Changes in Charge Density of Heparan Sulfate Isolated from Cancerous Human Liver Tissue

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ABSTRACT

Heparan sulfate fractions were isolated from three normal human livers and three cancerous human liver tissues, and their polyanionic properties were examined using electrophoresis, sequential partition fractionation, and chemical analyses. More than 60% of total glycosaminoglycans from normal human liver and about 30% from cancerous liver tissue were found to be heparan sulfate from their resistance to exhaustive digestion with chondroitinase ABC and their susceptibility to nitrous acid treatment. The heparan sulfate isolated from cancerous liver tissue afforded a lower sulfate/uronic acid molar ratio (0.58 to 0.65) than did normal human liver heparan sulfate (0.76 to 0.80). Also, the former showed lower electrophoretic mobility in 0.1 M HCl and a different partition fractionation profile in comparison with the latter. These differences in charge density of the macromolecules were not detected on the chondroitin sulfate and/or dermatan sulfate fractions isolated from normal human liver and cancerous liver tissue.

INTRODUCTION

Heparan sulfate is a common component of the surface of cells from various tissues (6, 8, 9, 20) and is distinguishable from all other kinds of glycosaminoglycans isolated from animal tissues by its structural complexity (10). Consequently, this glycosaminoglycan has been implicated in some cellular functions, such as cell growth and cell interaction. Several investigators found the changes in cellular sulfated glycosaminoglycans including heparan sulfate to be associated with the virus-induced transformation of cultured cells (3, 11, 16, 17); furthermore, Underhill and Keller (23, 24) and Winterbourne and Mora (25) reported the difference in charge density of cell surface heparan sulfate between normal and transformed mouse cells.

We previously identified heparan sulfate as a major component of the glycosaminoglycans isolated from rat liver (13, 21), human liver (7), and rat ascites hepatoma cells also (13). The heparan sulfate fraction obtained from AH-130 ascites hepatoma cells was extremely low sulfated, showing a molar ratio of sulfate/uronic acid of less than 0.4 (13). However, little is known about the structural alterations of heparan sulfate isolated from solid cancer tissue. In the present paper, the heparan sulfate fractions were isolated from normal human liver and human hepatocellular carcinoma tissue, and some of their biochemical properties were investigated mainly from the point of charge density of the macromolecule.

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pronase E were added after 6 hr from the start of the incubation. After digestion, the flask was cooled in ice water, 0.1 volume of 100% trichloroacetic acid was added, and the mixture was kept at 4° for 2 hr. The precipitated material was removed by centrifugation at 1000 x g for 10 min, and the supernatant solution was dialyzed against running tap water overnight followed by 2 changes of deionized water at 4° for 24 hr.

Precipitation of the glycosaminoglycans from the dialyzed solution was carried out using hexadecylpyridinium chloride as described previously (13). An aliquot of the 2.1 m NaCl eluate was used for the uronic acid assay to determine the concentration of total glycosaminoglycans, and the remainder was mixed with 2 volumes of ethanol and maintained at 4° overnight. The precipitated total glycosaminoglycan fraction was recovered by centrifugation at 1500 x g for 20 min, washing with 95% ethanol, and drying with ether. The dried material was dissolved in 2.0 ml of 0.1 M Tris-HCl buffer, pH 7.4, and incubated with 10 mg of DNase I and 10 mg of RNase A at 37° for 18 hr. After incubation, the nucleic acid-free total glycosaminoglycan fraction was obtained by the hexadecylpyridinium chloride precipitation and the ethanol precipitation as mentioned above. The product thus obtained was dissolved in distilled water to a concentration of 5 μmol uronic acid/ml and used for further analyses, such as enzymic digestion with mucopolysaccharidases, electrophoresis, and sulfate degradation.

Enzymic Digestion with Mucopolysaccharidases. The enzymic digestion to determine the composition of glycosaminoglycans from human liver samples was performed using Streptomyces hyaluronidase, testicular hyaluronidase, and chondroitinase ABC as described previously (13). The complete digestion with each enzyme was confirmed by both electrophoresis of the undigested portion and digestion of the standard mixture of reference glycosaminoglycans (7).

The heparan sulfate fraction was obtained by digestion of the total glycosaminoglycan fraction with chondroitinase ABC (1.0 unit of enzyme/glycosaminoglycans equivalent to 1.0 μmol uronic acid) in 0.1 M enriched Tris-HCl buffer, pH 8.0 (18), at 37° for 18 hr. The undigested portion was recovered by hexadecylpyridinium chloride precipitation and ethanol precipitation as described above.

Electrophoresis. Electrophoresis of glycosaminoglycans on a cellulose acetate membrane was carried out with 0.1 M HCl at a constant current of 2 ma/cm for 1 hr as described by Hsu et al. (4) and with 0.2 M calcium acetate solution at a constant current of 1 ma/cm for 3 hr as described by Seno et al. (19). Strips were stained with 0.5% Alcian Blue 8GS in 3% acetic acid for 15 min, rinsed with 1% acetic acid, and scanned by a Quick Scan densitometer (Helena Laboratories, Beaumont, Texas) at 610 nm.

Sequential Partition Fractionation of Glycosaminoglycans. An aliquot of glycosaminoglycan solution containing 1 to 2 μmol uronic acid was fractionated by the sequential partition procedure reported previously (14). The major fractions were recovered by ethanol precipitation and used for electrophoresis and sulfate assay.

RESULTS

Composition of Total Glycosaminoglycan Fraction from Human Liver Samples. The total amounts and composition of the glycosaminoglycans isolated from 3 normal human livers and three cancerous human liver tissues are shown in Table 1. For the preparations from normal human liver, the variation among 3 samples was extremely small so that the average values are listed in the table.

The glycosaminoglycan content of cancerous liver tissue is 7- to 10-fold higher than that of normal human liver. Enzymic digestion showed that approximately two-thirds of the total glycosaminoglycan fraction from normal human liver was chondroitinase ABC resistant. On the other hand, in cancerous liver tissue, chondroitin sulfate was the major constituent of the total glycosaminoglycan fraction, and the relative share of the fraction that was chondroitinase ABC resistant was considerably decreased. Electropherograms of the total glycosaminoglycan fractions in 0.2 M calcium acetate solution are shown in Fig. 1. The composition of the total glycosaminoglycan fraction as obtained by enzymic digestion was well confirmed by the electrophoretic findings.

These results are in fairly good agreement with our previous report (7).

Heparan Sulfate Fraction from Human Liver Samples. The glycosaminoglycan fractions obtained by the exhaustive digestion with chondroitinase ABC afforded single bands with a similar mobility to reference heparan sulfate on electrophoresis in both 0.2 M calcium acetate solution and 0.1 M HCl as shown in Chart 1.
in Fig. 2. Interestingly, in 0.1 M HCl, which is known to be a charge-dependent electrophoretic system, all the preparations obtained from 3 cancerous liver samples showed slightly but significantly less electrophoretic mobility than did the fraction from normal human liver. The difference in the mobilities is illustrated in Chart 1 by densitometric scanings of the electropherogram. These heparan sulfate-like bands completely disappeared after the preparations were treated with nitrous acid.

The effect of nitrous acid degradation on the chondroitinase ABC-resistant fractions and the molar ratio of sulfate/uronic acid in these preparations were summarized in Table 2. The effect of nitrous acid degradation was expressed as the decrease of uronic acid in ethanol-precipitable fraction after nitrous acid treatment (14). The chondroitinase ABC-resistant fractions from both normal human liver and cancerous liver tissue were susceptible to nitrous acid to the same extent as was the reference heparan sulfate. From these results, namely, nitrous acid degradation and the electrophoretic findings mentioned above, the chondroitinase ABC-resistant fractions can be identified as heparan sulfates.

As listed in Table 2, heparan sulfates from cancerous liver samples showed a significantly lower molar ratio of sulfate/uronic acid than did those from normal liver samples. The molar ratio obtained from reference beef lung heparan sulfate was similar to that from cancerous liver tissue heparan sulfates and was lower than that from normal human liver heparan sulfate. The difference in molar ratios of sulfate/uronic acid corresponds well to the difference in electrophoretic mobilities in 0.1 M HCl. Therefore, heparan sulfate isolated from cancerous liver tissue was considered to be lower sulfated and to have less charge density than normal human liver heparan sulfate.

Sequential Partition Fractionation of Heparan Sulfates. Heparan sulfate fractions isolated from 3 normal human livers and 3 cancerous liver samples were fractionated by the sequential extraction procedure with a constant interval of 0.01 M NaCl between successive extracting aqueous phases. Some reference glycosaminoglycans were also fractionated simultaneously. The uronic acid distributions obtained by this procedure are illustrated in Chart 2. The preparations isolated from 3 individual normal livers showed very similar distribution patterns with a peak at the 0.08 M NaCl fraction in which approximately 30% of the total uronic acid was recovered. On the other hand, the preparations from 3 cancerous liver samples were distributed in a lower salt concentration area, from 0.04 M NaCl to 0.08 M NaCl. Two of 3 cancerous liver heparan sulfate preparations showed a peak at 0.06 M NaCl, and their distribution patterns were almost identical. The third one (Chart 2b, Case 3) had a peak at 0.07 M NaCl, and the whole distribution pattern was slightly shifted to a higher salt concentration area compared with the other 2 but still clearly different from the distribution patterns of normal human liver heparan sulfates. Since Hurst and Sheng (5) showed that the sequential partition fractionation was primarily dependent on the charge density of the glycosaminoglycan samples, these results indicate that heparan sulfate fractions from cancerous liver tissue contain more glycosaminoglycan chains with less charge density than do heparan sulfate fractions from normal human liver. The fractionation pattern of reference beef lung heparan sulfate was similar to that of heparan sulfate from cancerous liver tissue rather than to that of heparan sulfate from normal human liver.

Each of the fractions containing significant quantities of uronic acid from 3 normal human liver heparan sulfate samples and from 2 cancerous liver heparan sulfate samples (Chart 2b Cases 1 and 2) was pooled and analyzed further. Table 3 shows the yield and the molar ratio of sulfate/uronic acid determined on the major fractions from each preparation. As the concentration of NaCl in extracting aqueous phase is increased, the molar ratio increases, demonstrating that the fractionation procedure depends on the charge density of the glycosaminoglycans. More than one-half of normal liver heparan sulfate was extracted at the salt concentration higher than

<table>
<thead>
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<th>Case</th>
<th>Decrease after nitrous acid treatment (%)</th>
<th>mol sulfate/mol uronic acid</th>
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<tbody>
<tr>
<td>Case 1</td>
<td>61.7</td>
<td>0.79</td>
</tr>
<tr>
<td>Case 2</td>
<td>63.1</td>
<td>0.76</td>
</tr>
<tr>
<td>Case 3</td>
<td>63.4</td>
<td>0.60</td>
</tr>
<tr>
<td>Case 2</td>
<td>62.0</td>
<td>0.58</td>
</tr>
<tr>
<td>Case 3</td>
<td>66.0</td>
<td>0.65</td>
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<tr>
<td>Reference glycosaminoglycans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>64.6</td>
<td>0.62</td>
</tr>
<tr>
<td>Heparin</td>
<td>77.6</td>
<td>2.31</td>
</tr>
<tr>
<td>Chondroitin 4-sulfate</td>
<td>&lt;1.0</td>
<td>0.93</td>
</tr>
</tbody>
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Table 2: Effect of nitrous acid treatment on chondroitinase ABC-resistant fractions of glycosaminoglycans isolated from normal human liver and cancerous human liver tissue, and molar ratio of sulfate/uronic acid of these preparations.

*Not determined.

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0.08 M NaCl; however, in the preparation from cancerous liver tissue, one half was extracted below 0.06 M NaCl. Electrophoretic behaviors of the major fractions from each preparation in 0.1 M HCl are shown in Fig. 3. The higher the salt concentration at which the fraction was extracted, the greater electrophoretic mobility the fraction showed.

**Sequential Partition Fractionation of Nitrous Acid-resistant Glycosaminoglycan Fractions.** To know whether the changes in charge density of glycosaminoglycans are specific to heparan sulfate fraction, total glycosaminoglycan fractions from normal human liver (a pooled material from 3 preparations) and from a cancerous liver tissue (Case 1) were treated with nitrous acid, and the nitrous acid-resistant fractions were fractionated by the sequential partition procedure. The results are indicated in Chart 3. The uronic acid distribution of the preparations from both normal human liver and cancerous liver tissue afforded 2 peaks, one at 0.03 M NaCl and the other at 0.09 M NaCl. The former was coincident with the distribution pattern of reference hyaluronic acid and the latter with reference chondroitin sulfate and dermatan sulfate. Since this partition procedure is not able to separate chondroitin sulfate from dermatan sulfate, the peak at 0.09 M NaCl is thought to consist of both chondroitin sulfate and dermatan sulfate. As shown in Chart 3, the distribution profile around 0.09 M NaCl of normal human liver preparation was quite similar to that of the preparation from cancerous liver tissue. This seems to indicate that the chondroitin sulfate and/or dermatan sulfate fraction from cancerous liver tissue possesses similar charge density to that from normal human liver. Electrophoresis in 0.1 M HCl was also performed on these preparations, and the mobilities of chondroitin sulfate and/or dermatan sulfate fractions from each preparation did not differ significantly from each other (data not shown).

**DISCUSSION**

The first report on the transformation-dependent change in charge density of cell surface heparan sulfate was made by Underhill and Keller (23) using 3T3 cells and their SV402 transformants. They suggested that the alteration in heparan sulfate from trypsin digest of SV40/3T3 was possibly related to the altered cell interaction and growth of the transformed cells. Recently, Winterbourne and Mora (25) demonstrated that similar alterations in heparan sulfate from cell and cell surface, as well as from medium, were observed in SV40-transformed cloned mouse cells and that there was a close relationship between these changes and the expression of SV40-specific T-antigen (25). In these reports, however, the difference between heparan sulfate from transformed cells and that from nontransformed cells was demonstrated mainly by the altered elution position of radiolabeled preparations from ion exchange chromatographic column or by the $^{35}$S/$^3$H ratio in heparan sulfate fraction isolated from the cells doubly labeled with $[^35]$Sulfate and $[^3]$Hglucosamine. Any results from direct chemical analysis on the isolated heparan sulfate were not shown. In the present paper, we actually isolated heparan sulfates from both normal human liver and cancerous human liver tissue. The preparations obtained as the chondroitinase ABC-resistant fractions were susceptible to nitrous acid degradation to the same extent as reference beef lung heparan sulfate, and heparan sulfate from cancerous liver tissue showed a clearly lower molar ratio of sulfate/uronic acid, lower electrophoretic mobility in 0.1 M HCl, and a different partition fractionation profile in comparison with heparan sulfate from normal human liver. These results demonstrate that the alterations in heparan sulfate can be detected in spontaneously occurring human solid cancerous tissue as well as in transformed cultured cells.

Heparan sulfate has been considered the major component of glycosaminoglycans in liver tissue (7, 13, 21) and seems to be generally present in a cell-associated form. Gressner et al. (2) demonstrated that approximately 90% of glycosaminoglycans synthesized by normal rat liver slices was heparan sulfate, and Oldberg et al. (15) reported that heparan sulfate was the only sulfated glycosaminoglycan synthesized by the isolated rat liver cells in primary cultures. Also, the occurrence of heparan sulfate on the cell surface or in the plasma membrane...
fraction of rat ascites hepatoma cells has been described (12, 26). Recently, we compared heparan sulfate isolated from normal rat liver tissue with heparan sulfate from normal rat liver cells isolated by a perfusion technique both qualitatively and quantitatively, and we obtained preliminary results that these 2 heparan sulfates showed identical electrophoretic behaviors and that almost 90% of heparan sulfate in normal rat liver tissue was derived from the liver cells.3 These results are not available for human hepatoma tissue; however, microscopic examination revealed that the cell populations of the hepatocellular carcinoma tissues used in this study were fairly homogeneous and that the percentage of cell types other than hepatoma cells would be less than 10%. Therefore, the observed alterations in heparan sulfate can be attributed mainly to the changes in cellular properties of malignant liver cells. Though some minor portion of the heparan sulfate we have isolated might not be cell associated or might be derived from cell populations other than liver cells or hepatoma cells (i.e., blood vessel cells, infiltrating inflammatory cells), the portion would not be significant enough to contribute substantially to the observed differences. We reported previously that heparan sulfate isolated from AH-130, an ascites hepatoma cell line, was remarkably low sulfated compared with that from normal rat liver (13), and it is interesting that similar results were obtained on the cancerous human liver tissue. The occurrence of heparan sulfate with decreased charge density seems to be closely related to the cancerous alterations of liver cells. It is possible that the observed changes in heparan sulfate fraction were only the result of nonspecific excessive cleavage of sulfate moieties by degradative enzymes in cancerous liver tissue, which frequently accompanies cell breakage and tissue necrosis. If that is the case, other glycosaminoglycans should also be influenced more or less similarly. However, chondroitin sulfate and/or dermatan sulfate fraction from cancerous liver tissue afforded a sequential partition fractionation profile very similar to that of the fraction from normal human liver as shown in Chart 3. This finding seems to indicate that the difference in sulfation is specific to heparan sulfate and not the result of nonspecific degradative processes. Furthermore, it suggests the possibility that heparan sulfate and chondroitin sulfate and/or dermatan sulfate have different origins in cancerous tissue; namely, the former may originate from cancerous cells, and the latter may be derived mainly from the surrounding mesenchymal tissue stimulated by growth of the tumor.

In the present experiments, heparan sulfate from normal human liver showed more anionic properties and higher molar ratio of sulfate/uronic acid than reference beef lung heparan sulfate, which afforded anionic properties similar to those of heparan sulfate from cancerous liver tissue. Oldberg et al. (15) reported that heparan sulfate synthesized by the isolated rat liver cells showed more retarded elution behavior on ion exchange chromatography than did reference heparan sulfate. Also, a heparan sulfate fraction with a molar ratio of sulfate/hexosamine of more than unity was isolated from normal rat liver (21). Therefore, the possibility that normal liver heparan sulfate possesses higher average sulfate content than do heparan sulfates isolated from other tissues cannot be ruled out, and the cancer-associated changes in heparan sulfate in tissues other than liver remain to be investigated.

REFERENCES


**Heparan Sulfate from Human Liver Cancer**

Fig. 1. Electrophoresis of glycosaminoglycans isolated from normal human liver and cancerous human liver tissue in 0.2 M calcium acetate solution. 1, mixture of reference glycosaminoglycans (from origin to anode) hyaluronic acid, dermatan sulfate, chondroitin 4-sulfate, and chondroitin 6-sulfate; 2, reference heparan sulfate; 3, total glycosaminoglycan fraction from normal human liver, Case 1; 4, total glycosaminoglycan fraction from cancerous liver tissue, Case 1; 5, total glycosaminoglycan fraction from cancerous liver tissue, Case 2; 6, total glycosaminoglycan fraction from cancerous liver tissue, Case 3.

Fig. 2. Electrophoresis of chondroitinase ABC-resistant fractions of glycosaminoglycans isolated from normal human liver and cancerous human liver tissue, a, (in 0.1 M HCl), reference hyaluronic acid (slower component) and chondroitin 4-sulfate (1); total glycosaminoglycan fraction from normal human liver, Case 1 (2); chondroitinase ABC-resistant fraction from normal human liver, Case 1 (3); total glycosaminoglycan fraction from cancerous liver tissue, Case 1 (4); chondroitinase ABC-resistant fraction from cancerous liver tissue, Case 1 (5); chondroitinase ABC-resistant fraction from cancerous liver tissue, Case 2 (6); chondroitinase ABC-resistant fraction from cancerous liver tissue, Case 3 (7); reference heparan sulfate (slower component) and heparin (8). b, (in 0.2 M calcium acetate solution), mixture of reference glycosaminoglycans (from origin to anode) hyaluronic acid, dermatan sulfate, chondroitin 4-sulfate, and chondroitin 6-sulfate (1); reference heparan sulfate (2); chondroitinase ABC-resistant fraction from normal human liver, Case 1 (3); chondroitinase ABC-resistant fraction from cancerous liver tissue, Case 1 (4); chondroitinase ABC-resistant fraction from cancerous liver tissue, Case 2 (5); chondroitinase ABC-resistant fraction from cancerous liver tissue, Case 3 (6).

Fig. 3. Electrophoresis of the major fractions of heparan sulfates isolated from normal human liver and cancerous human liver tissue obtained by sequential partition fractionation. Electrophoresis was carried out in 0.1 M HCl. a, reference heparan sulfate (1); heparan sulfate from cancerous liver tissue before fractionation (2); heparan sulfate from normal human liver before fractionation (3); 0.05 M, 0.06 M, 0.07 M, 0.08 M, and 0.09 M NaCl fractions of normal human liver heparan sulfate (4 to 8, respectively). b, reference heparan sulfate (1); heparan sulfate from normal human liver before fractionation (2); heparan sulfate from cancerous liver tissue before fractionation (3); 0.04 M, 0.05 M, 0.06 M, 0.07 M, and 0.08 M NaCl fractions of cancerous liver heparan sulfate (4 to 8, respectively).
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