Ganglioside Patterns of Metastatic and Non-Metastatic Transplantable Hepatocellular Carcinomas of the Rat

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In previous investigations, we correlated levels of sialic acid, gangliosides, and ganglioside glycosyltransferases with tumorigenesis over a 24-week continuum of growth of hepatocellular neoplasms of the rat induced by the carcinogen N-2-fluorenylacetamide. However, metastatic tumors developed only rarely and were not analyzed. To investigate surface changes associated with metastasis, well-differentiated and poorly differentiated hepatocellular carcinomas were transplanted to syngeneic recipient rats. From those, several metastatic and nonmetastatic isolates were obtained and compared. Both total and ganglioside sialic acid amounts in transplantable hepatomas were elevated above control liver values but were significantly lower for metastatic lines than for nonmetastatic lines. The nonmetastatic lines were characterized by ganglioside patterns depleted in the precursor ganglioside G_{M3} (sialic acid-galactose-glucose-ceramide) and elevated in the products of the monosialoganglioside pathway. In contrast, metastatic isolates exhibited a restoration of G_{M3} and nearer normal amounts of other gangliosides. The findings point to differences in sialic acid-containing glycolipids, comparing metastatic and nonmetastatic hepatocellular carcinomas, and further extend the concept that ganglioside alterations do not cause tumorigenesis but are the end result of a cascade of events which apparently continue beyond the onset of metastasis.

Both neutral glycosphingolipids and sialoglycosphingolipids (gangliosides) appear altered in amount and composition when a cell undergoes neoplastic transformation [1, 2]. The alterations frequently correlate with changes in activity of one or more glyco-

syltransferases responsible for the single sugar additions required for the biosynthesis of these glycoconjugates [3]. Not infrequently, the alteration appears as a deletion or reduction of the complex glycolipids concomitant with an increase in the simpler, precursor glycolipids [4, 5]. Particularly with liver cancer, the overall effect is to increase total glycolipid constituents including total ganglioside sialic acid [6] and neutral glycolipids [7].

While ganglioside amount and composition have been analyzed for a variety of hepatic neoplasms and contrasted with values from normal rat liver [4, 7–15], comparisons of metastatic and nonmetastatic hepatoma lines have not been reported. Metastasis of tumors is a phenomenon related to the cell surface [16, 17]. Gangliosides represent informational-type molecules enriched at the cell surface [18, 19], which function as receptors and antigens [20] and exhibit compositional alterations during the course of the tumorigenic progression [1–15]. In the present study, ganglioside profiles of metastatic and nonmetastatic transplantable hepatoma lines were compared in an effort to extend information on the tumorigenic cascade of glycolipid changes in liver cancer to and beyond the initial metastatic events.

MATERIALS AND METHODS

Animals and Tumors

The carcinogen, N-2-fluorenylacetamide (Aldrich) was administered orally at a level of 0.025% in a low-protein diet to male inbred rats (CDF; Charles River Breeding Laboratories, Wilmington, Massachusetts). After 6–12 months, hepatocellular carcinomas were removed from the liver, minced into 1- to 2-mm fragments and injected subcutaneously using a trocar into syngeneic recipients or processed for tissue culture (see Kloppel et al [21]; and Kloppel and Morré, submitted for publication). Tumorigenicity of tissue culture cells was monitored by injecting saline-washed cells into syngeneic animals. Pulmonary metastasis was confirmed by observing hepatocellular foci on the surface of the lungs. Tumor growth rates were estimated from measurements of tumor dimensions [22].

Hepatomas growing subcutaneously were aseptically removed and cleared of necrotic tissue prior to mincing for subsequent passages. Nonnecrotic samples for biochemical analysis were frozen at −20°C. All tissues were fixed with 10% buffered formalin for light microscopy. Regenerating liver was obtained 1 week following partial hepatectomy of sodium pentobarbital-anesthetized rats.

Analysis of Sialic Acid and Gangliosides

Hepatomas, control livers, or regenerating livers were homogenized in 1 volume of 1 mM sodium bicarbonate prior to protein [23] and total sialic acid [24] determinations. Homogenates were extracted over night at 4°C with 10 volumes of chloroform: methanol (CM) (1:1, v/v), filtered over medium porosity sintered glass, and the residue reextracted with CM (2:1) to remove gangliosides. Gangliosides were purified from the combined filtrates using DEAE-Sephadex (Sigma) and Unisil (Clarkson) chromatography [25]. The ganglioside content of purified fractions was determined by sialic acid quantitation [24] prior to composition analysis by thin-layer chromatography (Fig. 1) [26].
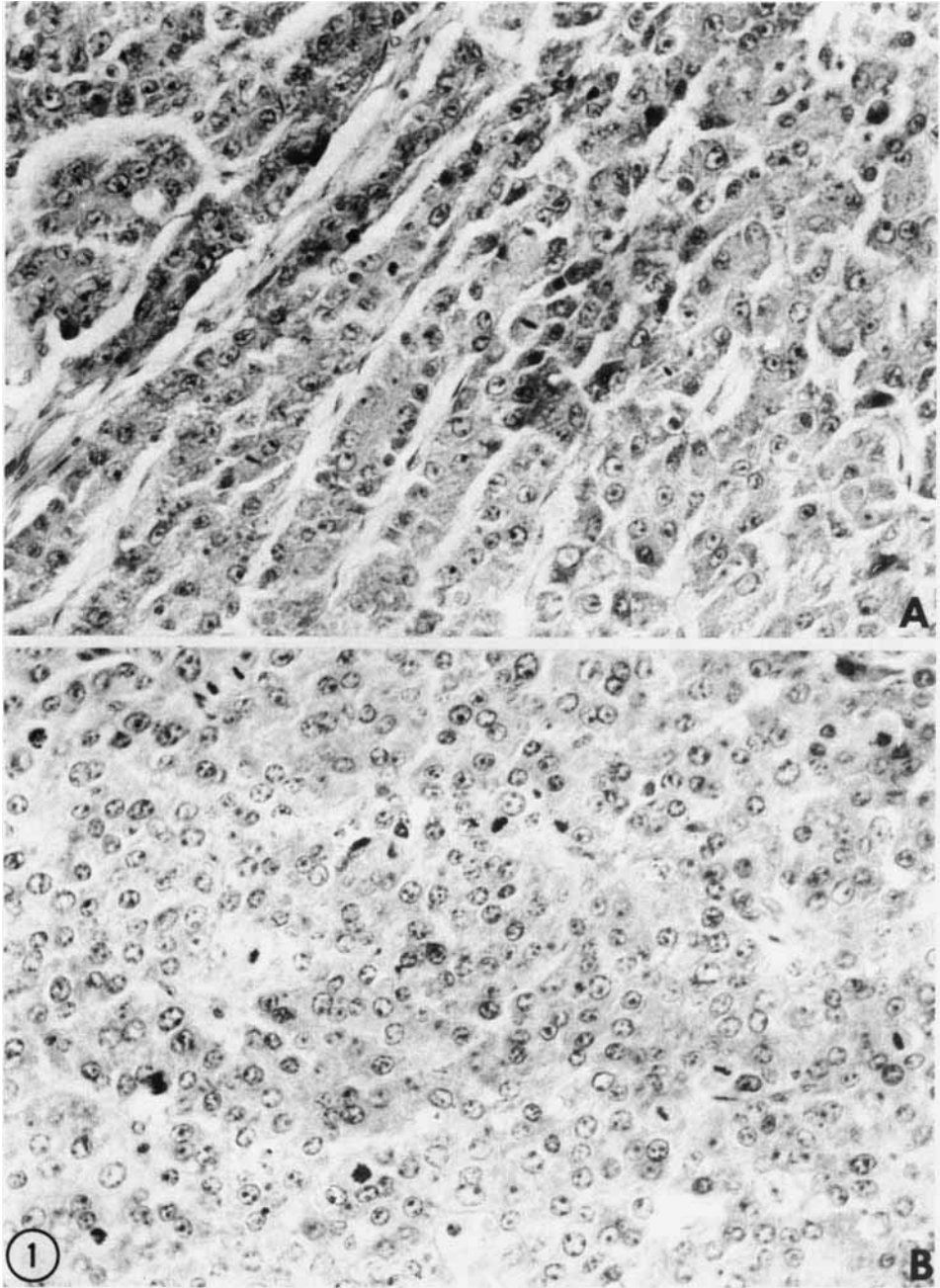
Fig. 1. A: Well-differentiated hepatocellular carcinoma with cellular organization reminiscent of normal liver; cells and nuclear sizes are near normal in appearance. B: Poorly differentiated hepatoma with overall loss of cellular organization, irregular cell and nuclear sizes, and multiple and prominent nucleoli. Hematoxylin and eosin; × 700.

TABLE I. Total and Ganglioside Levels of Sialic Acid in Liver and Transplantable Hepatomas

Tissue[b]	Sialic acid (nmoles/mg protein)[a]	
	Total	Ganglioside
Normal liver	4.5 ± 1.5 (12)	0.22 ± 0.08 (5)
Regenerating liver	3.4 ± 0.4 (3)	0.27 ± 0.12 (3)
Transplanted hepatomas		
Nonmetastatic		
T_2 (PD)	16.0 ± 6.9 (4)	1.12 ± 0.08 (4)
T_7 (PD)	9.2 ± 2.9 (3)	0.76 (1)
T_{10} (WD)	19.2 (1)	0.40 (1)
H_7 (PD)	25.3 ± 9.8 (3)	
Ave	17.9 ± 8.0 (11)[c]	0.94 ± 0.31 (6)[d]
Metastatic		
T_1 (PD)	15.6 ± 3.9 (3)	0.43 (1)
T_3 (HD)	8.3 (2)	0.28 (1)
T_8 (WD)	6.2 (2)	0.55 (1)
Ave	11.8 ± 4.4 (7)[c]	0.42 ± 0.14 (3)[d]

[a]Values are means ± standard deviations. Numbers in parentheses indicate number of samples analyzed in duplicate.

[b]Letters following hepatoma designations indicate histologic classification.
PD = poorly differentiated; WD = well-differentiated; HD = highly differentiated.

[c]Means are significantly different $(P < 0.10)$.

[d]Means are significantly different $(P < 0.01)$.

RESULTS

Characteristics of Transplantable Hepatomas

A variety of hyperplastic nodules and hepatocellular carcinomas of the liver were obtained following carcinogen treatment. However, only the hepatomas were successfully transplanted to syngeneic recipients. Following the scheme of Reuber [27], those hepatomas scored as well-differentiated displayed some cell order reminiscent of normal liver and cells and nuclei of relatively normal size and appearance (Fig. 1A). Poorly differentiated hepatomas lacked cell order, demonstrated irregular cell and nuclear sizes, and contained multiple and prominent nucleoli (Fig. 1B).

Growth rates of the transplanted hepatomas ranged from 0.1 to 0.8 mm/day. There was no obvious correlation between growth rate and degree of differentiation or between either of these two parameters and the ability to metastasize. Both slow- and fast-growing hepatomas of either well-differentiated or poorly differentiated lines demonstrated the ability to metastasize during the course of this study. Correspondingly, there was no correlation between ability to metastasize and degree of histologic deviation from normal.

Total Sialic Acid and Gangliosides

Sialic acid was increased in total amount in most transplantable hepatocellular carcinomas compared to normal or regenerating livers (Table I). Interestingly, total sialic

TABLE II. Percentage Composition of Gangliosides in Liver and Transplantable Hepatomas

Gangliosides[a]		Liver[b]		Transplantable hepatomas[b]	
		Normal (N = 4)	Regenerating (N = 2)	Nonmetastatic (N = 4)	Metastatic (N = 3)
G_{M3}	Gal-Glc-Cer NAN	46 ± 8	63	7 ± 4	40 ± 8
Monosialoganglioside pathway					
G_{M2}	GalNAc-Gal-Glc-Cer NAN	10 ± 2	10	2 ± 2	5 ± 5
$G_{M1} + G_{D1a}$	Gal-GalNAc-Gal-Glc-Cer + Gal-GalNAc-Gal-Glc-Cer NAN NAN NAN	30 ± 3	16	77 ± 6	37 ± 6
Disialoganglioside pathway					
G_{D3}	Gal-Glc-Cer NAN-NAN	7 ± 3	3	7 ± 4	16 ± 8
$G_{D1b} + G_T$	Gal-GalNAc-Gal-Glc-Cer + higher homologs NAN-NAN	8 ± 6	8	8 ± 4	2 ± 1
$\dfrac{G_{M2} + G_{M1} + G_{D1a}}{G_{D3} + G_{D1b} + G_T}$ Ratio[c]		2.6	2.4	5.3	2.5

Based on densitometer (Varicord) tracings of resorcinol-positive bands corrected for number of sialic acid residues per molecule. Values represent the mean ± standard deviation.

[a]Identified by comparison with standards. Cer = ceramide; Gal = galactose; Glc = glucose; GalNAc = N-acetylgalactosamine; NAN = N-acetylneuraminic acid (sialic acid); G_T = trisialogangliosides and higher homologs of G_{D1b} and G_{T3}. Use of a different solvent system (n-propanol: ammonium hydroxide; 7:3) resolved the G_{M1} and G_{D1a} bands; spots were approximately 70–80% G_{D1a}.

[b]N = number of samples analyzed in duplicate.

[c]Ratio of monosialoganglioside pathway products and intermediates to those of the disialoganglioside pathway.
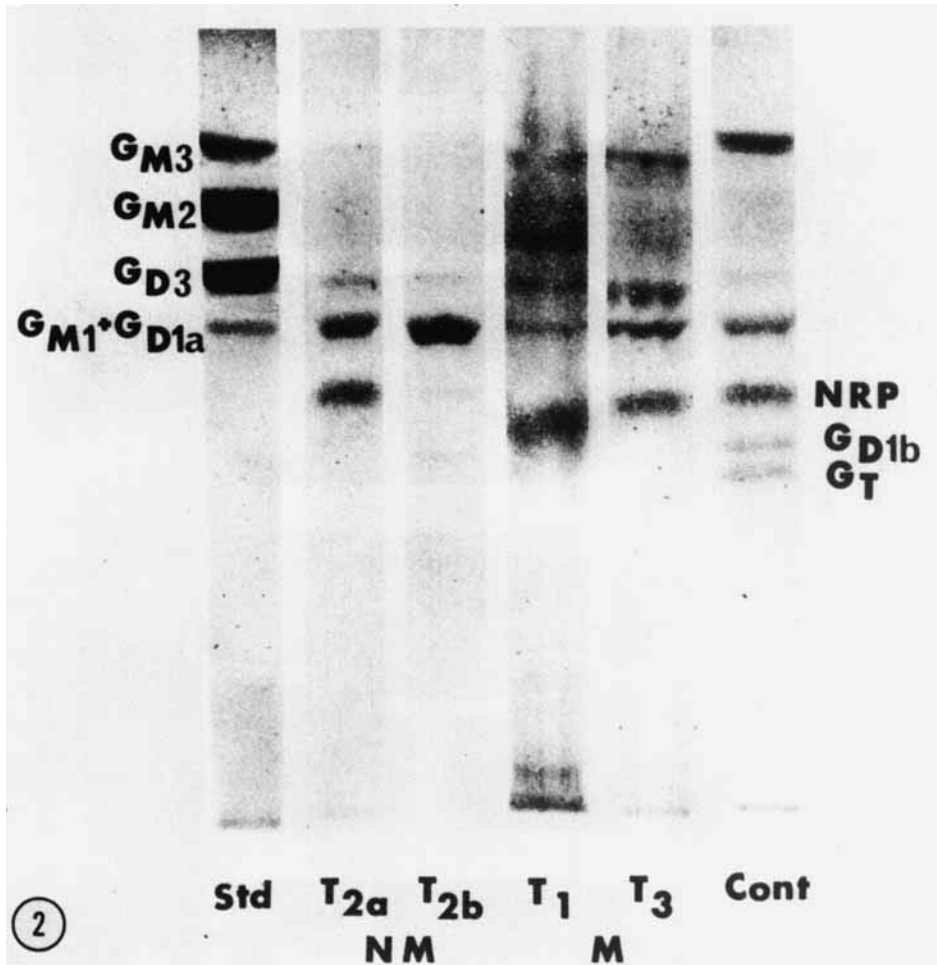
Fig. 2. Gangliosides from normal rat liver and metastatic (M) and nonmetastatic (NM) transplanted hepatomas. Tumors 2a and 2b represent the same transplantable line at two different generations. Silica gel G (Analtech) thin-layer plates were developed in chloroform:methanol:ammonium hydroxide:water (65:30:7:2.5, v/v) and sprayed with resorcinol reagent to detect sialic acid-containing glycolipids. NRP, nonresorcinol-positive band. Std, ganglioside standards. See Table II for an explanation of ganglioside terminology.

acid was lower in two or three metastatic lines and the mean for the metastatic lines was significantly lower than the mean of the nonmetastatic lines.

Ganglioside sialic acid was increased at least two-fold in all transplantable hepatomas when contrasted with the control average of 0.22 nmoles ganglioside sialic acid per milligram protein (Table I). As with total sialic acid, ganglioside sialic acid was significantly reduced in the metastatic lines.

Total and ganglioside sialic acid levels were similar comparing control and regenerating livers (Table I).

Ganglioside Composition

All transplantable hepatoma tissues exhibited different ganglioside patterns when contrasted with control tissues. A major difference, however, was observed between metastatic and nonmetastatic lines. Nonmetastatic lines were depleted in G_{M3} and enriched in G_{D1a}, while metastatic lines, although different from normal, exhibited a more normal pattern (Table II; Fig. 2). An elevation of the ratio of the gangliosides in the monosialoganglioside pathway to those in the disialoganglioside pathway was observed only in the nonmetastatic lines.

Regenerating liver yielded a relatively normal ganglioside pattern. The differences noted were, if anything, an enrichment in G_{M3} and a decrease in $G_{M1} + G_{D1a}$.

DISCUSSION

The development of hepatocellular carcinomas of N-2-fluorenylacetamide-treated livers was slow and progressive as observed by previous investigators [28]. That only certain hepatocellular foci of livers of carcinogen-treated animals were capable of transplantation is indicative of a critical transformation event which separates or distinguishes transplantable hepatomas from other premalignant forms of neoplasms [29]. Within the population of transplantable hepatomas, some eventually formed metastases while others did not.

Ganglioside patterns of rat liver change markedly during both development and tumorigenesis [4, 6]. The patterns of change are relatively complex in an apparent cascade of events in which levels of various ganglioside intermediates vary depending on the stage of development. Deletions or reductions in G_T and accumulations of G_{M3} or G_{D1a} [10–12] have been noted; G_{D1b} has also been observed to be increased in tumor tissue [8] as has G_{M1} [4]. There appears to be no common alteration in ganglioside profiles that characterizes all hepatomas in all stages of development [13–15]. What may happen is a sequence of ganglioside changes as summarized by Merritt et al [4]. In this sequence, a "cross-over" point was predicted at or about the time of metastasis where the levels of monosialogangliosides, at least, should approach control levels. The lower total and ganglioside sialic acid in metastatic lines has also been observed in metastatic and nonmetastatic cell lines derived from murine lung tumors (unpublished results). In this context, the apparent "return to normalcy" of the metastatic lines of transplantable hepatomas is of interest. To what extent the relatively normal ganglioside pattern of metastatic lines observed in the present study relates to the cells' ability to dislodge from the primary tumor mass and survive in metastatic isolation remains to be investigated.

ACKNOWLEDGMENTS

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