviously, the final effect of sphingolipids depends on their balance in the cell.

Tumor cells are characterized by disorders in proliferation, differentiation, and survival compared to normal homologous cells, and this is suggested to be accompanied by changes in the balance of biologically active sphingolipids. Although there are many data on changes in the sphingolipid composition of tumors compared to normal tissues [6], concurrent changes in the effector sphingolipids modulating cell growth and survival have not been analyzed. Therefore, in the present work the difference in contents of sphingomyelin, ceramide, glucosyl- and lactosylceramides, and gangliosides involved in cell proliferation and apoptosis was studied in hepatoma 27 and normal rat liver.

MATERIALS AND METHODS

Random-bred rats with body weight of 80-120 g were used. The hepatoma was transplanted subcutaneously (six animals), and the tumor was isolated 15-16 days after the transplantation. Livers from six healthy animals were isolated concurrently.

Lipids were isolated from the tissues by multiple extraction with a mixture of $CHCl_3$–CH_3OH (2 : 1 and 1 : 2 v/v) until their complete withdrawal. A small aliquot of extracts from the hepatoma and the normal liver were taken for determination of total lipid phosphate and sphingomyelin, and the main part of the extracts was washed five times with water to isolate gangliosides as described in [7].

The amount of sphingomyelin was determined as described earlier [8] after thin-layer chromatography (TLC) of phospholipids on silica gel-coated glass

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HPTLC-plates (10 × 10 cm) (Merck, Germany) in the system $CHCl_3$–CH_3OH–HCOOH (65 : 25 : 4).

The aqueous phase resulting from washing of lipid extracts and containing gangliosides was evaporated in a rotary evaporator, and the total content of lipid-bound sialic acids was determined in the residue after purification on a Sep-Pak C_{18} cartridge [9]. Gangliosides were separated and identified by TLC in the system $CHCl_3$–CH_3OH–0.02% $CaCl_2$ (60 : 40 : 9 v/v) (A) in the presence of brain gangliosides and GD_3 as marker substances. Gangliosides were cleaved with neuraminidase from *Vibrio cholerae* (Serva, Germany) at 37°C for 24 h [9]. The resulting products of enzymolysis were chromatographed on HPTLC-plates (5 × 5 cm) in the abovementioned system A and also on plates (5 × 5 cm) impregnated with NaH_2PO_4 in the system *n*-C_3H_7OH–H_2O–28% NH_4OH (6 : 2 : 1 v/v), in the presence of N-acetyl- and N-glycolylneuraminic acids as marker substances. Sialo-containing compounds were detected with resorcinol reagent [10]. After TLC on HPTLC-plates (10 × 10 cm) and detection with resorcinol reagent, the relative contents of gangliosides were determined by densitometry at 580 nm on a CS-920 scanimeter (Shimadzu, Japan).

Lipids prepared by evaporation of the organic layer were methanolyzed in 0.2 M NaOH in CH_3OH for 24 h at 20°C to cleave glycerolipids, and afterwards the reaction mixture was neutralized with 0.35 M AcOH in CH_3OH and evaporated.

The amount of ceramides was determined by densitometry on a CS-920 scanimeter at 630 nm [11] in an aliquot of the residue resulting after two-stage TLC on HPTLC-plates (10 × 10 cm) first in ether, then in the system $CHCl_3$–CH_3OH (9 : 1 v/v), and subsequent detection with 5% phosphomolybdenic acid.

Neutral glycosphingolipids were isolated by a modification of method [12]. The main part of the organic residue was acetylated overnight with the mixture acetic anhydride–pyridine (3 : 2 v/v) at 20°C. After termination of the reaction, the mixture was thrice evaporated with toluene. Then the dry residue was dissolved in the mixture hexane–1,2-dichloroethane (1 : 4 v/v) and placed onto a column filled with florisil (60-100 mesh; Merck) (at the residue/florisil ratio of 1 : 50 w/w) suspended in the same mixture of solvents. Then the column was washed with 50 ml of following eluents: a) hexane–1,2-dichloroethane (1 : 4 v/v); b) 1,2-dichloroethane; c) 1,2-dichloroethane–acetone (1 : 1 v/v). Each fraction was evaporated, the residue was dissolved in the mixture $CHCl_3$–CH_3OH (2 : 1 v/v), deacetylated with 0.5 ml of 0.5% sodium methylate for 1 h at 20°C, and evaporated in the presence of ethyl acetate. Glycosphingolipids were found only in fraction "c". Glycosphingolipids were identified and determined quantitatively by TLC on HPTLC-plates (10 × 10 cm) in the system $CHCl_3$–CH_3OH (5 : 1 v/v). For quantitative determination, 1 μl of glycosphingolipid solution (five parallel applications) and 1 μl (2 nmol) of solution of standard brain cerebrosides (three parallel applications) were applied onto a plate. After chromatography, the plate was sprinkled with phosphomolybdenic acid in alcohol and heated for 15 min at 160°C. The revealed spots were subjected to densitometry on a CS-920 scanimeter at 630 nm. The quantity of glycosphingolipids was calculated based on data obtained for the standard brain cerebrosides. On application of 1 μl of the experimental solution (the concentration no more than 3 nmol/μl), data were well reproducible, while application of greater volumes distorted the results.

The protein was determined by the method of Lowry et al. [13].

The significance of data was evaluated using Student's *t*-test.

RESULTS AND DISCUSSION

Compared to normal liver, in hepatoma 27 absolute contents of all of the studied sphingolipids is increased (Table 1); this seems to be due to increase in the activity of dihydroceramide synthase (dihydroceramide is an intermediary product in biosynthesis of all sphingolipids) in tumors compared to normal tissues [14].

The contents of sphingomyelin, ceramide, and glucosyl-, galactosyl-, and lactosylceramides in hepatoma 27 are increased twofold (Table 1). The content of gangliosides is also significantly increased. The total content of lipid-bound sialic acids is increased about 3.5-fold, and contents of gangliosides GM3 and GM1 in the hepatoma is, respectively, 4.5 and 20 times higher than in the liver (Table 2). But no gangliosides GD1a, GD1b, and GT1b are present in the hepatoma. However, TLC of the hepatoma gangliosides revealed a sialoglycosphingolipid with the chromatographic mobility similar to that of ganglioside GD3, but this compound was unchanged by

Table 1. Contents of sphingomyelin, ceramide, and neutral glycosphingolipids in normal rat liver and rat hepatoma 27 (nmol per mg protein; mean values of four-five determinations ± mean deviations are presented)

Sphingolipids	Liver	Hepatoma 27
Sphingomyelin	15.08 ± 0.19	31.88 ± 1.48*
Ceramide	4.18 ± 0.15	10.91 ± 0.48*
Glucosylceramide	0.45 ± 0.02	0.97 ± 0.14*
Galactosylceramide	0.12 ± 0.04	0.27 ± 0.04**
Lactosylceramide	0.13 ± 0.04	0.25 ± 0.02*

* $p < 0.001$.
** $p < 0.01$.

Table 2. Contents of gangliosides in normal rat liver and rat hepatoma 27 (nmol per mg protein; mean values of four-five determinations ± mean deviations are presented)

Gangliosides	Liver	Hepatoma 27
Lipid-bound sialic acids	0.39 ± 0.02	1.46 ± 0.02*
GM3	0.15 ± 0.006	0.70 ± 0.04*
GM2	0.019 ± 0.001	0.038 ± 0.004*
GM1	0.036 ± 0.004	0.72 ± 0.04*
GD1a	0.042 ± 0.004	—
GD1b	0.032 ± 0.003	—
GT1b	0.011 ± 0.001	—

* $p < 0.001$.

enzymolysis of the ganglioside mixture with neuraminidase from *Vibrio cholerae*, which cleaves all compounds except GM1. The enzymolysis products contained two sialic acids, N-acetyl- and N-glycolylneuraminic; therefore, this sialoglycosphingolipid chromatographically similar to the ganglioside GD3 was concluded to be the ganglioside GM1 containing N-glycolylneuraminic acid and displaying a similar chromatographic mobility.

Thus, absolute contents of all sphingolipids are increased in hepatoma 27, both those which stimulate (sphingomyelin, glucosyl- and lactosylceramides, gangliosides) and those which inhibit (ceramides) the tumor growth.

There are numerous literature data on increase in the sphingomyelin level in tumor compared to normal tissues (see review [6]), and the increase in its content was established to correlate with malignant transformation and activity of metastasizing [15, 16]. As to ceramide, changes in its content seemed to depend on the tumor type: the content of ceramide was increased in tumor cells compared to normal homologous cells [14, 17, 18], but its decreased level was also recorded, especially in highly malignant tumors [19, 20]. Note that the pool of tumor ceramides contains a significant amount of biologically inactive dihydroceramide [17, 21] that decreases the antiproliferative activity [22].

Glycosphingolipids (including gangliosides) influence not only proliferation and apoptosis of tumor cells but also their properties. Thus, the metastasizing ability of the melanoma B16 cells correlates with increase in contents of gangliosides GM2 and GM3 [23]. The effect of glycosphingolipids significantly depends on their location and expression and also on expression of enzymes responsible for their metabolism. Thus, in drug-resistant (unlike drug-sensitive) tumor cells the expression of lactosylceramide was decreased, the expression of gangliosides GM2 and GM3 was increased [24], and the activity of glucosylceramide synthase was also increased [25]. The high expression of sialidase in tumor cells was shown to prevent their apoptosis [26]. Since glycosphingolipids are concentrated in "glyco-synapses", i.e., domains located in the plasma membrane, it was recently suggested that tumor progression should significantly depend on the intimate interaction between glycosphingolipids of the tumor cell and those of the host cell [27].

In the present work the contents of all biologically active sphingolipids studied were significantly increased in the hepatoma 27 compared to the normal homologous liver; thus, the tumor was suggested to have increased ability for angiogenesis (sphingomyelin), increased cell proliferation (glucosyl- and lactosylceramides), and increased antiapoptotic activity (glucosyl- and lactosylceramides, gangliosides GM1, GM2, GM3). Although the contents of effectors stimulating cell growth and inhibiting apoptosis increase concurrently with the content of ceramide, which inhibits the proliferation and stimulates apoptosis, the balance of these compounds seems to be toward promoting the tumor growth.

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