Tissue and Urinary Glycosaminoglycan Patterns Associated with a Fast, an Intermediate, and a Slow-growing Morris Hepatoma

Charles E. Kupchella, E. Elaine Drake, Jeffrey Kennedy, Kevin L. Curran, Raya Warick, and H. P. Morris

Cancer Center, University of Louisville, Louisville, Kentucky 40201 [C. E. K., E. E. D., J. K., K. L. C., R. W.], and the Department of Biochemistry, Cancer Research Unit, College of Medicine, Howard University, Washington, D. C. [H. P. M.]

ABSTRACT

The purpose of this investigation was to evaluate the glycosaminoglycans (GAG's) in different behavioral-histological types of l.m.-transplanted hepatomas and in the liver and urine of animals bearing these tumors. Groups of 10 Buffalo rats carrying fast-growing (7777), intermediate (5123tc), and slow-growing (9618A) Morris hepatomas were studied as the tumors reached 3 cm. Urinary and tissue GAG's were isolated by proteolysis, separated as cetylpyridinium complexes, and measured as uronic acid. The GAG's were further purified using anion-exchange chromatography and characterized with mucopolysaccharidases. Tissue GAG's were also evaluated histochemically using Alcian blue staining and mucopolysaccharidases. Tissue GAG's were also evaluated histochemically using Alcian blue staining and mucopolysaccharidases. Tissue from fast-growing, intermediate, and slow-growing tumors exhibited greater GAG levels than did normal liver in the hyaluronic acid (0.4 M NaCl-soluble) fraction and in the chondroitin sulfate-heparan sulfate (1.2 M NaCl-soluble) fraction. The livers of tumor-bearing animals exhibited GAG levels similar to those of normal liver. Increased urinary GAG excretion was appreciated in animals bearing Tumors 5123tc and 9618A but not in those bearing Tumor 7777.

INTRODUCTION

An increasing number of reports cite the presence of comparatively high levels of GAG's in both animal tumors (4, 7, 18, 22) and human tumors (5, 12, 16). There also have been reports citing qualitatively and quantitatively abnormal urinary GAG excretion in association with malignant tumors (6, 10, 11, 23-25). Although there have been attempts to establish any functional relationship between tumor GAG's and tumor cell properties (23, 25-27, 30, 31), the significance of elevated GAG's in malignant tumors remains obscure.

In view of the possibility that GAG's play an important role in the expression of one or more malignant cell properties, our purpose here was to evaluate the GAG patterns associated with transplantable hepatomas exhibiting different growth rates and metastatic properties. Because it has been suggested that tumor GAG may be contributed by normal host tissue in response to the presence of hepatic tumor (7), a secondary purpose was to evaluate the influence, if any, of "remote" hepatomas on the GAG's of the host liver; a third purpose was to evaluate the urinary GAG patterns in hepatoma-bearing animals.

1 Supported by American Cancer Society Grant IN-111B, by a grant from the Manufacturing Chemists Association, and in part by USPHS Grants CA 10729 and CA 246201. 2 Present address: Biological Sciences, Murray State University, Murray, Ky. 42071. To whom requests for reprints should be addressed. 3 The abbreviation used is: GAG, glycosaminoglycan.

MATERIALS AND METHODS

Materials. Hyaluronic acid (umbilical cord) was purchased from Nutritional Biochemical Corp. (Cleveland, Ohio); chondroitin sulfate (whale and shark cartilage) and sodium heparin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Authentic samples of heparin, chondroitin 4-sulfate, heparan sulfate, and hyaluronate were also kindly supplied by Dr. M. B. Matthews, University of Chicago. Bovine testicular hyaluronidase was purchased from ICN Pharmaceuticals (Cleveland, Ohio), Streptomyces hyaluronidase was obtained from Calbiochem (La Jolla, Calif.), and Proteus vulgaris chondroitinase ABC was purchased from Sigma.

Experimental Design. Forty male Buffalo rats were shipped from Lab Supply Company (Indianapolis, Ind.) to Washington, D. C., where 10 were inoculated bilaterally (thigh) with Tumor 7777, 10 were inoculated with Tumor 5123tc, and 10 were inoculated with Tumor 9618A. These were shipped to Louisville with 10 controls. Throughout the study, animals were provided free access to food and water even when placed in metabolic cages for urine collections.

Urine collections were made twice each week from animals bearing Tumor 7777 and once each week from animals bearing Tumors 5123tc and 9618A. Collections were made alternatively on one-half of the animals in each group, with 5 control animal collections made each time a collection was made from tumor-bearing animals.

Tumors. Line 5123tc is a tissue culture variant of a moderately differentiated trabecular hepatocellular carcinoma induced with dietary administration of N-2-fluorenylphthalamic acid. Tumors were received and studied here in the 165th generation. Tumor line 7777 is a poorly differentiated hepatocellular carcinoma induced with dietary administration of N-2-fluorenylphthalamic acid. This line was studied in the 159th transplant generation. Tumor line 9618A is a well-differentiated hepatocellular carcinoma induced by dietary administration of N-2-fluorenylphthalamic acid. This line was studied in the 13th generation.

Characteristics of these 3 tumors observed in our laboratory and selected characteristics reported by Hruban et al. (13, 14) are summarized in Table 1 (see also Fig. 1).

Extraction and Purification of GAG's from Liver and Tumor Tissue. Dry defatted tissue was subjected to proteolysis, trichloroacetic acid precipitation, and dialysis, and the GAG's were separated as cetylpyridinium chloride complexes into 0.03 M NaCl-soluble, 0.4 M NaCl-soluble, 1.2 M NaCl-soluble, and 2.1 M NaCl-soluble fractions as described by Schiller et al. (28) and measured as uronic acid (see Schiller et al.) by the carbazole method of Bitter and Muir (1).
ANION-EXCHANGE CHROMATOGRAPHY. Following uronic acid measurement, the uronic acid-positive material was pooled by tumor line with tumor and liver tissue material pooled separately. The cetylpyridinium chloride was removed as described by Korn (17), and then each fraction was dialyzed and subjected to Dowex 1-X2 (200 to 400 mesh) anion-exchange chromatography as described by Schiller et al. (28). The 0.0, 0.5, 1.0, 1.25, 1.5, and 2.0 M NaCl eluate fractions were eluted stepwise in 15 to 30 fractions (10 ml) each. One ml samples were assayed for uronic acid to produce an elution profile. Chromatographic fractions were pooled across all tissue groups, dialyzed, and concentrated, yielding 6 pooled fractions which were then subjected to enzymatic characterization.

ENZYMATIC CHARACTERIZATION. Each of the fractions were subjected to digestion by Streptomyces hyaluronidase (digests only hyaluronic acid), bovine testicular hyaluronidase (digests hyaluronic acid, chondroitin, and chondroitin sulfate but not heparin, dermatan sulfate, or heparan sulfate), and chondroitinase ABC (digests hyaluronic acid, chondroitin, chondroitin sulfate, and derma sulfate but not heparin or heparan sulfate) as described by Kojima et al. (16).

HISTOCHEMISTRY. Small pieces of liver and tumor as well as lung and intestine in selected animals were fixed in Zenker’s fluid, embedded in paraffin, cut at 6 µm, and subjected to hematoxylin and eosin, Alcian blue-periodic acid-Schiff (21), and Masson’s trichrome (21) staining. Alcian blue-periodic acid-Schiff staining was also carried out with and without prior digestion in chondroitinase ABC and bovine testicular hyaluronidase (9). For the chondroitinase ABC study, hydrated tissue sections were incubated with enzyme (16) for 2 hr at 37°; control sections were incubated in buffer only.

STATISTICAL ANALYSIS. Statistical analysis of tissue GAG data was carried out in a sequential format starting with an analysis of variance. Significant differences among the means were further evaluated using the Newman-Keuls procedure described by Snedecor and Cochran (29).

RESULTS

The amounts of GAG in Tumors 7777, 5123tc, and 9618A and in normal liver are presented by fractions in Chart 1. "Uronic acid" levels in the 0.03 M NaCl fraction were similar in Tumors 7777 and 5123tc but were significantly (p < 0.05) lower than the levels found in tumor 9618A and in normal liver. Uronic acid levels in the 0.04 M NaCl fraction isolated from the 3 tumors were similar and significantly higher (p < 0.05) than the levels found in normal liver. In the 1.2 M NaCl fraction, tumors exhibited 5- to 6-fold greater (p < 0.05) uronic acid levels than normal liver. In the 2.1 M NaCl fraction, uronic acid levels were similar for all tissues except that Tumor 9618A had significantly higher (p < 0.05) levels than did Tumor 5123tc.

There was a statistically significantly greater (p < 0.05) level of uronic acid in the 0.03 M NaCl fraction for the livers of animals bearing Tumor 5123tc compared to normal liver and the livers of animals bearing the other 2 lines. Except for this, differences among livers were unremarkable.

Table 1

<table>
<thead>
<tr>
<th>Tumor line</th>
<th>Growth rate designation</th>
<th>Time (days) until tumor reached 3-cm-long axis</th>
<th>Histology</th>
<th>Metastatic potential</th>
<th>General metastatic characteristic observed here</th>
<th>Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>7777</td>
<td>Fast</td>
<td>18</td>
<td>Poorly differentiated</td>
<td>++ +</td>
<td>Scattered lung micro-metastases but no gross metastases evident at sacrifice</td>
<td>+</td>
</tr>
<tr>
<td>5123tc</td>
<td>Intermediate</td>
<td>35</td>
<td>Moderately differentiated</td>
<td>++</td>
<td>Multiple large metastases to lungs grossly evident in lungs of all animals at sacrifice</td>
<td>0</td>
</tr>
<tr>
<td>9618A</td>
<td>Slow</td>
<td>89–99</td>
<td>Well differentiated</td>
<td>0</td>
<td>No lung metastases evident by sampling at sacrifice</td>
<td>0</td>
</tr>
</tbody>
</table>

4 According to the data of Hruban et al. (14).
Chromatographic data for the 0.4 M NaCl and 1.2 M NaCl GAG fractions, those fractions which were appreciably larger in tumor tissue versus normal liver, are presented in Chart 2 together with the patterns obtained for these same fractions isolated from normal liver and the liver of tumor-bearing animals. These data indicate that, even though the uronic levels in the initial fractions were similar from tumor line to tumor line, there were some qualitative GAG differences in the fractions isolated from different tumor lines. This is even more apparent in composite Chart 3, which was derived by multiplying the mean of the individual uronic acid levels shown in Chart 1 by the percentage of distribution shown in Chart 2 and then summing within each chromatographic fraction.

Enzymatic Characterization of Tumor GAG's

The results of the enzymatic characterization of GAG's isolated from tumor tissue are summarized in Table 2. On the basis of the elution patterns reported by Kao and Leslie (15) and corroborated in our own study of authentic GAG's and on the enzyme susceptibilities for each fraction given in Table 2, we arrived at the identities of the predominant GAG in each chromatographic fraction given in Table 2, Column 7.

Histochemical Observations

Tumors. Each of the 3 tumor types exhibited significantly more intense Alcian blue staining than did normal liver or host liver; GAG's were generally distributed throughout the tumor tissue. Tumor sections subjected to hyaluronidase or to chondroitinase ABC exhibited reduced levels of Alcian blue staining consistent with biochemical measurements and the enzymatic characterization of chemically isolated fractions.

Host Livers. The livers of animals bearing Tumors 7777 and 9618A were histologically indistinguishable from normal liver. Liver tissue from animals bearing Tumor 5123tc consistently exhibited a slightly greater vacuolar appearance than did normal liver (Fig. 1B).

Urinary GAG Excretion

Over the entire study, mean total uronic acid excreted per pair of animals per 24 hr for control animals and animals bearing Tumors 7777, 5123tc, and 9618A were 118 ± 7 (S.E.), 116 ± 3, 203 ± 12, and 201 ± 7 μg, respectively. GAG excretion by animals bearing Tumors 5123tc and 9618A were statistically significantly (p < 0.05) elevated over controls and animals bearing Tumor 7777. There were 22, 8, 8, and 27 twenty-four-hr collections assayed, respectively. The small number of 7777 and 5123tc samples was due to the rapid growth of these tumors and time in transit after inoculation.

Urinary excretion profiles for animals bearing Tumor 5123tc over time and in relation to tumor size are illustrated in Chart 4. A similar pattern over a longer time span was observed in animals bearing Tumor 9618A. In both 5123tc and 9618A-bearing animals, uronic acid excretion appeared to be greater after the tumors reached larger sizes, but no regression with tumor size was apparent in either case.
Table 2
Identification of the predominant GAG in each of our anion-exchange chromatographic fractions

The identification (Column 7) of the predominant GAG or GAG's present in each of our anion-exchange chromatographic fractions (Column 1) was deduced from (a) solubilities of cetylpyridinium chloride complexes, (b) anion-exchange chromatographic patterns compared to those that we obtained for authentic GAG's and those reported by Kao and Leslie (15) (Columns 2 and 3), and (c) the susceptibility of each fraction to mucopolysaccharidases (Columns 4, 5, and 6).

<table>
<thead>
<tr>
<th>Dowex 1-X2 fraction (M NaCl)</th>
<th>GAG's reported by Kao and Leslie (15) to be primarily eluted in this fraction</th>
<th>Other GAG's reported to be partially eluted in this fraction</th>
<th>% digested by Streptomyces hyaluronidase</th>
<th>% digested by bovine testicular hyaluronidase</th>
<th>% digested by chondroitinase ABC</th>
<th>Predominant GAG in our fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>Hyaluronic acid (84)</td>
<td>None</td>
<td>70-100</td>
<td>80-100</td>
<td>100</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>0.5</td>
<td>Heparan sulfate (78)</td>
<td>Heparan sulfate (12)</td>
<td>35-61</td>
<td>61</td>
<td>&lt;2</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>1.0</td>
<td>Chondroitin 4-sulfate (66)</td>
<td>Heparan sulfate (14)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Chondroitin 4-sulfate (13)</td>
</tr>
<tr>
<td>1.25</td>
<td>Heparitin sulfate (63)</td>
<td>Chondroitin sulfate (19)</td>
<td>30</td>
<td>25</td>
<td>22</td>
<td>Heparan sulfate and/or heparin</td>
</tr>
<tr>
<td>1.5</td>
<td>Heparitin 6-sulfate (67)</td>
<td>Dermatan sulfate (67)</td>
<td>&lt;2</td>
<td>0</td>
<td>0</td>
<td>Heparin</td>
</tr>
<tr>
<td>2.0</td>
<td>Heparin (72)</td>
<td>Chondroitin 6-sulfate (19)</td>
<td>0</td>
<td>?</td>
<td>0</td>
<td>Heparan sulfate</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage eluted per fraction.

**DISCUSSION**

The results depicted in Charts 1 to 3 conform to the generalization (7, 16, 18) that tumors, including hepatic tumors, exhibit high levels of GAG's relative to the tissue of origin. Charts 2 and 3 suggest that differences between tumors may be related to the behavioral properties of the tumors. Chart 3 reveals a gradation from normal liver, through the liver of tumor-bearing animals, well-differentiated, slowly growing tumor tissues to faster-growing, metastatic tumor lines. The gradation shown in Chart 3 is even more striking if the patterns for tumor lines 7777 and 5123tc are inverted. Since tumor line 5123tc was more highly metastatic than was line 7777 (Table 1), such an inversion would arrange the tissues according to metastatic potential and raises the possibility that the patterns, particularly Fraction 1.0 M, are related in some way to metastatic potential.

It is postulated that the 1.0 M NaCl chromatographic fraction contains an undersulfated form of heparan sulfate such as that described by Kuroda et al. (18) in AH109 hepatomas. Both Kuroda et al. (18) and Saito (25) reported that heparan sulfate is the major GAG constituent in AH109A hepatic tumors. Kuroda et al. also reported that most of this heparan sulfate is eluted in 1.0 M NaCl in anion-exchange chromatography and that heparan sulfate is also the predominant GAG in normal liver and suggested that this may mean that the tumor heparan sulfate comes from tumor cells and not from connective tissue elements within the tumors.

The predominance of heparan sulfate in Morris hepatomas and in AH109A hepatomas do not conform with the fact that hyaluronic acid and chondroitin sulfate have generally been identified as the predominant GAG's in animal tumors (4, 7). These findings likewise do not conform with the report by Kojima et al. (16) that chondroitin sulfate and hyaluronic acid are the predominant GAG's in human hepatocellular cancer.

The possibility that tumor heparan sulfate is related in some direct way to tumor behavior has been raised by others. A role for sulfated GAG's in cell recognition and adhesion has been proposed by Dietrich et al. (8), and Chiarugi and Vannucchi (3) have proposed that cell surface heparan sulfate regulates both cell division and transport.

The histological appearance of the tumors studied here and the histological normalcy of the livers of tumor-bearing animals agree with the findings reported by Hruban et al. (14). Although the vacuolar appearance of the livers of animals bearing Tumor 5123tc is not abnormal in rodents, this characteristic was uniformly present in all Tumor 5123tc-bearing animals and was found in no others. The possibility exists that this structural feature is related to the high concentrations of the uncharac-
terized, 0.03 M NaCl-soluble, uronic acid-positive-material found in these livers.

The fact that urinary GAG excretion was elevated in animals bearing Tumors 5123tc and 9618A but not in animals bearing Tumor 7777 suggests that urinary GAG excretion may reflect properties of certain tumors and is not simply an indirect result of the presence of tumor. Overall levels of excretion of GAG’s in our control and experimental animals agree with levels reported for rats by Lehtonen et al. (19).

It remains to be determined what the source(s) of the elevated urinary GAG is (are). There have been reports of striking increases in GAG synthesis in tumor cells (see Ref. 20), but Kojima et al. (16) have suggested that decreased degradation may account for increased GAG in some tumors. Our investigations provide no evidence that the source of the abnormal GAG increases in tumor tissue and urine is anything other than the tumor cells themselves; however, a possibility that these results stem from an impaired ability of the liver to degrade circulating GAG’s cannot be ruled out.

CONCLUSION

Our data clearly show that hepatomas 7777, 5123tc, and 9618A have GAG compositions that are appreciably different than that of normal liver. These data show that there are also qualitative differences in GAG between tumor lines and that heparan sulfate patterns parallel growth rate and possible degree of malignancy. Heparan sulfate is the predominant GAG in the hepatomas studied. The increased urinary excretion of GAG’s for 2 of the 3 tumor lines examined suggests that urinary GAG analysis may prove useful in the detection and diagnosis of some hepatic tumors.

REFERENCES

Fig. 1. A, typical lung metastases in animals bearing Tumor 7777. These were found more frequently and earlier and were larger in animals bearing Tumor 5123tc. No lung metastases were found in any of the animals bearing Tumor 9618A ×50. B, liver of animals bearing Tumor 5123tc showing clear cytoplasm-vacuolar appearance characteristic of such livers. ×50.
Tissue and Urinary Glycosaminoglycan Patterns Associated with a Fast, an Intermediate, and a Slow-growing Morris Hepatoma

Charles E. Kupchella, E. Elaine Drake, Jeffrey Kennedy, et al.

*Cancer Res* 1981;41:419-424.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/41/2/419

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.