Sialic Acid Elevated in Experimental Liver Cancer[1]

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Introduction

Constituents of cell surfaces of mammalian cells which may be important to cancer-related properties are glycoproteins and glycolipids (14, 15). Sialic acid is a common terminal saccharide on many of these glycoproteins and glycolipids.

Some tumors have been reported to have elevated sialic acid content, including human tumors of the colon, stomach, breast and other tissues (1, 2, 6) and experimental liver tumors of the rat (8-10). Some authors, however, have concluded that specific cell surface sialic acid changes are not a general property of neoplastic cells (11, 17). In cultured transformed cells, the most frequent change is a lowering of the membrane sialic acid content (3, 12, 13).

As part of a continuing study to determine to what extent sialic acid changes are associated with experimental liver cancer, the present study compares the sialic acid content of several transplantable hepatomas and normal livers of animals bearing transplantable hepatomas to those of carcinogen-induced squamous cell carcinomas and normal and regenerating liver. The results, although preliminary, suggest a pattern of sialic acid change which may be a property of neoplastic cells when considered in the context of previously published results from our laboratory and work of others.

Materials and Methods

To obtain hyperplastic and neoplastic liver tissues, inbred (CDF) male Wistar rats (Carworth Farms, New City, N.Y.) weighing 150 to 170 g were fed a low protein basal diet (Carcinogenic Basal Diet, Teklad Mills, Madison, Wisconsin) containing 0.05% N-2-fluorenylacetamide (Aldrich Chemical Co.) according to the schedule of Merkow et al. (7). Control rats received basal diet without added carcinogen. At the end of a 13-week feeding schedule, all rats were fed the basal diet for two additional weeks. Rats were killed by cervical dislocation after a 24 hr fast and bled.

Transplantable tumors originated from carcinogen treated livers and were harvested 6 to 10 months after the beginning of carcinogen administration. Hyperplastic nodules and hepatomas appearing in tissues were removed, washed, minced in sterile salt solution and injected subcutaneously into syngeneic recipients. At the same time, a portion of each tumor was fixed in Bouin's fixative solution or buffered 2% glutaraldehyde for histopathological analysis. Any remaining tissue was stored at -20° C for determination of sialic acid and protein. Once the transplanted tumors had reached a diameter of approximately 3 cm, they were removed aseptically, minced and transplanted

131

into a second syngeneic recipient or processed for tissue culture as outlined below.

To initiate tumor cell lines in culture, the transplantable hepatomas were rinsed in calcium- and magnesium-free balanced salt solution and necrotic areas were removed. Finely minced portions of the tumor were added to growth media or incubated at 37° C in a trypsinizing flask with either 1% collagenase, 0.25% trypsin, or 0.05% trypsin containing 0.02% EDTA for intervals of 15 to 20 min. After incubation, cells were removed, washed and placed in growth medium. The growth medium was a minimum essential medium with Earles salts or Ham's F-10 nutrient mixture supplemented with 15 to 20% fetal calf serum, or 10% donor horse serum and 10% fetal calf serum. No single combination of processing, medium, or serum was successful with all tumors processed. Once the cultures had become confluent, or when dense colonies developed, subcultures were obtained by routine procedures. Tumorigenicity was monitored by harvesting cells and injecting the saline washed cells into syngeneic recipient animals.

Regenerating liver was induced by surgical removal of one or two liver lobes of sodium pentobarbital anesthesized animals. Ligation with suture was used to prevent hemorrhaging. Hyperplastic liver tissue was removed one week later and frozen for later analysis.

For biochemical analysis, tissues were minced, rinsed to remove residual blood, and homogenized in four volumes of ice-cold distilled water with a Polytron tissue homogenizer (Kinematica, Lucerne, Switzerland). The resulting homogenates were sampled for determination of protein (5) and sialic acid (16). For sialic acid determinations, samples were hydrolyzed with 0.5 ml 0.1 N HCl for 1 hour at 80° C, and the sialic acid determined by the thiobarbituric acid procedure of Warren (16). In order to reduce interference from the crude homogenates, values of sialic acid were calculated by recording absorbance at 2 wavelengths and substituting these values into the following equation: nmoles sialic acid = 90 $(O.D._{549})$ - 33 $(O.D._{532})$. The value reported is the mean of triplicate determinations.

Results

The transplantable hepatomas studied were well differentiated hepatomas derived from primary tumors induced in the rat by oral administration of the carcinogen N-2-fluorenylacetamide. Tumors were analyzed after the third transfer in syngeneic recipients.

On a protein basis, levels of total sialic acid were elevated 1.4 to 4.0 times control liver in the four transplantable hepatomas analyzed (Table 1). Additionally sialic acid levels were significantly elevated in livers from carcinogen treated animals prior to the appearance of either hepatomas or hyperplastic nodules and even in apparently normal livers of animals bearing transplantable hepatomas subcutaneously implanted. In contrast, regenerating liver showed no elevation in sialic acid. A transplantable squamous cell carcinoma from the jaw region (*in vivo* and *in vitro*), derived from rats fed the carcinogen N-2-fluorenylacetamide, showed sialic acid values similar to those of hepatomas (Table 1).

TABLE 1. *Sialic acid from experimental tumors and control tissues.*

Tissue Source	Total Sialic Acid (nanomoles per mg protein) ± Standard Deviation
Control Liver	4.5 ± 0.3
Liver from Carcinogen-Treated Animals	7.0 ± 1.0
Regenerating Liver	3.8
Transplantable Hepatomas	
$\quad$ RLT1	18.0
$\quad$ RLT2	16.4
$\quad$ RLT3	6.3
$\quad$ RLT7	10.3
Liver from Rats Bearing Transplantable Tumors	5.9 ± 0.7
Transplantable Squamous Cell Carcinomas from Jaw Region	
$\quad$ JT1	18.0
$\quad$ JT2	16.9
$\quad$ In Cell Culture	21.6

When sialic acid content was expressed as a function of growth rate for the four transplantable hepatomas and two jaw tumors, an optimum curve was obtained (Fig. 1). Sialic acid content was greatest on a protein basis (or fresh weight basis) with tumors of intermediate growth rate. The slowest and most rapidly growing hepatomas had specific sialic acid values of lesser magnitude although still elevated relative to control liver.

TABLE 2. *Characteristics of experimental tumors cultured in vitro.*

Tissue Source	Derived from Transplantable Tumor	Transfer Number *In Vivo*	Predominant *In Vitro* Cel Type	Tumor Production *In Vivo*
Liver	RLT1	1	epithelial-like	yes
		2	fibroblast-like	no
	RLT2	1	fibroblast-like	
		2	fibroblast-like	
	RLT3	1	fibroblast-like	no
	RLT4	1	fibroblast-like	
Jaw Region	JT1	1	epithelial-like	yes
	JT2	1	epithelial-like	yes

Those hepatoma and squamous cell carcinoma lines successfully carried in cell culture are summarized in Table 2. Thus far, only the squamous cell carcinoma and one hepatoma line have been successfully transplanted back into an animal.
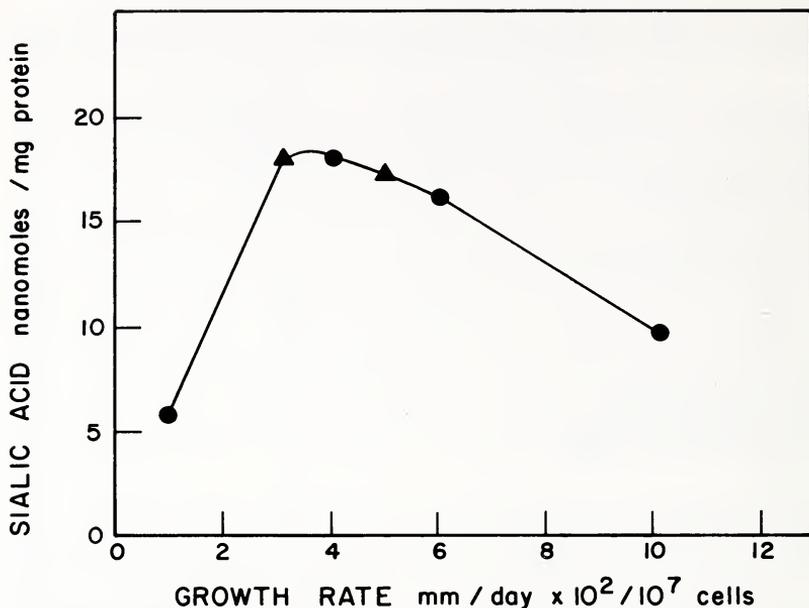
FIGURE 1. *Relationship between specific sialic acid content and growth rate of transplantable hepatomas (▲) and squamous cell carcinomas of the jaw region (●) originally induced in the rat by administration of the carcinogen N-2-fluorenylacetamide.*

Discussion

As emphasized in the Introduction, sialic acid alterations during tumorigenesis have been questioned as to general significance because, while elevations have been recorded for solid tumors, a frequent change in transformed cells in culture is a lowering of sialic acid content (11, 17). Our findings taken together with previous work from our laboratory and work of others and summarized in Figure 2, however, suggest that sialic acid changes may exhibit a more meaningful pattern than previously suspected.

Merritt et al. (8) reported increased sialic acid in preneoplastic hyperplastic nodules of rat liver induced by administration of the N-2-fluorenylacetamide carcinogen. Later studies (9) compared pooled small hepatomas most of which were classified as well differentiated as well as livers from carcinogen-treated animals, liver tissue surrounding nodules and hepatomas, fetal liver and livers of developing animals.

Sialic acid levels are elevated in fetal liver and at birth, drop sharply in the week after birth and remain more or less constant in the adult. Following administration of carcinogen, sialic acid values once again begin to increase with a nearly 2-fold elevation in hyperplastic liver nodules. Maximum values are attained in well differentiated hepatomas with a decline in invasive, poorly differentiated and poorly circumscribed hepatomas.
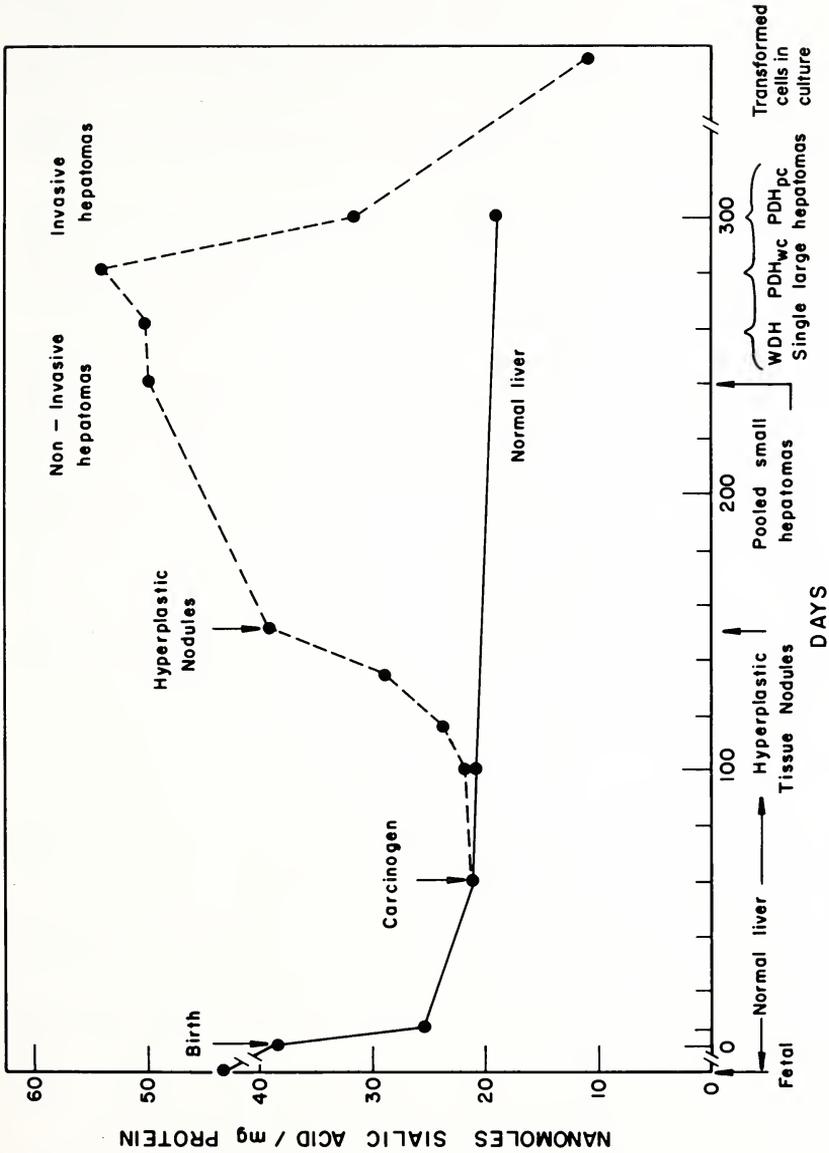
FIGURE 2. *Summary of changes in total specific sialic acid content during liver development and N-2-fluorenylacetamide-induced tumorigenesis in rat liver and cells in culture. Indications for cells in culture are based on information from the literature (see discussion). Other values are from the studies of Merritt et al. (8, 9) in our laboratory.*

The present results augment and extend these observations. It should be noted that the absolute values obtained are less than those reported by Meritt et al. (8, 9). However a different method of sialic acid determination was utilized in the present investigation along with a procedure to correct for interference from non-sialic acid sources of absorbing chromogens. However, the relative values obtained are comparable to those of Merritt et al. (89) and show an optimum curve with maximum sialic acid content on a protein basis with transplantable tumors of intermediate rates of growth.

These findings point to an explanation for changes in sialic acid of opposite sign previously obtained. Cell lines of fibroblast origin exhibit extremely rapid rates of growth. Transformation serves to give an even more rapid rate of growth. Thus a decrease in sialic acid under such conditions would follow the same pattern we have observed with the transplantable hepatomas.

Although we have not studied the cause of the increased total sialic acid content in hyperplastic tissues and well differentiated hepatomas, other studies from our laboratory indicate that similar increases in lipid-associated sialic acid (sialic acid-containing glycolipids = gangliosides) are attributable to increased specific activities of glycolipid biosynthetic enzymes (10). Recently Kloppel et al. (4) reported a biochemical method of cancer detection based on serum analysis of lipid-associated sialic acid in mice and humans bearing mammary and colonic carcinomas. In this regard, the elevated sialic acid levels in livers of rats bearing transplantable hepatomas are of interest. Taken with the observations of Kloppel et al. (4) that a serum sialic acid fraction is elevated, the findings point to elevations in sialic acid as a primary and early tissue response to the presence of cancer or to specific lesions of a potentially precancerous nature.

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