Variation in Glycosaminoglycan Components of Breast Tumors

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SUMMARY

The correlation between the content of individual glycosaminoglycans and the histological patterns are studied on breast tumor tissues. The myxomatous stroma of intracanalicular fibroadenoma contained a large amount of glycosaminoglycans, which were mainly hyaluronic acid. The chondroitin 4- and 6-sulfate level was also high. As the supporting stroma of this tumor became denser and more fibrous, the level of hyaluronic acid content was reduced. In the case of pericanalicular fibroadenoma, glycosaminoglycans were small in amount and the levels of hyaluronic acid and chondroitin sulfate were low, but the ratio of dermatan sulfate content was higher. In the case of gynecostasia, the content was almost the same as that of pericanalicular fibroadenoma. Scirrhous carcinoma tissues contained a relatively large amount of hyaluronic acid and chondroitin sulfate. No remarkable differences in heparan sulfate content were observed in any one of the breast tumors tested. Dermatan sulfate-chondroitin sulfate copolymers were detected in all the tumors. The presence of dermatan sulfate seemed to have an intimate relation with the fibrogenesis in the interstitial stromal element of the tumor tissues.

INTRODUCTION

The epithelial tumor cells are more or less intermingled with connective tissue that consists mainly of mesenchymal cells, fibers, and ground substance. The characteristic stroma of neoplasms is produced by the connective tissue in the course of its destruction and proliferation, and most of the stroma is considered to proliferate either from the surrounding tissue or specific connective tissue (as "specific stroma") growing with tumor cells. A fibroadenoma of the breast is a growth composed of epithelial and connective tissue components, and the connective tissue elements in the tumor vary from the myxoid to the densely collagenous. Ozzello and Speer (17) investigated the distribution and interrelations of the acid and neutral mucopolysaccharides in the breast by means of histochemical technique, and Ozzello et al. (16) also found that acid mucopolysaccharides, especially hyaluronic acid, are essential to the growth of human mammary carcinoma cells in vitro.

In a previous paper we reported that pleomorphic adenoma, the so-called mixed tumor, of the salivary gland could be classified into 2 types based on the morphology of tumor cells and the biochemical analysis of glycosaminoglycans (24). As the precise nature of stroma in breast tumors has not been ascertained, a chemical analysis of glycosaminoglycans and histochemical studies of breast tumors have been carried out by means of the same procedure as described in the earlier paper (24). In this present paper, the content of the individual glycosaminoglycans in small pieces of tumor tissues, which showed a monotonous pattern histologically, is described, and the relationship between the glycosaminoglycan component and the histological and histochemical findings is studied.

MATERIALS AND METHODS

Each case of breast tumor examined is shown in Table 1. Each tumor mass was cut into 2 parts. One part was fixed in 95% aqueous ethanol and kept in a refrigerator, and the other was fixed in 10% formalin, then embedded in paraffin, and sectioned. The cut surface of the formalin-fixed tumor tissue was observed histologically, and the small pieces of tissue that were coincident with the histological section were then taken from the corresponding cut surface of the ethanol-fixed tissue. The tissue element on the surface of the ethanol-fixed small pieces was also ascertained histologically, and the undesired areas were cut out. Thus, only desired areas of the small pieces of tumor tissue remained. Those tissues that showed a monotonous pattern histologically were used for the chemical analysis of glycosaminoglycans.

Histochemical Studies

Each tissue fixed in formalin and/or alcohol was embedded in paraffin and sectioned. The sections were stained with each of the following dyes: hematoxylin and eosin, Alcian blue, toluidine blue, and periodic acid-Schiff. In order to detect the components of glycosaminoglycans, the digestion test with chondroitinase ABC (pH 8.0, 10 units/ml, 37°C, 1 hr) or Streptomyces hyaluronate lyase (pH 5.0, 100 turbidity reducing units/ml, 37°C, 1 hr) was performed.

Biochemical Analysis of Glycosaminoglycans

Extraction and Purification of Glycosaminoglycans from Tumor Tissue. The small pieces of tissue placed in 95%
aqueous ethanol were dried with acetone, weighed, and crushed. The segments were put into a test tube containing 0.3 M NaOH and were kept at 4° overnight. The mixture was then adjusted to pH 8.0 with 4 M HCl and then 0.05 volume of 1 M Tris-HCl (pH 8.0) and Pronase-P (5 mg/g of dry tissue) was added. This mixture was incubated at 40° for 20 hr with toluene as preservative. The resulting mixture was centrifuged at 8000 x g for 15 min and the supernatant fluid was collected. Glycosaminoglycans in the supernatant were precipitated with 3 times the volume of 95% aqueous ethanol containing 1% potassium acetate. The precipitate was washed with 80% aqueous ethanol and acetone dried. The residue (from 0.1 g of dry tissue) was dissolved in 1 ml of water and treated with RNase and DNase at 37° for 10 hr. Afterwards, glycosaminoglycans were precipitated with 0.20 volume of 10% CPC solution in the presence of 0.03 M NaCl. The CPC-glycosaminoglycan complex that was formed was collected by centrifugation at 8000 rpm for 20 min, then washed with 0.1% CPC solution, and extracted twice with 1-ml aliquots of 3 M NaCl solution. Glycosaminoglycans in the extract were precipitated with 3 times the volume of 95% aqueous ethanol containing 1% potassium acetate. The precipitate was dissolved in 1 ml of water, and the precipitation with 95% aqueous ethanol containing 1% potassium acetate was repeated 2 additional times. The resulting precipitate was washed with 80% aqueous ethanol, acetone dried, and dissolved in water to give a concentration of 1 mg/ml.

Identification of Individual Glycosaminoglycans. For the identification of individual glycosaminoglycans, the digestion of mucopolysaccharides with chondroitinase ABC, chondroitinase AC II, and hyaluronate lyase was carried out as described in a previous paper (24). In order to determine whether a given mucopolysaccharide component was sensitive or resistant to these enzymes, the digest was examined by electrophoresis on a cellulose acetate membrane with both buffer systems, pyridine-acetic acid buffer, pH 3.5, and 0.2 M calcium acetate. In order to identify the digestion products with chondroitinase ABC and chondroitinase AC II, paper chromatography was carried out according to the method of Saito et al. (20).

Quantitative Analysis of Individual Glycosaminoglycans. Contents of the individual glycosaminoglycans, i.e., chondroitin 4-sulfate and 6-sulfate, dermatan sulfate, hyaluronic acid, and heparan sulfate, were determined according to Hata and Nagai (6) as follows. (a) After electrophoresis (see above), the cellulose acetate membrane was stained with Alcian blue (0.2 g/100 ml of 0.1% acetic acid solution) and, as a result, the colored spots were shown on the strip corresponding to the individual standard glycosaminoglycans. (b) Each colored spot on the cellulose acetate strip was cut out and extracted with 1 ml of 5% CPC in a boiling water bath for 15 min. The absorbance of Alcian blue dye in the extract was measured at 615 nm. Calibration curves of each glycosaminoglycan were obtained from the absorbance of the standards run concurrently. Hexuronic acid was assayed either by the orcinol method (2) or by the carbazole method (3) using glucuronic acid as standard.

The following commercial materials were used: chondroitinase ABC (from Proteus vulgaris) (27), chondroitinase AC II (from Arthrobacter aurescens), hyaluronate lyase (from Streptomyces hyaluronicus nov. sp.) (14), and chondroitin 4-sulfate, chondroitin 6-sulfate, and hyaluronic acid from Seikagaku Kogyo Co. Ltd., Tokyo, Japan. Generous gifts of dermatan sulfate (hog intestinal mucosa) and heparan sulfate (beef lung) were made by Dr. M. B. Mathews, Dr. J. A. Cifonelli, and Dr. L. Roden, University of Chicago.

RESULTS

A direct relationship was observed between the increased concentration of standard glycosaminoglycan and the increase in absorbance at 615 nm, as shown in Chart 1. The amount of individual glycosaminoglycan in each breast tumor is summarized in Chart 2.

Intracanalicular Fibroadenoma

In the case of intracanalicular fibroadenoma, it was easy to distinguish macroscopically the myxomatous area from the ethanol-fixed tumor tissue. The tumor tissue consisting of the myxomatous stroma contained a large amount of glycosaminoglycans, which were mainly hyaluronic acid, and the content of both chondroitin 4- and 6-sulfate was also large, as shown in Chart 2, Cases ta, 2, and 3a. As the stromal element became denser and more fibrous, the levels of hyaluronic acid fell (Chart 2, Cases tb and 3b). In the

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Table 1

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Size of tumor (cm)</th>
<th>Histological diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31</td>
<td>F</td>
<td>1.8 x 1.8</td>
<td>Intracanalicular fibroadenoma</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>F</td>
<td>1.5 x 1.5</td>
<td>Intracanalicular fibroadenoma</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>F</td>
<td>3.0 x 3.0</td>
<td>Intracanalicular fibroadenoma</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>F</td>
<td>Size of the tip of an index finger</td>
<td>A sclerosed type of intracanalicular fibroadenoma</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>F</td>
<td>1.5 x 0.7</td>
<td>Pericanalicular fibroadenoma</td>
</tr>
<tr>
<td>6</td>
<td>34</td>
<td>F</td>
<td>1.5 x 1.5</td>
<td>Pericanalicular fibroadenoma</td>
</tr>
<tr>
<td>7</td>
<td>42</td>
<td>F</td>
<td>Size of the tip of a small finger</td>
<td>Pericanalicular fibroadenoma</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>M</td>
<td>5.0 x 5.0</td>
<td>Gynecomastia</td>
</tr>
<tr>
<td>9</td>
<td>45</td>
<td>F</td>
<td>2.0 x 1.5</td>
<td>Scirrhus carcinoma</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>F</td>
<td>3.0 x 2.5</td>
<td>Scirrhus carcinoma</td>
</tr>
<tr>
<td>11</td>
<td>62</td>
<td>F</td>
<td>7.0 x 8.0</td>
<td>Medullary carcinoma</td>
</tr>
</tbody>
</table>

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The abbreviation used is: CPC, cetylpyridinium chloride.
Glycosaminoglycan in Breast Tumor

A 615 nm

C-4-S, C-6-S

OS

0.10

HS

0.05 ~ HA

I I

1.0 2.0 3.0 4.0 l~g

Chart I. Calibration curves for the determination of individual glycosaminoglycans by the method using paper electrophoresis. After electrophoresis, the strip was stained in Alcian blue and destained. The dye bound to glycosaminoglycans was extracted with CPC solution, the absorbance of which was measured at 615 nm. C-4-S, chondroitin 4-sulfate; C-6-S, chondroitin 6-sulfate; DS, dermatan sulfate; HS, heparan sulfate; HA, hyaluronic acid.

capsule of the tumor tissue, the amount of glycosaminoglycan was scanty (Chart 2, Case 1c). Histologically, the stromal components consisted of stellate cells and myxomatous substance, and there was little fibrous element (Fig. 1). Hyaluronate lyase digestion decreased the metachromasia in the myxomatous area most intensely in the histological sections. In the case of the sclerosed type of this tumor, consisting of densely fibrous and hyalinized stroma (Fig. 2), the amount of glycosaminoglycan was small, and the levels of hyaluronic acid and chondroitin 4- and 6-sulfate were markedly reduced, but the rate of dermatan sulfate to the other glycosaminoglycans increased (Chart 2, Case 4).

Pericanalicular Fibroadenoma

In this tumor, the glycosaminoglycans were fewer than those in intracanalicular fibroadenoma (Chart 2, Cases 5, 6, and 7). The levels of hyaluronic acid and chondroitin 4- and 6-sulfate were low, but the ratio of dermatan sulfate content was higher. Histologically, the cellular fibrous tissue showed a tendency to be arranged in concentric fashion around the epithelial tubules, and the basement membrane stained with periodic acid-Schiff could be distinctively observed between the epithelial and stromal tissues (Figs. 3 and 4). The digestion tests with hyaluronate lyase and chondroitinase were slightly positive.

In the case of gynecomastia, which consisted of dense fibrous connective tissue, the glycosaminoglycan content was almost the same as that in the pericanalicular fibroadenoma (Chart 2, Case 8; Fig. 5).

Carcinoma

Two cases of scirrhous carcinoma were examined. Histologically, the amount and quality of the interstitial connective tissue element varied in different regions of the same carcinoma tissues. The stroma showed the process of hyaluronic acid digestion in some areas while it was looser in texture, more cellular in composition, and smaller in amount in other portions. These carcinoma tissues contained a relatively large amount of hyaluronic acid and chondroitin 4- and 6-sulfate (Chart 2, Cases 9 and 10). The hyaluronic acid content was almost the same as that in chondroitin sulfate. The amount of glycosaminoglycans, however, was scanty in the case of medullary carcinoma, which consisted of carcinoma cells arranged in irregular masses with a delicate connective tissue stroma (Chart 2, Case 11).

Characterization of the Individual Glycosaminoglycans

The identification of the glycosaminoglycans in this study was as follows.

Hyaluronic Acid. The identification of hyaluronic acid was based on the susceptibility to the Streptomyces hyaluronidase and the mobilities corresponding to standard hyaluronic acid by the membrane electrophoresis with both buffer systems (pyridine:acetic acid, pH 3.5, and 0.2 M calcium acetate).

Chondroitin 4- and 6-Sulfate. By the electrophoresis with pyridine-acetic acid buffer system, the mobility of the material obtained from the tumor tissues was the same as that of standard chondroitin 4-sulfate or 6-sulfate, which could not be separated with this buffer system. By the electrophoresis with 0.2 M calcium acetate system, it was possible to

mg/mg dry weight

hyaluronic acid

chondroitin 4- and 6-sulfate

dermatan sulfate

heparan sulfate

1a 1b 1c 2 3a 3b 4 5 6 7 8 9 10 11 (case)

Chart 2. The amount of individual glycosaminoglycans in the breast tumors. Cases 1a, 2, and 3a, the myxomatous area of intracanalicular fibroadenoma; Cases 1b and 3b, the more fibrous and denser stroma of intracanalicular fibroadenoma; Case 1c, the capsule of intracanalicular fibroadenoma; Case 4, sclerosed type of intracanalicular fibroadenoma; Cases 5, 6, and 7, pericanalicular fibroadenoma; Case 8, gynecomastia; Cases 9 and 10, scirrhous carcinoma; Case 11, medullary carcinoma.
separate the standard chondroitin 4-sulfate and 6-sulfate, but the material from the tumor tissue appeared as 1 band extending from standard chondroitin 4-sulfate zone to 6-sulfate zone. The glycosaminoglycan was completely depolymerized with chondroitinase ABC or chondroitinase AC II, and unsaturated disaccharides corresponding to 3-O-β-D-4,5-glucuronosyl-N-acetylgalactosamine 4-sulfate and 3-O-β-D-4,5-glucuronosyl-N-acetylgalactosamine 6-sulfate were detected by descending paper chromatography in butyric acid:0.5 M ammonia (5:3).

Since chondroitin 4- and 6-sulfate could not be separated by the electrophoresis, their contents were shown together as chondroitin 4- and 6-sulfate.

**Heparan Sulfate.** The identification of the heparan sulfate was based on the undigestability with chondroitinase ABC, chondroitinase AC II, and *Streptomyces* hyaluronidase and on the mobilities corresponding to the standard heparan sulfate by the electrophoresis with both buffer systems (see above).

**Dermatan Sulfate.** The identification of the dermatan sulfate was based on the susceptibility to chondroitinase ABC and on the mobilities corresponding to standard dermatan sulfate by the electrophoresis with both buffer systems (see above). By the pretreatment of the materials obtained from all cases tested with chondroitinase AC II, the color intensity of the spot was partially diminished on the electrophoretogram. The products of the enzymatic reaction were analyzed by subjecting the digest of the glycosaminoglycan mixture to paper chromatography in butyric acid:0.5 M ammonia (5:3). Chart 3 shows a UV-absorption print of the chromatogram obtained from the case of gynecomastia. Spots could be seen corresponding to oligosaccharides when the material was completely digested with chondroitinase AC II. No such components were produced when the same sample was treated with chondroitinase ABC, indicating that the spot corresponding to standard dermatan sulfate was composed of dermatan sulfate-chondroitin sulfate copolymer.

The total hexuronic acid contents of the glycosaminoglycans mixture obtained from each tissue were analyzed by the carbazole method as well as orcinol method. Table 2 shows the ratio of uronic acid contents measured by the carbazole to those by the orcinol. It is also shown that the iduronic acid level was relatively high in Cases 6 and 8. The data support further the suggestion that the proportion of dermatan sulfate is relatively high in these cases.

### DISCUSSION

The present results indicate that the myxomatous stroma of intracanalicular fibroadenoma contained a large amount of glycosaminoglycans that were mainly hyaluronic acid. An increase of hyaluronic acid has been reported in the interfibrillar ground substance of myxedematous skin, in edematous laryngeal polyp, and in the first period of granulomatous evolution (8, 13, 18). It was noted that a striking increase in the hyaluronic acid synthesis was observed after transformation of fibroblasts induced by virus (7, 21). In the intracanalicular fibroadenoma, when viewed micro-
scopically, there was a remarkable proliferation of connective tissue, which projected into the ducts in the form of polypoid masses, producing great elongation and distortion of the ducts as shown in Fig. 1. The stromal element, consisting of stellate cells, was rich in vascularity. Murad et al. (12), when studying mammary fibroadenoma, suggested that certain stromal cells, derived from the pericyte, appeared to be responsible for the type of neoplasm, whereas the ductular proliferation was secondary to the stromal tissue overgrowth. Again, the present study shows that the myxomatous area contained a large amount of hyaluronic acid, which decreased as the stromal elements became dense and fibrous. From this result it may be considered that the stromal cells proliferating vigorously produced a large quantity of hyaluronic acid that inhibits the fibrogenic reaction. In his experimental study, Rydell (19) demonstrated that the injection of hyaluronic acid showed the decreased fibrotic wound reaction.

As shown in Fig. 6, the scirrhous carcinoma tissue examined in this study consisted of connective tissue intermingled with a large number of carcinoma cells. The tissue pieces, however, contained a large amount of glycosaminoglycans. The hyaluronic acid content was almost the same as that in chondroitin 4- and 6-sulfate, and the dermatan sulfate level was not as low. Microscopic views showed that the basement membrane stained with periodic acid-Schiff was indistinct, and the extensions of the epithelial processes into the adjacent stromal tissue could be seen. These histological features seem to indicate that the glycosaminoglycan synthesis is due to mesenchymal cell activity associated with the presence of carcinoma cells. In other words, the carcinoma cells might be able to promote the glycosaminoglycan production of mesenchymal cells. By electron microscopic studies on the epithelial stromal junction of mammary glands, Ozzello (15) demonstrated that, in the more advanced manifestation of the dysplasia, the delimiting fibroblasts showed disarray in their orderly distribution. Tarin (26) demonstrated an accumulation of fragmented basement membrane-like material and the extension of epithelial processes into the adjacent tissue as the fine structural changes at the junction between epithelium and connective tissue in mammary carcinoma. It was reported that scirrhous carcinoma cells did not have a surface function of the cells.

The present result shows also that the level of dermatan sulfate was higher in pericanalicular fibroadenoma and gynecomastia, which consisted of the denser and more fibrous stroma, than in intracanalicular fibroadenoma as shown in Figs. 3, 4, and 5. It has been reported that hyaluronic acid decreased whereas the sulfated mucopolysaccharides increased during the evolution of the granulomatous tissue (18) and that the rate of dermatan sulfate exceeded that of chondroitin 6-sulfate as the granulomatous tissue matured (10). By the chemical assay of salivary gland tumor, Lovell et al. (9) found that the level of dermatan sulfate was not related to the levels of the other mucopolysaccharides but was linked to the hydroxyproline contents of the tumor, which was related to the collagen in the capsule and septa. In the present study, the level of dermatan sulfate was not as high as in the scirrhous connective tissue compared with that in the myxomatous stroma, although the rate of dermatan sulfate content to the content of other glycosaminoglycans increased. Furthermore, evidence is presented in this paper to indicate that the component corresponding to standard dermatan sulfate on the electrophoretogram actually represents a dermatan sulfate-chondroitin sulfate copolymer. The data in Table 2 indicate that the carbazole to orcinol ratios of glycosaminoglycans from Cases 4, 9, and 10 are all 0.8, but the ratio of hyaluronic acid + chondroitin sulfate to dermatan sulfate is much less in Case 4 compared to Cases 9 and 10 as shown in Chart 2. It may be considered that iduronic acid content in the dermatan sulfate is larger in the case of carcinoma (Cases 9 and 10) compared to the other tumors. Several investigators (4, 5) have shown that the dermatan sulfates of skin, umbilical cord, aorta, meniscus, and intestinal mucosa are copolymers composed of 3 types of repeating disaccharide units in different proportion. Thus, the skin dermatan sulfate is reported to consist of L-iduronosyl-N-acetylgalactosamine 4-sulfate and D-glucuronosyl-N-acetyl-galactosamine 6-sulfate, whereas umbilical cord dermatan sulfate contains L-iduronosyl-N-acetylgalactosamine 4-sulfate, D-glucuronosyl-N-acetylgalactosamine 4-sulfate, and D-glucuronosyl-N-acetylgalactosamine 6-sulfate (4).

Based on these observations and studies on biosynthesis of dermatan sulfate, Malmström et al. (11) described that 4-sulfation of N-acetylgalactosamine moieties is coupled with the C-5 inversion of the adjacent glucuronic acid. The present observation that the iduronic acid containing region is rich in 4-sulfated N-acetylgalactosamine (Chart 3) is compatible with the synthetic mechanism proposed by Malmström et al. (11). It is conceivable that 4-sulfotransferase is a key enzyme involved in the synthesis of dermatan sulfate-chondroitin sulfate copolymer and that this synthetic process may be related with the dense and fibrous stroma formation or with the scirrhous change.

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Fig. 1. Microscopic section of tumor tissue from Case 1 showing myxomatous stromal element of intracanalicular fibroadenoma. H & E, × 40.

Fig. 2. Sclerotic type of intracanalicular fibroadenoma. Case 4. H & E, × 60.

Fig. 3. Pericanalicular fibroadenoma. Case 6. The fibrous tissue arranges in concentric fashion around the epithelial tubules, and the basement membrane is distinct. Periodic acid-Schiff, × 100.

Fig. 4. Pericanalicular fibroadenoma. Case 7. H & E, × 60.

Fig. 5. Gynecomastia. Case 8. Dense fibrous connective tissue can be seen. H & E, × 40.

Fig. 6. Scirrhous carcinoma. Case 9. H & E, × 100.
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