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Neoplastic Alteration of a Membrane-associated Sialidase of Rat Liver

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Rat liver particulate fraction contains two types of membrane-associated and gangliosides-hydrolyzing sialidase, which have been shown to be identical to two membrane-associated sialidases of rat brain (I and II) chromatographically, immunologically and in substrate specificity. Chromatography on AH-Sepharose 4B of the membrane sialidases of rat primary hepatoma induced by 3′-methyl-4-dimethylaminoazobenzene (MeDAB) further revealed that hepatocarcinogenesis induces a marked decrease in sialidase II but no decrease in sialidase I. Using antisera against sialidases I and II of rat brain, immunoprecipitation studies of the solubilized particulate fractions of rat liver and MeDAB-hepatoma gave results similar to those obtained chromatographically. Using the same immunological technique, sialidase II but not sialidase I was found to be decreased in AH109 A hepatoma and in regenerating and fetal liver.

Key words: Membrane sialidase — Rat liver — Hepatoma — Gangliosides

We have previously elucidated that rat liver possesses at least three types of sialidase, localized mainly in the lysosomal matrix, cytosol and plasma membrane, respectively.[1-3] These sialidases also differ in substrate specificity, and the plasma membrane enzyme is distinguished from others by its preference for gangliosides.[3] On the basis of these findings, we have compared sialidase activities between rat liver and hepatomas[4, 5]; we have found that hepatocarcinogenesis lowers the activity of the membrane-associated sialidase.[5] Subsequent studies on rat brain particulate fraction, however, have revealed that the fraction contains two types of ganglioside sialidase, sialidases I and II, which are located mainly in the synaptosomes and lysosomal membrane, respectively.[6] In addition to the different subcellular locations, these sialidases differ from each other immunologically. Thus an antibody to sialidase I raised in the rabbit immunoprecipitated sialidase I and did not cross-react with sialidase II, while antibody to sialidase II precipitated sialidase II but not sialidase I.[6] These findings prompted us to reinvestigate the membrane-associated sialidase(s) of rat liver and its neoplastic alteration(s). We report here the results obtained.

MATERIALS AND METHODS

Materials Bovine brain mixed gangliosides (type II) and lactosylceramide were purchased from Sigma (St. Louis, MO). GM_3 ^{14}C-labeled in the NeuAc moiety (^{14}C-labeled GM_3) was synthesized from lactosylceramide and CMP-[^{14}C]NeuAc (New England Nuclear, Boston, MA) using the Golgi fraction of AH-109A ascites hepatoma (solid type) as the source of GM_3 synthase and diluted with non-radioactive GM_3 (dog erythrocyte or bovine brain) to give a final radioactivity of 1000 cpm/nmol. Whichever source of GM_3 was used to dilute the radioactivity, ^{14}C-labeled GM_3 thus prepared was a good substrate for sialidase. The procedure was described in detail previously.[3] DEAE-cellulose (DE-52) and AH-Sepharose 4B were the products of Whatman (Kent) and of Pharmacia (Uppsala), respectively. Antisera were produced in rabbits by immunizing them with the membrane sialidase I (anti-I) or II (anti-II) of rat brain as described elsewhere.[6] *Staphylococcus aureus* cells (Protein A-Bacterial Adsorbent) were obtained from Seikagaku Kogyo (Tokyo).

Tissues and hepatomas Normal brain and liver were obtained from male Wistar rats (150–200 g) fed *ad libitum*, and fetal liver from Wistar fetuses at 18–19 days of gestation. Regenerating liver was obtained from partially hepatectomized male Wistar rats 24 h and 48 h after the operation.[7] Primary hepatocellular carcinoma was induced in male Wistar rats by feeding them with MeDAB.[8] Transplantable AH 109A hepatoma was inoculated into male Donryu rats subcutaneously and harvested 12–14 days later.[4]

Solubilization of membrane-associated sialidase Tissues were homogenized in 4 vol of 0.25 *M* sucrose/1 m*M* EDTA using a glass/Teflon homogenizer. The homogenate was centrifuged at 600*g* for 10 min and the supernatant was centrifuged at 105,000*g* for 1 h. The resulting pellet was suspended in 1 ml/g tissue of the homogenizing buffer and used as the particulate fraction.

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Abbreviations used: MeDAB, 3′-methyl-4-dimethylaminoazobenzene; 4MU-NeuAc, 4-methylumbelliferyl-α-D-N-acetylneuraminic acid.

Membrane sialidase was then solubilized from the particulate fraction by using deoxycholate and Triton X-100 at final concentrations of 0.5 and 0.1% (w/v), respectively. After homogenization in a glass/Teflon homogenizer, the mixture was centrifuged at 105,000*g* for 1 h and the supernatant was saved.

AH-Sepharose 4B chromatography of the solubilized sialidase Brain enzyme (50 ml) solubilized as above was applied to a AH-Sepharose column (2.5×7 cm) equilibrated with 20 m*M* potassium phosphate (pH 6.8)/1 m*M* EDTA/0.1% Triton X-100 (buffer A) and eluted with a linear 0–0.4 *M* NaCl gradient in 400 ml of buffer A, collecting 10-ml fractions. The sialidase I or II eluted from the column was separately diluted with an equal volume of buffer A, and each was applied to a second column of AH-Sepharose 4B (two-thirds of the volume of the first column) and developed with a linear 0–0.4 *M* NaCl gradient in 300 ml of buffer A, collecting 7.5-ml fractions. When the chromatographic analysis of membrane-associated sialidases solubilized from rat liver and hepatomas (each 20 g) was being carried out, however, chromatography on a DEAE-cellulose column (2.5×7 cm) was substituted for the first AH-Sepharose 4B chromatography. The solubilized particulate fraction was applied to a DEAE-cellulose column equilibrated with buffer A, washed with 2 vol of the buffer and eluted with 100 ml of the buffer containing 0.2 *M* NaCl. The active fractions were pooled and diluted with 3 vol of buffer A, and applied to an AH-Sepharose column (1.5×10 cm) equilibrated with buffer A. The column was developed with a linear 0–0.4 *M* NaCl gradient in 280 ml of buffer A, collecting 7-ml fractions.

Sialidase assay The assay mixture contained 100–200 nmol (as bound sialic acid) of mixed gangliosides, 50 m*M* sodium acetate (pH 4.5), 0.05% (w/v) sodium deoxycholate and enzyme in a final volume of 0.2 ml. After incubation at 37°C for 1–2 h, the reaction mixture was immediately passed through an AG1X-2 column[4] and the sialic acid released was determined by the thiobarbituric acid method.[9] When the enzyme had been solubilized, sodium deoxycholate was omitted from the assay mixture. The enzymes that had been purified by column chromatography were assayed without passage through an AG1X-2 column. The activity toward 4MU-NeuAc was determined by measuring 4MU released spectrofluorometrically.[2] To test the immuno-reactivity of sialidase to the rabbit antisera, ^{14}C-labeled GM_3 was used as the substrate: the reaction mixture contained 5–10 nmol of the substrate, 0.1% (w/v) Triton X-100, 50 m*M* sodium acetate (pH 4.5), and enzyme in 0.1 ml. The assay procedure was described in detail elsewhere.[6] One unit of sialidase was defined as the amount of enzyme which hydrolyzed 1 nmol of sialic acid/h.

Immunoprecipitation studies Protein A-Bacterial Adsorbent (60–100 μl) was preincubated with various amounts of antiserum at 37°C for 30 min. The mixture was centrifuged at 1000*g* for 5 min, and the pellet was mixed with sialidase (2–5 units). After incubation at 37°C for 30 min with occasional shaking, the mixture was centrifuged at 10,000*g* for 10 min and the resulting supernatant was assayed for sialidase using ^{14}C-labeled GM_3 as the substrate. Percent activity precipitated by antiserum was expressed as:

$$[(A-B)/A]\times 100$$

where A is sialidase activity remaining in the supernatant after treatment with non-immune serum, and B is the activity in the supernatant after the treatment with anti-I or anti-II.

RESULTS

In earlier studies, we obtained membrane-associated sialidases I and II solubilizing rat brain particulate fraction with Triton X-100 plus deoxycholate followed by chromatography on AH-Sepharose 4B: under the conditions used, sialidase I emerged in front of sialidase II (Fig. 1a). When the two sialidases were individually applied to the second column of AH-Sepharose, however, sialidase I was found to require a greater concentration of NaCl for elution than sialidase II (Figs. 1b and 1c). That the major peaks in Figs. 1b and 1c are actually sialidases I and II, respectively, has been ascertained by their activity toward 4MU-NeuAc (Fig. 1) and immunoprecipitability with antibodies to sialidases I and II (Table I). While the reason for the changes in the elution position of sialidases observed at the second chromatography is still obscure, it is true that several contaminants including deoxycholate, which might disturb chromatographic analysis, should have been eliminated from the sialidases upon the first chromatography. After the second chromatography, sialidases I and II were no longer contaminated with each other and their elution positions became stable.

In order to examine the chromatographic pattern of the sialidase(s) of rat liver particulate fraction as quantitatively as possible, we substituted a stepwise DEAE-cellulose chromatography for the first AH-Sepharose chromatography so that sialidases I and II could be applied together to "the second" column of AH-Sepharose. As shown in Fig. 2a, first a minor then a major peak of ganglioside sialidase activity emerged from the column, their elution positions being identical to those of rat brain sialidases II and I, respectively. Then the antibodies to rat brain sialidases I and II raised in rabbits were used to identify these liver sialidases more conclusively: we found that the antibody to sialidase I totally and specifically precipitated fraction 30 (the

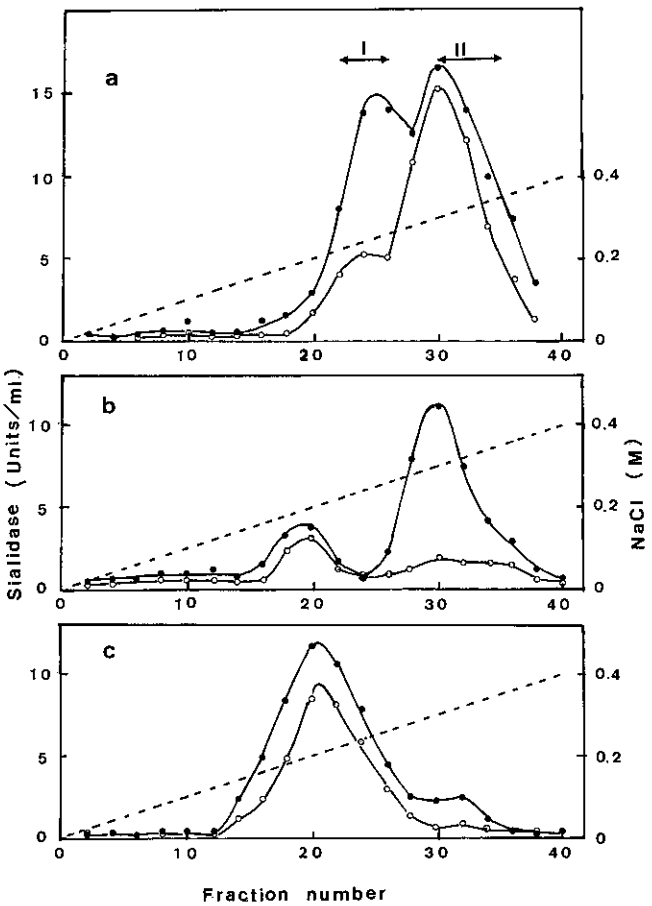

Fig. 1. Resolution of two membrane-associated and ganglioside-hydrolyzing sialidases of rat brain by AH-Sepharose 4B column chromatography. (a) The solubilized particulate fraction was chromatographed. (b) The pooled active fractions indicated as I in (a) were rechromatographed. (c) The pooled active fractions indicated as II in (a) were rechromatographed. The procedures are described in detail under "Materials and Methods." Sialidase activity was assayed with mixed gangliosides (●) or 4MU-NeuAc (○) as the substrate. ----, NaCl concentraton.

major peak) while the antibody to sialidase II precipitated fraction 20 (the minor peak) to the extent of 72% (Table I). It was confirmed by further separation on an AH-Sepharose 4B column that partial precipitation of fraction 20 by anti-I was due to contamination of sialidase I (data not shown). We may therefore conclude that rat liver particulate fraction possesses both sialidases I and II.

Fig. 2b shows the data obtained when the particulate fraction from MeDAB-induced hepatoma was treated in exactly the same manner. More than 90% of the ganglioside sialidase activity was eluted in the same position as

Table I. Immunoprecipitation of AH-Sepharose Eluates by Antisera against Membrane Sialidases I and II

Tissue	Sialidase fraction from AH-Sepharose tested	Activity precipitated[a] (%) by	
		anti-I	anti-II
Brain	Fr. 20 of Fig. 1c	0	95
	Fr. 30 of Fig. 1b	100	0
Liver	Fr. 20 of Fig. 2a	22	72
	Fr. 30 of Fig. 2a	97	0

a) The values were determined as described in the text.

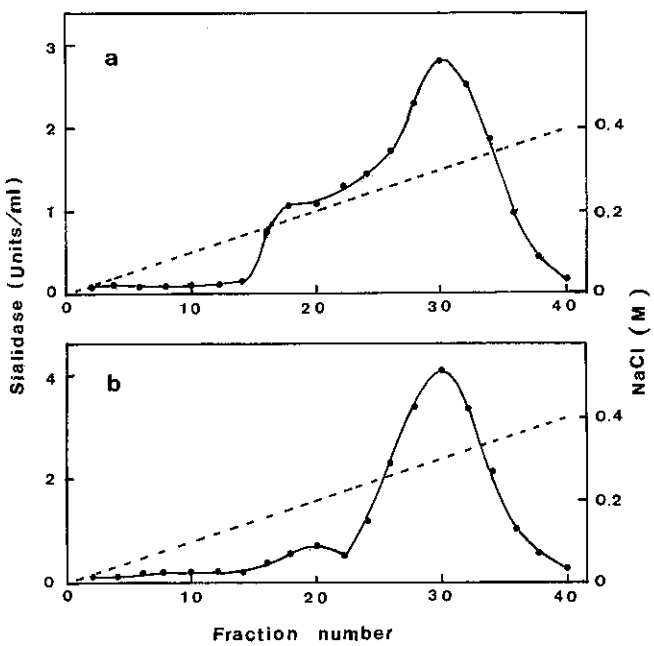

Fig. 2. AH-Sepharose 4B column chromatography of membrane-associated and ganglioside-hydrolyzing sialidases of rat liver (a) and hepatoma (b). The pooled active fractions from a DEAE-cellulose column were chromatographed on an AH-Sepharose column as described under "Materials and Method." The fractions were assayed with mixed gangliosides (●). ----, NaCl concentration.

sialidase I. It thus appears that the hepatoma does not contain sialidase II in a significant amount.

To compare the levels of liver and MeDAB-induced-hepatoma sialidases I and II in a more quantitative manner, the particulate fractions from these tissues were subjected to solubilization and the solubilized fractions were tested for immunoreactivity with the antibodies to sialidases I and II (Table II). The activities of sialidases

Table II. Immunoprecipitation of Solubilized Particulate Fractions of Liver and Hepatomas by Antisera against Membrane Sialidases I and II

	Ganglioside-hydrolyzing sialidase (unit/mg protein)[a]	Activity precipitated (%)[a,b] by		Sialidase (unit/mg protein)[c]	
		anti-I	anti-II	I	II
Control liver	12.9	61	38	7.9	4.9
	15.3	59	29	9.0	4.4
	11.2	73	36	8.2	4.0
Fetal liver	5.5	94	7	5.2	0.4
	7.1	91	3	6.5	0.2
Regenerating liver					
24 h	5.8	85	22	4.9	1.3
48 h	8.4	73	21	6.1	1.8
MeDAB-hepatoma	8.5	98	5	8.3	0.4
	8.9	85	10	7.6	0.9
	10.2	89	0	9.1	0
AH-109A	7.9	88	11	7.0	0.9
	8.2	99	10	8.1	0.8

a) The values are means of 2–3 experiments; for each experiment, the solubilized particulate fraction obtained from 3 rats (or 2 litters for fetal liver) was used. The ganglioside-hydrolyzing sialidase was quantitatively solubilized from the particulate fractions of livers and hepatomas listed above.[5]
b) The values were calculated as described in the text.
c) The values were calculated as follows: (ganglioside-hydrolyzing sialidase) × (% activity precipitated/100).

I and II in Table II were individually computed from the immunological data and the total activity of ganglioside sialidase shown in the table. Confirming the chromatographic data (Fig. 2), the data in Table II make it clear that the level of sialidase II is extremely low in the hepatoma as compared with liver. Interestingly, the level of sialidase I is not lowered in the hepatoma. Table II includes the results of a similar analysis made for AH-109A, a transplantable rat hepatoma, and fetal and regenerating liver. A marked decrease in sialidase II was observed particularly for AH-109A and fetal liver; the level of sialidase I was hardly affected in AH-109A, as in MeDAB-hepatoma, but a considerable decrease of sialidase I occurred in fetal and regenerating liver.

DISCUSSION

We have previously reported that hepatocarcinogenesis lowers the level of membrane-associated sialidase on the basis that the ganglioside sialidase activity of hepatoma particulate fraction is lower than that of hepatic particulate fraction.[5] However, it is now clear that hepatic particulate fraction contains two types of ganglioside sialidase, sialidases I and II, and that it is sialidase II that is decreased upon hepatocarcinogenesis. Since sialidase I is not particularly decreased, the membrane sialidase pattern of hepatomas shows a predominance of sialidase I (Fig. 2b). It is of interest that, unlike hepatomas, regenerating liver and fetal liver possess lowered sialidase I as well as markedly decreased sialidase II. We have also investigated the effects on membrane-associated sialidase of mouse JB6 cells of TPA, which induces anchorage-independent growth of these cells and tumorigenicity. After TPA treatment, sialidase II was found to be decreased (as it was in hepatomas) but sialidase I was substantially increased (unpublished results). This suggests that in certain cases, neoplastic transformation may be accompanied by an increase in sialidase I. This view is further supported by the data of Schengrund *et al.*,[10] who reported that virally transformed BHK cells but not their normal counterpart hydrolyzed exogeneously added gangliosides.

The present study together with our previous studies[1–3, 11] establishes that rat liver contains at least four types of sialidase, intralysosomal, cytosolic, membrane-I and membrane-II. Intralysosomal sialidase is a catabolic enzyme judging from its subcellular location, optimal pH and substrate specificity. This judgement is also compatible with the neoplastic increase of this enzyme,[4] since numbers of lysosomal glycosidases have been shown to be increased in tumors.[12–15] Membrane sialidase I is located in the plasma membrane and attacks only gangliosides including GM_1 and GM_2.[6] In splenic macrophages activated with OK-432, this type of enzyme

is increased more than 10-fold concomitantly with the appearance of cell surface antigen asialo-GM_1,[16] thereby suggesting that the major function of membrane sialidase I may be to modify the cell surface gangliosides. If this is true, the present results indicate that modification of cell surface gangliosides occurs actively in tumors.

In contrast to membrane sialidase I, membrane sialidase II, whose major site appears to be the lysosomal membrane,[6] is markedly decreased upon hepatocarcinogenesis, as is a previously reported cytosolic sialidase.[4] The two sialidases are capable of attacking intact glycoproteins, which are only poor substrates for intralysosomal and plasma membrane sialidases. This may have a bearing on the data of Warren *et al.*,[17] who reported that neoplastic hypersialylation occurs mainly if not exclusively in the sugar chains of glycoproteins.

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REFERENCES

1) Miyagi, T. and Tsuiki, S. Rat liver-lysosomal sialidase. Solubilization, substrate specificity and comparison with the cytosolic sialidase. *Eur. J. Biochem.*, **141,** 75–81 (1984).
2) Miyagi, T. and Tsuiki, S. Purification and characterization of cytosolic sialidase from rat liver. *J. Biol. Chem.*, **260,** 6710–6716 (1985).
3) Miyagi, T. and Tsuiki, S. Evidence for sialidase hydrolyzing gangliosides GM_2 and GM_1 in rat liver plasma membrane. *FEBS Lett.*, **206,** 223–228 (1986).
4) Miyagi, T., Goto, T. and Tsuiki, S. Sialidase of rat hepatomas: qualitative and quantitative comparison with rat liver sialidase. *Gann,* **75,** 1076–1082 (1984).
5) Sagawa, J., Miyagi, T. and Tsuiki, S. Membrane-associated sialidase of rat liver and its decrease in hepatomas. *Jpn. J. Cancer Res.*, **79,** 69–73 (1988).
6) Miyagi, T., Sagawa, J., Konno, K., Handa, S. and Tsuiki, S. Biochemical and immunological studies on two distinct ganglioside-hydrolyzing sialidases from the particulate fraction of rat brain. *J. Biochem.*, **107,** 787–793 (1990).
7) Miyagi, T. and Tsuiki, S. Glucosamine 6-phosphate synthase of regenerating rat liver. *Cancer Res.*, **39,** 2783–2786 (1979).
8) Miyagi, T. and Tsuiki, S. Studies on UDP-N-acetylglucosamine: α-mannoside β-N-acetylglucosaminyltransferase of rat liver and hepatoma. *Biochim. Biophys. Acta,* **668,** 148–157 (1981).
9) Warren, L. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.*, **234,** 1971–1975 (1959).
10) Shengrund, C-L., Lausch, R. N. and Rosenberg, A. Sialidase activity in transformed cells. *J. Biol. Chem.*, **248,** 4424–4428 (1973).
11) Miyagi, T., Sagawa, J., Konno, K. and Tsuiki, S. Immunological discrimination of intralysosomal, cytosolic and two membrane sialidases present in rat tissues. *J. Biochem.*, **107,** 794–798 (1990).
12) Bosmann, H. B. Elevated glycosidases and proteolytic enzymes in cells transformed by RNA tumor virus. *Biochim. Biophys. Acta,* **264,** 339–343 (1972).
13) Bosmann, H. B., Lockwood, T. and Morgan, H. R. Surface biochemical changes accompanying primary infection with Rous sarcoma virus. II. Proteolytic and glycosidase activity and sublethal autolysis. *Exp. Cell Res.*, **83,** 25–30 (1974).
14) Bosmann, H. B. and Hall, T. C. Enzyme activity in invasive tumors of human breast and colon. *Proc. Natl. Acad. Sci. USA,* **71,** 1833–1837 (1984).
15) Chatterjee, S. K., Bhattacharya, M. and Barlow, J. J. Glycosyltransferase and glycosidase activities in overian cancer patients. *Cancer Res.*, **39,** 1943–1951 (1979).
16) Sagawa, J., Miyagi, T. and Tsuiki, S. Effects of OK-432 activation on the sialidase activities of rat peritoneal macrophages. *Tohoku J. Exp. Med.*, **160,** 149–156 (1990).
17) Warren, L., Buck, C. A. and Tuszynski, G. P. Glycopeptide changes and malignant transformation, a possible role for carbohydrate in malignant behaviour. *Biochim. Biophys. Acta,* **516,** 97–127 (1978).