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URINARY GLYCOSAMINOGLYCAN PATTERNS IN ANGIOSARCOMA OF THE LIVER

KEVIN L. CURRAN, BA, MS, CHARLES E. KUPCHELLA, PHD, AND CARLO H. TAMBURRO, MD

Glycosaminoglycans extracted from 24-hour urine specimens from patients with hepatic angiosarcoma and from normal/controls were separated as cetylpyridinium complexes into "hyaluronic acid," "chondroitin sulfate," and "heparin" fractions, then further separated and characterized by anion-exchange chromatography and hyaluronidase susceptibility. The chromatographic pattern of the urinary chondroitin sulfate fraction in patients with angiosarcoma of the liver differed from those of controls in that there was a relative increase in the total amount of uronic acid in a hyaluronidase-resistant fraction and a decrease in a fraction susceptible to hyaluronidase digestion. These changes appeared to become more pronounced with advancing disease. Chromatographic patterns and determinations of hyaluronidase susceptibility indicated that the resistant fraction was heparan sulfate and that the susceptible fraction was chondroitin-4-sulfate and/or chondroitin-6-sulfate.

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THE EMERGENCE OF ANGIOSARCOMA OF THE liver and its relationship to vinyl chloride exposure[4,7] has prompted a search for methods to detect this lesion. Although systematic screening programs are currently in operation,[10,16] there is still no single chemical indicator which is specific for angiosarcoma or for changes which may precede this disease.

The association of elevated tissue glycosaminoglycans (GAG) with tumors, including angiosarcoma, has been established.[1,8,18,19,22] Glycosaminoglycans are also known to be involved in normal connective tissue synthesis and collagen deposition and are elevated in connective tissue disorders.[12] Since angiosarcoma of the liver has both neoplasia and fibrogenesis in its etiology[17] GAG changes could be expected to serve to signal the appearance of early lesions and may be useful in evaluating advanced lesions.

Galambos[8] suggested that since the liver contributes very little to the overall connective tissue of the body, hepatic fibrogenesis should not be expected to result in significant increases in urinary GAG or collagen degradative or synthetic products. Preliminary studies in our laboratory, however, demonstrated an increase in both liver and urinary GAG in patients with angiosarcoma, chronic active hepatitis, and cirrhosis.[18] Most of the increase in urinary GAG occurred in the chondroitin sulfate fraction and, in contrast to what was found for normal and other diseases, the urinary chondroitin sulfate fraction was the *only* uronic acid positive fraction found in the urine of seven of nine cases of vinyl-chloride-exposure-associated liver injury other than angiosarcoma. This study was undertaken to characterize more completely the urinary "chondroitin sulfate" fraction in hepatic angiosarcoma.

From the University of Louisville, Cancer Center, and the Price Institute for Surgical Research Health Sciences Center, Louisville, Kentucky 40201.

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Address for reprints: C. E. Kupchella, Cancer Center, University of Louisville, Louisville, KY 40201.

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CLINICAL SUMMARIES

Case 1—(Hepatic Angiosarcoma—advanced)

A 46-year-old white male worked as a chemical helper in a vinyl chloride polymerization plant for thirteen years prior to the diagnosis of angiosarcoma. Twelve years after initial employment, the patient exhibited a persistent elevation of lactic dehydrogenase and underwent angiographic studies which demonstrated multiple areas of scattered tumor stain throughout both lobes of the liver with areas of central translucency consistent with the diagnosis of angiosarcoma of the liver.

Exploratory laparotomy and liver biopsy confirmed this diagnosis, and the patient was treated with adriamycin, cyclophosphamide, and methotrexate; an

initial response was associated with a decrease in the alkaline phosphatase activity, improvement in indocyanine green clearance and an increase in radioisotopic uptake in areas of previously defective uptake. After completion of the chemotherapy course, hepatic function deteriorated and the patient underwent partial hepatic lobe radiation (total dose of 5,000 rads) over a two-month period. Despite radiation therapy, the clinical course continued to deteriorate with the development of ascites, peripheral edema, increasing jaundice, hypoalbuminemia, and marked elevations of transaminases and alkaline phosphatase activities. This was followed by progressive hepatic failure, hepatorenal syndrome and hepatic coma. Autopsy findings showed extensive involvement of the liver with angiosarcomatous tissue extending into the diaphragm and metastasis to retroperitoneal and mediastinal lymph nodes, lungs, right adrenal gland and cerebellum. The right lobe of the liver demonstrated near elimination of the angiosarcoma, presumably due to the radiation treatment. Urinary GAG assays reported here were made on 24-hour urine specimens collected over the two-week period before death (Fig. 1).

Case 2 (Hepatic Angiosarcoma—moderately advanced)

A 54-year-old vinyl chloride polymerization worker was first employed as a polymerization vat cleaner 28 years prior to the diagnosis of angiosarcoma. Two years prior to diagnosis, the patient had persistent biochemical liver, abnormalities although he was otherwise asymptomatic with a normal liver-spleen scan. Angiographic studies showed peliosis hepatis. A liver biopsy revealed focal sinusoidal dilatation, mild chronic inflammatory reaction with portal fibrosis, Kupffer cell hyperplasia and dysplasia. Subsequent biopsies demonstrated continued sinusoidal dilitation, atypical and dysplastic Kupffer cells with premalignant changes. The peliosis hepatis pattern became more pronounced and multiple radioisotopic defects were evident on liver scan. A repeat biopsy one year after initial biochemical abnormality demonstrated malignant sinusoidal cells. The patient was treated with a combination of adriamycin, cytoxan, and methotrexate with limited clinical and biochemical response. Death was preceded by peripheral edema, ascites, progressive hepatic failure, and coma. The GAG analyses reported here were made 10 and 6 months before death (Fig. 1, [middle]).

MATERIALS AND METHODS

Twenty-four hour urine specimens were collected from two patients with angiosarcoma of the liver, and from two normal controls.

Urine specimens were stored at −70°C until analysis. Cetylpyridinium chloride (Sigma Chemical Company, St. Louis) was added to the entire 24-hour volume to precipitate the GAGs

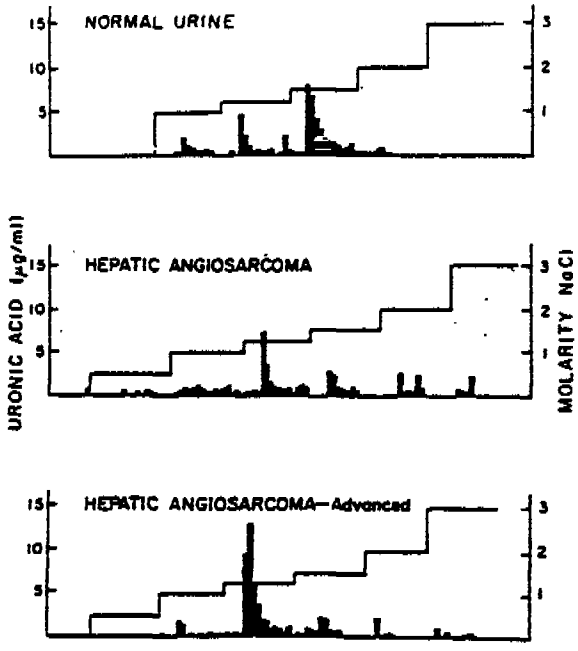

FIG. 1. Elution Patterns of the Urinary Chondroitin Sulfate Fraction. The glycosaminoglycans (GAG) in a 24-hour urine specimen were precipitated with cetylpyridinium chloride (CPC) and separated as 0.4 M NaCl soluble ("hyaluronic acid"), 1.2 M NaCl soluble ("chondroitin sulfate"), and 2.1 M NaCl soluble ("heparin") fractions. Each fraction was then subjected to anion-exchange chromatography. Shown here are typical 1.2 M (chondroitin sulfate) fraction elution patterns (Advanced = case 1).

according to the method of DiFerrante.[6] The hyaluronic acid, chondroitin sulfate, and heparin fractions were eluted individually according to the method of Schiller *et al.*[10] Cetylpyridinium chloride was removed[14] and the GAGs were subjected to anion-exchange chromatography as described by Schiller *et al.*[10] Glycosaminoglycan fractions were applied to 1.0 × 44 cm AG1-X2 (200–400 mesh, chloride form) columns Bio Rad Laboratories, Richmond, California) and eluted stepwise with 0.0, 0.5, 1.0, 1.25, 1.50, 2.0, and 3.0 M NaCl. At a flow rate of 1.0 ml/min, approximately sixteen 10.3 ml fractions of each molar strength of NaCl were collected and a sample of each fraction was analyzed for uronic acid by the method of Bitter and Muir.[3] Standards of heparin (Nutritional Biochemical Company), chondroitin sulfate (Sigma Chemical Company), and hyaluronic acid (Nutritional Biochemical Company) were also evaluated by ion exchange chromatography.

The uronic-acid-positive fractions within each individual salt fraction were pooled, dialyzed to remove salt, and concentrated. The fractions eluted by 1.25 or 1.50 M NaCl were tested for

TABLE 1.

Source	Ratio of Total Uronic Acid Eluted in 1.25 M/1.5 M NaCl
Normal	0.364
Normal	0.316
Angiosarcoma, case 2, pre-chemotherapy[1]	0.843
Angiosarcoma, case 2, post-chemotherapy[2]	0.971
Angiosarcoma, case 1, advanced	5.000

NOTE: The glycosaminoglycans (GAG) in a 24-hour urine specimen were precipitated with cetylpyridinium chloride (CPC) and separated as 0.4 M NaCl soluble ("hyaluronic acid"), 1.2 M NaCl soluble ("chondroitin sulfate"), and 2.1 M NaCl soluble ("heparin") fractions. The CPC was removed from the 1.2 M NaCl-CPC-solubilized fraction and the GAGs further purified by anion-exchange chromatography. The total amount of GAG in the resulting 1.25 M and 1.50 M NaCl column-eluted fractions was determined and the ratio of the two fractions was calculated. ([1]One day prior to beginning of chemotherapy; [2]Two days following chemotherapy initiation.)

susceptibility to testicular hyaluronidase (Nutritional Biochemical Company) using a modification of the cetyltrimethylammonium-bromide, turbidimetric assay described by DiFerrante.[6]

RESULTS

The major GAG fraction observed in all urines—both from normal controls or from patients with angiosarcoma—was the fraction solubilized by 1.2 M NaCl/1% cetylpyridinium chloride (the "chondroitin sulfate" fraction). Anion exchange chromatography of hyaluronic acid and heparin fractions revealed no qualitative differences between controls and angiosarcoma patients. The anion exchange column patterns of the 1.2 M NaCl solubilized GAGs are shown in Fig. 1. Chromatography of the urinary "chondroitin sulfate" fractions of patients with angiosarcoma yielded a comparatively large, uronic-acid positive peak in 1.25 M NaCl. The ratios of the total amount of uronic acid-positive material eluted with 1.25 M NaCl to the total amount eluted with 1.50 M NaCl are given in Table 1.

TABLE 2. Hyaluronidase Susceptibility

Source	Depolymerization %[1]
Heparin, standard	5.0
Hyaluronic acid, standard	93.1
Chondroitin sulfate, standard	97.6
1.25 M NaCl column-eluate, pooled fractions from angiosarcomatous patients	43.5
1.50 M NaCl column-eluate, pooled fractions from angiosarcomatous patients	100.0
1.50 M NaCl column-eluate, normal	100.0

[1] Glycosaminoglycans isolated from urine were tested for hyaluronidase susceptibility by measuring changes in turbidity developed with the addition of cetyltrimethylammonium bromide following incubation with hyaluronidase. Normal controls exhibited only minor amounts of 1.25 M NaCl column-eluted GAG and consequently do not appear in this table.

The susceptibility of the GAGs eluted with 1.25 or 1.50 M NaCl to hyaluronidase degradation is given in Table 2. The GAG eluted with 1.25 M NaCl was resistant to hyaluronidase, the enzyme producing only a 43% reduction in turbidity. The 1.50 M NaCl-eluted GAG fraction was 100% susceptible to hyaluronidase degradation.

DISCUSSION AND CONCLUSION

The chromatographic pattern found here for controls conforms to urinary glycosaminoglycan distributions reported by others.[12,21] These patterns suggest that there was a relative increase in urinary heparan sulfate and a decrease in chondroitin-4- and/or -6-sulfate in patients with hepatic angiosarcoma. This interpretation agrees with the Dowex 1-X2 chromatographic patterns reported by Kao and Leslie[12] and by others.[2,19]

Heparan sulfate is reported to be partially susceptible to hyaluronidase digestion,[20] and this correlates well with the observed 43% digestion of the GAG in our 1.25 M NaCl fraction. Since heparan sulfate has been shown to be associated with blood vessels,[20] an increase in the urinary excretion of this GAG is not surprising in this vascular lesion. Also, chondroitin-4- and -6 sulfates are reportedly eluted from Dowex 1-X2 columns with 1.50 M NaCl[2,19] and are susceptible to hyaluronidase[20] suggesting that our 1.5 M fraction is chordroitin-4- and/or chondroitin-6-sulfate.

Assuming that urinary GAG patterns described here are reflections of hepatic changes, it will be important to determine what processes these changes reflect, *i.e.*, those of neoplastic growth, fibrogenesis, or cell death. In this regard, it should be noted that 1) the ratio of heparan sulfate to chondroitin sulfate reported here for angiosarcomatous urine is similar to that reported for cirrhotic human liver *tissue* by Becker,[2] and 2) the shift from a hyaluronidase-

susceptible to a hyaluronidase-resistant GAG is consistent with the suggestion by Hutterer and Rubin[11] that the stabilization of collagen depends on a shift to a hyaluronidase-resistant GAG envelope surrounding the collagen bundle. Although Hutterer and Rubin attribute this to an augmentation of dermatan sulfate, Becker[2] reported that the GAG pattern in human cirrhosis was characterized by the augmentation of dermatan sulfate *and* heparan sulfate. If the observed changes in urinary GAG are reflective of vinyl chloride-exposure-associated fibrosis, the fact that fibrosis is a precursor of angiosarcoma[17] indicates that the observations reported here constitute a promising lead in early detection of vinyl-chloride-induced liver disease.

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