Urinary Glycosaminoglycan Excretion as a Biochemical Marker in Patients with Bladder Carcinoma

Patrick T. Hennessey, Robert E. Hurst, George P. Hemstreet III, and Gary Cutter

School of Public Health [R. E. H., P. T. H., and G. C.] and Division of Urology [G. P. H.], Department of Surgery, University of Alabama in Birmingham School of Medicine, Birmingham, Alabama 35294

ABSTRACT

Urinary glycosaminoglycan excretion was examined in 25 individuals with bladder cancer in comparison to glycosaminoglycan excretion by eight normal individuals. Urinary glycosaminoglycan was isolated by gel filtration and quantified as macromolecular uronate concentration. Electrophoresis in calcium acetate and densitometry of Alcian blue-stained electrophoreograms were used to separate and quantify the relative amounts of individual glycosaminoglycans. Elevated excretion of macromolecular uronate was noted in 53% of the cancer cases. The highest levels were found among individuals with metastatic disease. Three electrophoretic bands were always detected in the control and cancer groups: chondroitin sulfate, heparan sulfate (both confirmed by chemical and enzymatic degradation), and a third band (Band 1) of unknown composition. A fourth band, corresponding to dermatan sulfate, was detected in the cancer group. Diagnostic limits were drawn from the observed distributions of normals, and with these limits 92% of the cancer cases, including 12 of 12 metastatic cases, could be identified. The results strongly suggest noninvasive urinary glycosaminoglycan analysis may well provide a new biochemical approach for detecting and monitoring the pathogenesis of bladder cancer.

INTRODUCTION

The identification of readily measurable biochemical substances in urine has been pursued as one means to facilitate early detection of cancers. A recent review by Lessing (17) discussed a number of biochemical markers which have been investigated as possible indicators of bladder cancer. These include: enzyme levels such as alkaline phosphatase, lactic dehydrogenase, muramidase, β-glucuronidase, and creatinine phosphokinase; carcinoembryonic antigen; and nucleic acids and other high-molecular-weight substances in the macromolecular fraction of interfering low-molecular-weight urinary constituents. Recovery of urinary glycosaminoglycan with this method is complete. Selected samples were further purified by single extraction in a butyl alcohol/hexadecylpyridinium chloride:NaCl system (13). Excretion was measured as the uronic acid of the macromolecular fraction against a ω-glycuronlactone standard (Pfanstiehl Laboratories, Inc., Waukegan, Ill.), using a half-scale of the method of Blumenkrantz and Asboe-Hansen (2). Electrophoresis. Aliquots of macromolecular fractions were lyophilized and reconstituted in sufficient quantity of deionized water to yield a final concentration of 7 to 10 nmol of uronate per µl. Two µl were applied to the cellulose acetate strips (Separax; Jookoo Sangyo Ltd.,
Urinary Glycosaminoglycan in Bladder Carcinoma

Tokyo, Japan) 0.5 cm apart, and a current of 1 ma/cm (of strip width) was applied for 2 hr at room temperature in 0.2 M calcium acetate (16). All samples were run against a quality control sample of reference chondroitin sulfate, dermatan sulfate (Seikagaku Kogyo Ltd., Tokyo, Japan), and a beef lung heparan sulfate (Dr. J. A. Cifonelli), in a 2:1:1 ratio, determined by uronate assay of the individual solutions prior to combination. After electrophoresis, the cellulose acetate strips were air dried and stained with 0.5% Alcian blue in 5% acetic acid for 15 min, followed by destaining in 5% acetic acid and rinsing with deionized water.

The electrophorograms were analyzed by densitometry and electronic integration (Quick-Scan; Helena Laboratories, Beaumont, Texas) to measure individual glycosaminoglycans. It was not possible from these data to measure the mass or moles of individual glycosaminoglycan excreted; the absolute intensities of the bands were too variable to use directly, and moreover, the composition (and hence the amount of dye bound per polymer unit) of the urinary glycosaminoglycan species is different from that of the reference standard (12). For example, the chondroitin sulfate found in urine is generally less sulfated than is the reference standard (19). These problems were obviated by normalizing the absolute band densities. Two methods were used in order to emphasize different compositional aspects. When normalized to the sum of the absolute densities of all resolved bands, the resulting percentage band densities are representative of the percentage composition of the total glycosaminoglycan fraction in terms of its constituents. When the intensities of the other bands were normalized to the intensity of the chondroitin sulfate band, the resulting band ratios are representative of the concentration ratios of the individual species.

In order to further identify Band 1, selected samples were electrophoresed in 0.1 M HCl at a current of 2 ma/cm for 1 hr or in 0.2 M barium acetate at 6 ma/cm for 2 hr. Two other stains were also used: 0.04% Coomassie Brilliant Blue (Bio-Rad Laboratories, Rockville Centre, N. Y.) in 27% isopropyl alcohol:10% acetic acid; and 0.10% toluidine blue in 5% acetic acid with destaining in 5% acetic acid. Samples were run in both HCl and calcium acetate against reference standards of chondroitin sulfate, dermatan sulfate, heparan sulfate, and hyaluronate (Dr. J. A. Cifonelli), bovine serum albumin (Pentex, Inc., Kanakee, Ill.), salmon testis DNA (Worthington Biochemical Corp., Freehold, N. J.), and carcinoembryonic antigen (Dr. David Pritchard). These electrophorograms were analyzed by visual inspection.

Chemical and Enzymatic Identification of Bands. Glycosaminoglycans were identified in several selected samples from normal and cancer-affected individuals. The techniques used were modifications of those of Dietrich et al. (8) and Kojima et al. (16), as follows.

Sufficient quantity of macromolecular fraction to provide 30 nmol of uronate was aliquoted into each of 4 micro test tubes (400 µl polyethylene; Bio-Rad Laboratories). Reference chondroitin sulfate, heparan sulfate, dermatan sulfate, and hyaluronate were used as internal quality controls of enzymatic activity and specificity. To one of the aliquots was added 0.17 turbidity-reducing unit of Streptomyces hyaluronidase (Miles Laboratories, Inc., Elkhart, Ind.) in 5 µl of 0.01 M NaCl and 20 µl of 0.1 M acetic acid buffer, pH 5. To another of the aliquots was added 8 × 10⁻³ unit of chondroitinase AC-II (Seikagaku Kogyo Ltd.) in 5 µl of deionized water and 20 µl of 0.05 M ammonium acetate buffer, pH 7, at 37°C. To a third aliquot was added 8 × 10⁻³ unit of chondroitinase ABC (Seikagaku Kogyo Ltd.) in 5 µl of deionized water and 20 µl of 0.05 M Tris-HCl, pH 8, at 37°C. The fourth aliquot received 20 µl of 1.5 M NaNO₃ in deionized water and 10 µl of glacial acetic acid. All tubes were mixed well and incubated at 37°C; Aliquots 1, 2, and 3 were incubated for 4 hr, and Aliquot 4 was incubated for 90 min. Enzymatic degradation was terminated by boiling for 3 min; nitrous acid degradation was stopped by the addition of 20 µl of 2.0 M ammonium sulfamate solution. Samples were lyophilized to dryness, reconstituted in 10 µl of deionized water, and electrophoresed as above to identify individual bands.

Data Analysis. Preliminary analysis suggested nonnormal distribution of the cases. The third moment value of 4.87 (p < 0.01) and Geary's statistic of 15.5 (p < 0.01) indicated pronounced skewness and kurtosis. Therefore, the nonparametric Wilcoxon rank-sum test was used to analyze the uronate excretion and electrophoretic-densitometric data throughout. This technique evaluates the probability that the 2 samples are drawn from the same population, based on rank, without the assumption of underlying normality inherent in the t test. Differences in proportions (Table 2 and text describing Table 5) were analyzed for statistical significance using Fisher's exact test.

RESULTS

Excretion of Macromolecular Uronate. Chart 1 presents the excretion of urinary glycosaminoglycan, measured as the concentration of macromolecular uronate, for the normal group and the groups with localized or metastatic disease. Among the normal group, excretion tended to cluster between 60 and 120 nmol/ml, with only a single value of 170 nmol/ml being outside this range. In the group with apparently only localized tumors, a wider range of excretion levels was observed. The range was between 48 and 302 nmol/ml, and nearly 50% of the values exceeded 120 nmol/ml. An even wider range was observed in the group with metastatic disease, wherein values ranged from 27 nmol/ml to a high of almost 400 nmol/ml, with 58% of the individuals excreting more than 120 nmol/ml macromolecular uronate.

The mean uronate excretion increased progressively from 104 nmol/ml in the normal group to 323 nmol/ml in the localized-disease group to 175 nmol/ml in the group with metastatic disease. Glycosaminoglycan excretion by the various groups was compared by the Wilcoxon rank-sum test with the results shown in Table 1. The trend in mean values is suggestive; however, probably due to the considerable overlap of the groups, none of the differences was statistically significant at α = 0.05 when tested by the conservative Wilcoxon test.

Chart 1. Total urinary glycosaminoglycan excretion, expressed as nmol of macromolecular uronate per ml of urine for normals (N), individuals with localized cancer (LC; □), individuals with metastatic cancer (MC; △), and all bladder cancer combined (AC). Horizontal bars, mean values for each classification.
The value of 120 nmol/ml is the upper limit of normal macromolecular uronate excretion (14, 15). An alternate way of examining the significance of the uronate excretion data is to compare the proportions in each group (normal, localized, and metastatic) exhibiting elevated values. These comparisons are shown in Table 2. With this test, the differences in proportions of elevated values between the normal group and the metastatic and combined cancer groups closely approach significance (p = 0.054 and 0.056, respectively). Again, the considerable overlap of the various groups results in the lack of clear statistical significance. However, the number of elevated values observed within some of the groups of affected individuals is considerable and suggests a positive correlation between elevated glycosaminoglycan excretion and the presence of bladder cancer. Interestingly, the individual in the control group with an elevated uronate excretion was not the individual with urethritis; that person was found to have a perfectly normal excretion level.

Electrophoretic Characterization. The results of the electrophoretic and degradative analyses of the macromolecular fractions are shown in Table 3. The relative mobilities of the reference glycosaminoglycan preparations and their susceptibilities to the degradative treatments are shown in the top half of Table 3; the data for the urinary glycosaminoglycans are listed in the bottom half. The macromolecular fraction from normal individuals afforded 3 alcianophilic bands, which are labeled Bands 1, 2, and 3. Band 3 comigrated with reference chondroitin sulfate, and its susceptibilities to degradation were identical to those of chondroitin sulfate. Band 2 comigrated with reference heparan sulfate and was degraded by nitrous acid, but not by any of the enzymes. Band 1 did not comigrate with any of the reference preparations, nor was it degraded by any of the treatments. A fourth band was observed in the macromolecular fractions of 4 of the affected individuals, of whom all had high-grade (Grade 3 and 4) tumors. This band, when present, was the least intense. It comigrated with dermatan sulfate and, like reference dermatan sulfate, was susceptible only to digestion by chondroitinase ABC.

Table 1
Wilcoxon rank-sum analysis of macromolecular uronate

<table>
<thead>
<tr>
<th>Groups compared</th>
<th>T1</th>
<th>T2</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal vs. localized</td>
<td>64</td>
<td>112</td>
<td>NS</td>
</tr>
<tr>
<td>Normal vs. metastatic</td>
<td>62</td>
<td>196</td>
<td>NS</td>
</tr>
<tr>
<td>Normal vs. all cases</td>
<td>96</td>
<td>176</td>
<td>NS</td>
</tr>
</tbody>
</table>

a T1 = ∑Ri of the smaller of the 2 groups compared, where Ri = rank of individual observation.

b One-tailed (9).

c Not significant at α = 0.05.

Test of proportion of sample groups exceeding 120 nmol macromolecular uronate per ml in normal and bladder cancer-affected groups

<table>
<thead>
<tr>
<th>Groups compared</th>
<th>Proportions compared</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal vs. localized</td>
<td>1/8, 6/13</td>
<td>0.133</td>
</tr>
<tr>
<td>Normal vs. metastatic</td>
<td>1/8, 7/12</td>
<td>0.054</td>
</tr>
<tr>
<td>Normal vs. all cases</td>
<td>6/13, 7/12</td>
<td>&gt;0.25</td>
</tr>
<tr>
<td>Normal vs. all cases</td>
<td>1/8, 13/25</td>
<td>0.056</td>
</tr>
</tbody>
</table>

Using Fisher's exact test, where x/n is the proportion of sample >120 nmol uronate per ml and n is sample size in the (sub)groups compared.

Chart 2 displays the band densities of each of the 3 main electrophoretic bands in each sample within the indicated groups. The normal group clustered closely about their mean values of 15 ± 1.9% (S.D.) for Band 1, 27 ± 4.3% for Band 2, and 58 ± 4.2% for Band 3, but the groups affected with either localized or metastatic disease were much more variable. Table 4 lists the comparisons of band densities among the 3 groups of subjects. Band 1 was appreciably elevated in both affected groups compared to normals, but the difference between metastatic and localized cases is not statistically significant at α = 0.05. In contrast, Band 2 is statistically elevated in the metastatic-disease group compared to either the normal group or the localized-disease group. Since the band densities are expressed as the percentage of total density, the percentage density of Band 3 is decreased in affected individuals.

The reproducibility of the method itself was assessed from the 12 replicate analyses of the reference glycosaminoglycan.
sample. The means ± S.D. of the individual glycosaminoglycan bands were 25.5 ± 2.2% for reference heparan sulfate, 33.0 ± 2.0% for reference dermatan sulfate, and 42.0 ± 2.0% for reference chondroitin sulfate. The reproducibility is therefore ±2 percentage band density units.

Chondroitin sulfate excretion was compared in concentration units by multiplying the percentage band density of Band 3 of the individual by the corresponding macromolecular uronate concentration. The mean values were 60 nmol/ml for the normal group, 68 nmol/ml for the localized-disease group, 65 nmol/ml for the metastatic-disease group, and 67 nmol/ml for the group of all cancer patients; these differences were not statistically significant at α = 0.05. Thus, the concentration of excreted chondroitin sulfate does not appear to be affected by the presence of bladder cancer.

The densities of Bands 1 and 2 were also standardized to the percentage density of Band 3 of the individual, as displayed in Chart 3. These ratios are more sensitive to small changes in Bands 1 and 2 than are the corresponding percentage band densities. The main effect of this transformation is to further resolve the metastatic-disease group from the normal group. The this effect is particularly evident with regard to the Band 1: Band 3 ratio. Using a value of 0.33 as the upper limit of normal, 68% of Band 1: Band 3 ratios were above this value, while only 9% of Band 1: Band 3 ratios were above the value of 0.33 for reference heparan sulfate.

Diagnostic Validity of Glycosaminoglycan Analyses. Table 5 presents the validity of various diagnostic criteria based upon the analysis of glycosaminoglycan excretion. The criteria are based upon the distributions observed in the normal group and, with the exception of macromolecular uronate, were arbitrarily established at levels which exclude all of the normals. Uronate excretion alone (greater than 120 nmol/ml) will detect slightly more than one-half of the abnormal samples, while detecting only a single presumed normal (P = 0.056). Of the
measures dependent upon the percentage band densities, the
decrease in the percentage of Band 3 (chondroitin sulfate) is
most significant, detecting 10 of 12 of the metastatic disease
($p < 0.001$) and 14 of 25 of all cancers ($p < 0.005$).
Even better results are obtained by expressing the ratios of
Bands 1 and 2 to Band 3, with the Band 1 ratio being best in
this regard. If these 2 ratios are combined so that an elevated
value for either is considered abnormal, diagnostic validity is
improved further to 11 of 12 of the metastatic disease group
($p < 0.001$) and 19 of 25 of all the cancer patients ($p <
0.001$). Interestingly, 7 of 12 of the metastatic cases could be
detected by a Band 2:Band 3 ratio of greater than 0.62, while
only 2 localized cases and no normals were detected with this
criterion. Finally, if the 2 band ratio criteria are combined with
an abnormal uronate measurement, 23 of 25 of the cancer
patients are detected as abnormal, at the expense of a single
presumed normal individual ($p = 0.0001$).

**Properties of Band 1.** Band 1 was subjected to several
different stains and electrophoresed in other systems in order
to obtain further characterization. Electrophoresis in 0.1 M HCl
showed it to be an anion at low pH. Staining with toluidine blue
was much less intense than with Alcian Blue, in contrast to the
staining properties of either purified standards or Bands 2 and
3. Band 1 did not stain with the glycoprotein stain, Coomassie
Brilliant Blue. Several other substances were subjected to
electrophoresis in the calcium acetate system, including car-
cinoembryonic antigen, bovine serum albumin, DNA, and pur-
ified keratan sulfate II to determine whether any corresponded
to Band 1. The only one of these substances to stain with
Alcian Blue under the conditions used was keratan sulfate II,
which afforded a broad band spanning the region from the
chondroitin sulfate to dermatan sulfate bands.

**DISCUSSION**

One approach to the early detection of cancer has been
based upon the identification of biochemical markers which
are specific for the presence of cancer. Ideally, such sub-
stances should be present in readily obtained body fluids such as
urine, and their analysis should be simple, accurate, and
expensive. The results of the current study demonstrate
specific aberrations in both the quantitative levels and qualita-
tive pattern of excreted glycosaminoglycan which are associ-
ated with bladder cancer, and therefore, the possibility of using
urinary glycosaminoglycan analyses to detect bladder cancer
is suggested.

When total glycosaminoglycan excretion was examined,
some differences emerged, but there was considerable overlap
between normal individuals and those affected with bladder
cancer. However, when separated into their component mac-
molecular species by electrophoresis, the differences be-
tween normal and abnormal urine samples were much more
striking. Clearly bladder cancer results in increased excretion
of the as yet unidentified substances denoted as Band 1;
metastatic cancer results in increased heparan sulfate and
possibly dermatan sulfate in pronounced metastatic cases.
The excretion of chondroitin sulfate, which represents the main
product of normal glycosaminoglycan metabolism, does not
appear to be altered. These qualitative differences could be
quantified in terms of concentration-independent ratios of band
densities, and several such ratios were examined in order to
determine which could best separate normal from abnormal
and metastatic disease from localized disease.

Expressing the results as ratios greatly facilitates inter-
pretation by obviating the large variability due to concentration
differences, which acts to mask disease-related differences.
The use of these ratios is analogous to the use of creatinine
ratios to quantify low-molecular-weight urinary constituents in
concentration-independent terms. Creatinine ratios alone, how-
ever, do not reduce intersample variability in urinary glyco-
saminoglycan measurements (14, 15). The ratios to Band 3
afforded the best sensitivity in this respect. The reason may
well be that, since the estimates of absolute chondroitin sulfate
excretion are identical for normal and affected groups, ratios
to Band 3 compare abnormal excretion to the main product of
normal glycosaminoglycan metabolism, the chondroitin sulfate
band. Although the Band 3 ratios are valid for detecting bladder
cancer because chondroitin sulfate excretion was unaffected,
the ratio may not be useful for all cancers, since Hopwood and
Dorfman (11) reported elevated chondroitin sulfate excretion
in Wilms' tumor. Consequently, the percentage band densities
should also be examined, even though the sensitivity of bladder
cancer detection is somewhat reduced.

The glycosaminoglycan composition of normal urine shows
surprisingly little variability. In all the normal samples, the
standard deviations of the percentage of Band 2 (heparan
sulfate) and the percentage of Band 3 (chondroitin sulfate) are
only slightly (although significantly) larger than the inherent
variability in the method as measured by the standard devia-
tions of the reference bands in the standard sample. The
variance of Band 1 in the normal urine samples was also quite
small. When expressed as coefficients of variation, these vari-
ances are only 13, 16, and 7% for Bands 1, 2, and 3, re-
spectively. One unexplained finding is that the high macromolecular
uronate excretion (170 nmol/ml) by one of the supposed
normal subjects. This value is considerably higher than that
shown by any other normal in this study or in previous studies
with the same methods (14, 15). However, the pattern of band
densities of the sample of this individual upon electrophoresis
fell within the typical normal pattern.

In the abnormal group, a quite different picture emerges.
Several of the cancer-affected individuals afforded extremely
raised values, comparable to those found in genetic diseases
of glycosaminoglycan metabolism (15). These findings agree
with those of recent investigators with other cancers (6, 10)
and, wheremethodological differences permit comparison,
with earlier results as well (4). The variability in composition,
whether expressed as percentage band densities or ratios to
Band 3, is also much larger than is observed in the normal
group. The elevation in macromolecular uronate excretion does
not result simply from an enhancement of the glycosaminogly-
can normally excreted; rather, the qualitative pattern is altered,
and excretion of specific species is increased. Band 1, the
composition of which is currently unknown, was elevated irre-
spective of whether the tumor was localized or metastatic.
However, Band 2 was elevated in the metastatic group but not
in the localized group. One of the 2 individuals in the localized-
disease group with increased heparan sulfate (Table 5 and
Charts 2 and 3) was found to have bone metastases 3 months
later. The other such patient was lost to followup. Thus, meta-
tasis might well have been occult at the time of specimen
collection, or increased heparan sulfate excretion may be a
found in the urines of individuals affected with hemopoietic cancers in the current study involving solid tumors, heparan sulfate excretion was increased although elevated dermatan sulfate was found with 4 highly malignant tumors.

Three main mechanisms have been proposed to account for the increased glycosaminoglycan excretion in cancers. These mechanisms are not mutually exclusive, and more than one possibility is also suggested that combining glycosaminoglycan measurements with other biochemical tests may well afford a battery of tests which together could present a very powerful approach to biochemically detecting cancer by noninvasive procedures.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Candace Echols for her aid in reviewing clinical records, Balinda Perkins for her help in preparing this manuscript, and Dr. J. A. Citronelli of the University of Chicago and Dr. David Pritchard of the University of Alabama in Birmingham for their generous gifts of materials.

REFERENCES

Urinary Glycosaminoglycan Excretion as a Biochemical Marker in Patients with Bladder Carcinoma

Patrick T. Hennessey, Robert E. Hurst, George P. Hemstreet III, et al.


Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/41/10/3868

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.