Abnormal Accumulation of Proteoglycan in Human Thyroid Adenocarcinoma Tissue

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Abstract

Glycosaminoglycan (GAG) was extracted from thyroid tissue obtained at surgery for thyroid adenoma and adenocarcinoma and compared with that extracted from the thyroid tissue obtained at autopsy of non-thyroid disease. The amount of GAG was almost doubled in thyroid adenoma and increased from 6 to 15 fold in adenocarcinoma when compared with that of apparently normal thyroid tissue. Analysis by Sepharose 2B or 6B column chromatography revealed that at least a part of the GAG in thyroid carcinoma tissue was present in macromolecule form and the molecular size became smaller when treated with papain or alkaline borohydride. The results indicated that those fractions of GAG were present as proteoglycans. The GAG was composed of a mixture of heparan sulfate and chondroitin sulfate or dermatan sulfate in one carcinoma tissue and mainly composed of chondroitin sulfate or dermatan sulfate in the other. The mechanism of the increase in proteoglycan and GAG remains to be elucidated.

In our previous study, we have demonstrated the presence of heparan sulfate glycosaminoglycan (GAG) at least in part in a form of proteoglycan in normal thyroid tissue (Shishiba et al., 1983). As proteoglycans were known to provide a good milieu for cell growth and development, in the present study, we sought to determine whether GAG's in a form of proteoglycan were increased in human thyroid adenoma and adenocarcinoma tissue. We have observed a 2 to 3 fold increase in GAG in thyroid adenoma and a 6 to 15 fold increase in thyroid adenocarcinoma tissue.

Materials and Methods

Guanidine HCl and cesium chloride of the purest reagent grade were purchased from Calbiochem; 6-aminohexanoic acid and benzamidine hydrochloride from Eastman; papain (twice crystallized) from Sigma; chondroitinase ABC (proteus vulgaris) from Miles; Sepharose 2B and sepharose 6B from Pharmacia.
Human thyroid adenoma or carcinoma tissue was obtained at surgery. After trimming off the extraneous tissue and fat, the tissue was stored frozen at -80°C until use. The diagnosis was based on clinical factors and the histopathological findings on the excised tissue. The apparently normal portion of thyroid tissue was obtained at the autopsy of the cases which died from non-thyroid disease and kept frozen until analysis.

The specimen of thyroid tissue was thawed, weighed and chopped with scissors. An aliquot of distilled water was added and mixed with the tissue fragments. Two aliquots of 8M guanidine HCl, 0.2 M 6-aminohexanoic acid, 0.01 M benzamidine HCl, and 0.05 M EDTA, pH 5.8, were added and left for 48 h with continuous stirring at 4°C. After this extraction procedure, tissue fragments were removed by passing through a glass fiber filter. Dissociative CsCl density gradients (initial density 1.46 g/ml) were formed in a Hitachi 65P RPS-50 rotor by centrifugation at 37000 rpm at 10°C for 48 h as described for cartilage proteoglycans (Heingard, 1972; Hascall and Heingard, 1974). Four equal fractions designated as D1 through D4 from bottom to top were prepared using a Beckman tube slicer as described by Heingard (1972).

The bottom fourth fraction designated as D1 was dialyzed against 0.5 M sodium acetate, pH 7.0 for 48 h and then against distilled water for 48 h before lyophilization to obtain the proteoglycans as sodium salt.

Lyophilized specimens were reconstituted with an appropriate buffer and subjected to the following treatments. Papain digestion was carried out at 65°C containing 0.005 M sodium EDTA and 0.005 M cysteine hydrochloride. Digestion with chondroitinase ABC (0.25 unit/mg of sample) was performed in a buffer consisting of 0.1 M Tris and 0.1 M sodium acetate, pH 7.3 incubating for 3 h at 37°C.

Treatment with alkaline borohydride was carried out in 0.05 M NaOH at 45°C for 24 h with 1 M sodium borohydride, which prevented the degradation of sugar chains by the "peeling" reaction (Carlson, 1968). Excess borohydride was destroyed by neutralization of the solution with glacial acetic acid. Enzymatic digests or alkaline borohydride-treated samples were chromatographed immediately or after storage at -20°C. Nitrous acid treatment was carried out at room temperature with 0.24 M NaOH in 0.18 M acetic acid for 80 min (Lindahl et al., 1973).

Protein was estimated by the method of Lowry et al. (1951) with bovine serum albumin (BSA) as a standard. Hexuronic acid concentration was determined by the carbazol reaction (Dische, 1947) with glucuronic acid as a standard. Analytical Sepharose 2B and 6B columns (110×0.7 cm) were prepared and eluted with 0.5 M sodium acetate, pH 7.0.

Results

Table 1 summarizes the results of the analysis of D1 fraction of dissociative CsCl density gradient centrifugation performed on normal human thyroid, thyroid adenoma and thyroid adenocarcinoma. As summarized in the Table, the D1 fraction content of normal thyroid gland was less than 0.2 mg/g thyroid wet weight. That of thyroid adenoma from three patients was between 0.4 to 0.6 mg, being 0.5 mg/g wet weight in average. That of thyroid adenoma from 5 patients was 1.3 to 2.9 mg/g, being more than 6 to 15 fold that of normal subjects. The increase in D1 fraction content in thyroid carcinoma tissue was statistically significant (P <0.01) when compared either to normal or to adenoma. Hexuronic acid content in D1 fraction was 67.3±35.4 in normal, 69.6±28.6 in adenoma tissue, and 563.6±248 µg/g tissue in carcinoma tissue, respectively. The glycosaminoglycan (GAG) content was estimated from hexuronic acid content assuming that hexuronic acid comprises approximately 35% of the weight of GAG's, and listed in Table 1. As shown, in normal thyroid tissue, GAG comprises almost all the weight of the D1 fraction. Inaccuracy in weighing a small amount of lyophilized material from normal thyroid tissue caused a big variation. In three thyroid adenoma tissues, GAG comprises 17–60% of the weight of D1 fraction. In five cases of thyroid adenocarcinoma, GAG comprises 51–94% of the weight of D1 fraction, being 78.1% in average.

To examine the molecular moiety containing GAG in D1 fraction, column
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Table 1. DI Fraction Content

<table>
<thead>
<tr>
<th>Origin of thyroid</th>
<th>DI mg/g, thyroid</th>
<th>hexuronic acid μg/g, thyroid</th>
<th>GAG* mg/g, thyroid</th>
<th>% of GAG in DI</th>
<th>Constituents of GAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine normal thyroid</td>
<td>0.2</td>
<td>86.6</td>
<td>0.24</td>
<td>100%</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>Normal human thyroid (1)</td>
<td>0.2</td>
<td>89.0</td>
<td>0.25</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>Normal human thyroid (2)</td>
<td>0.2</td>
<td>26.4</td>
<td>0.07</td>
<td>38%</td>
<td>?</td>
</tr>
<tr>
<td>Follic. Adenoma (#804171)</td>
<td>0.4</td>
<td>84.6</td>
<td>0.24</td>
<td>61%</td>
<td>?</td>
</tr>
<tr>
<td>Follic. Adenoma (from Ito Hosp.)</td>
<td>0.5</td>
<td>87.6</td>
<td>0.24</td>
<td>50%</td>
<td>?</td>
</tr>
<tr>
<td>Follic. Adenoma (#810080)</td>
<td>0.6</td>
<td>36.6</td>
<td>0.11</td>
<td>17%</td>
<td>?</td>
</tr>
<tr>
<td>Papil. Adenoca (#792301)</td>
<td>2.0</td>
<td>660.0</td>
<td>1.89</td>
<td>94%</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>Papil. Adenoca (#800052)</td>
<td>2.9</td>
<td>937.0</td>
<td>2.68</td>
<td>92%</td>
<td>Chondroitin sulfate</td>
</tr>
<tr>
<td>Papil. Adenoca (#810232)</td>
<td>1.3</td>
<td>364.0</td>
<td>1.04</td>
<td>80%</td>
<td>Dermatan sulfate</td>
</tr>
<tr>
<td>Papil. Adenoca (#812398)</td>
<td>1.8</td>
<td>324.0</td>
<td>0.92</td>
<td>51%</td>
<td>?</td>
</tr>
<tr>
<td>Papil. Adenoca (#831287)</td>
<td>1.8</td>
<td>533.0</td>
<td>1.52</td>
<td>73%</td>
<td>?</td>
</tr>
</tbody>
</table>

* GAG content was estimated assuming hexuronic acid comprises approximately 35% of the weight of GAG.

Fig. 1. Elution pattern of GAG in DI fraction of patient #792301 by Sepharose 6B column. Panel A shows that untreated, and panel B shows that digested with papain.

Fig. 2. Elution pattern of GAG in DI fraction of patient #792301 by Sepharose 2B column. Panel A shows that untreated, and panel B shows that treated with alkaline borohydride.
chromatography was performed employing Sepharose 2B and 6B. When the D1 fraction obtained from patient #792301 with thyroid carcinoma was analyzed with Sepharose 6B columns, as shown in Fig. 1, A, hexuronic acid was eluted in two peaks, 22.9% at void volume (Vo) and another 77.7% at Kav=0.23. The protein peak was observed only at Vo (not shown in the figure). When the D1 fraction was digested with papain, the peak at Vo disappeared, a peak at Kav=0.23 was unchanged and a new peak appeared at Vt (Fig. 1, B). Thus, the peak eluted at void volume represents proteoglycan and the peak eluted at Kav=0.23 represents the single GAG chain because the elution position of the peak was not changed with papain digestion. When the D1 fraction was analyzed by Sepharose 2B column and was treated with alkaline borohydride, the peak at Vo (15.3% of hexuronic acid) disappeared, and a peak at Kav=0.64 was unchanged, and a peak appeared at Kav=0.9 (Fig. 2, A and B). The peak at void volume represents proteoglycan and the peak at Kav=0.64 probably represents the single GAG chain because the former disappeared and the latter stayed at the same elution position after alkaline borohydride treatment which is known to release GAG by elimination reaction. When the D1 fraction was treated with chondroitinase ABC and chromatographed with a Sepharose 6B column, there was a proportional reduction in hexuronic acid content in the fraction eluted at Vo (from 22.9 to 16.8%) and Kav=0.23 (from 77.7 to 34.7%) and a new peak (48.5% of hexuronic acid) appeared at Kav=0.9 (Fig. 3, A and B).

These results indicate that approximately 30% to 55% of GAG's are composed of chondroitin sulfate or dermatan sulfate. Treatment of D1 fraction with nitrous acid reduced the hexuronic acid peak at Vo considerably and a small peak (approximately 60%) remained at Kav=0.23, indicating that the remaining portion (approximately 40%) of GAG is composed of heparin or heparan sulfate, as shown in Fig 3, C.

The D1 fraction obtained from patient #800052 with thyroid carcinoma presented a different pattern of GAG distribution on chromatographic analysis. Hexuronic acid was eluted in a broad peak at Kav=0.13 (Fig. 4, A). A protein peak was observed at the same position. With the papain digestion, the hexuronic acid peak at Kav=0.13 decreased and a new peak appeared at Kav=0.47 (Fig. 4, B). Alkaline boro-
hydride treatment caused a similar change (Fig 4, C). Thus, the results suggested that at least a part of GAG (approximately 12%) existed as a form of proteoglycan. From the fact that the remaining 88% of GAG was eluted at $K_{av}=0.13$ even after the treatment with papain or alkaline borohydride, the following two explanations are possible: One is that the major portion of GAG (approximately 88%) existing as proteoglycan was rather resistant to the effect of papain or alkaline borohydride. Another explanation is that the size of GAG of this patient was heterogeneous. Smaller sized GAG consisted of proteoglycan and was eluted at $K_{av}=0.47$ when treated with papain or alkaline borohydride.

Fig. 4. Elution pattern of GAG in D1 fraction of patient #800052 by Sepharose 6B column. Panel A shows that of intact D1 fraction: panel B shows that treated with papain: panel C shows that treated with alkaline borohydride: panel D shows that treated with alkaline borohydride and nitrous acid in sequence.

Fig. 5. Elution pattern of GAG in D1 fraction of patient #800052 by Sepharose 2B column. Panel A shows that untreated and panel B shows that treated with chondroitinase ABC.
Larger sized GAG (eluted at $K_{av}=0.13$) existed as single GAG chain with very small amounts of covalently linked peptide, if any. Nitrous acid treatment after alkaline borohydride treatment caused only a minor change on the chromatographic elution pattern of hexuronic acid (Fig. 4, D). When the D1 fraction was treated with chondroitinase ABC, and analyzed with a Sepharose 2B column, the peaks presenting a high molecular form of hexuronic acid disappeared and the peak at $V_t$ was increased (Fig. 5, B). These results suggested that the GAG of this patient was mainly composed of chondroitin sulfate or dermatan sulfate.

In the remaining three cases, analysis of the GAG was not possible because of the paucity of excised surgical materials.

Discussion

The present study demonstrated a definite increase in GAG in human thyroid carcinoma tissue for the first time. The amount of GAG in thyroid carcinoma tissue ranged from 6 to 15 fold of that of normal (Shishiba et al., 1983), while that of thyroid adenoma tissue ranged from 2 to 3 fold. The present study also demonstrated that a part of the GAG (approximately 15–23% in case #792301 and at least approximately 12% in case #800052) was present as proteoglycans. From the elution position shown in Fig. 1A, 2A and 4A, the molecular weight of proteoglycan was estimated to be more than 4 million. The nature of GAG in thyroid carcinoma tissue was different from case to case. For example, in case #792301 approximately 40% of GAG was composed of chondroitin sulfate or dermatan sulfate (sensitive to chondroitinase ABC) and the remaining 60% was heparin or heparan sulfate (sensitive to nitrous acid), while that in case #800052 was almost exclusively composed of chondroitin sulfate or dermatan sulfate (sensitive to chondroitinase ABC).

Despite the use of strong denaturing (dissociative) conditions of 4M guanidine HCl and protease inhibitors throughout the extraction and purification procedures, approximately 60% to 80% of the GAG in case #792301 and possibly 88% of GAG in case #800052 were present as a single GAG chain, suggesting proteolytic degradation of proteoglycans. The nature of this proteolysis, whether this represents a physiological process or experimental artifacts remains to be studied.

The molecular weight of this single chain species of GAG was estimated to be more than 50,000 based on the published data on the calibration of Sepharose 6B column by chondroitin sulfate (Wasteson, 1971). Although it was not possible to analyze GAG’s in the remaining three cases because of the paucity of surgical materials we speculated that at least a part of the GAG was present as proteoglycans as in the two cases described.

The reason why proteoglycans increase in thyroid carcinoma tissue remains to be elucidated. As proteoglycans are the constituents of various biological materials such as connective tissue, basement membranes or plasma membranes of cells, there are number of possible explanations for its increase (Roden, 1980). Whatever the mechanism and the site of increase, the accumulation of proteoglycan in human thyroid carcinoma may possibly provide a good marker for this neoplasm.

Acknowledgment

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References


