ACID MUCOPOLYSACCHARIDES IN SILICONE-INDUCED TUMORS OF RATS*1

(TLATE XXI)

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Synopsis

Determination was made of the acid mucopolysaccharide content in a tumor induced by subcutaneous imbedding of silicone in Sprague-Dawley rats. The acid mucopolysaccharides in fibroma and fibroadenoma proved to be hyaluronic acid and chondroitinsulfate B. Fibrosarcoma and fibrosarcoma transformed or being transformed from fibroma contained, in addition to these substances, chondroitinsulfate A. Only fibroadenoma additionally contained a very small amount of heparin or heparin-like material. No difference was found in the amounts of acid mucopolysaccharides contained in tumor tissue among silicone-induced tumors. A ratio of chondroitinsulfate to hyaluronic acid was the highest in fibroadenoma.

INTRODUCTION

Takeuchi16) reported that chondroitinsulfate and crude hyaluronic acid promoted the growth of solid Ehrlich ascites tumor. The present authors12) also demonstrated that chondroitinsulfate A and C promoted the growth of solid Tawa sarcoma and that large amounts of hyaluronic acid and chondroitinsulfate were contained in solid Tawa sarcoma and they increased during the growth phase of this tumor. It is very probable that acid mucopolysaccharides are utilized for the growth of tumor. However, experimental evidence on this problem does not seem sufficient.

In the authors' laboratory, various tumors were induced in rats by subcutaneously imbedding of silicone which is commonly used for denture construction and subcutaneous prosthesis. In the present series of work, an attempt was made to determine the composition and amount of acid mucopolysaccharides in these tumors.

MATERIALS AND METHODS

Tumor What were employed consisted of three fibromas (Photo 1), three fibroadenomas (Photo 2), three fibrosarcomas (Photo 3), and two fibrosarcomas transformed or being transformed from fibroma (Photo 4). These tumors were obtained after subcutaneous imbedding or injection of various commercial silicone rubbers (Silascon RTV-6501 for industrial use and Phycon 6500 for medical use, Fuji Polymer Industries Co., Ltd., Japan; Silastic 390 Soft Liner for dental use, Dow Corning Co., U.S.A.) in 120 Sprague-Dawley rats in order to examine the carcinogenicity of these materials.7) For chemical analysis,

*1 A part of this study was reported at the 30th Annual Meeting of the Japanese Cancer Association, Tokyo, October 1971.

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silicone-induced tumor used in this study seems to be more favorable than chemically
induced tumor because, in the former tumor, influence of the carcinogenic agent can be
ignored and its growth is found only adjacent to the subcutaneously imbedded material
without metastasis.

**Extraction and Fractionation of Acid Mucopolysaccharides** The silicone-induced
tumor was removed 60 to 90 days after it became palpable. Any visible necrotic tissue
was separated and excluded from the extraction. The tumor tissue was homogenized
with acetone, filtered, dried, and the powder was suspended in water. The solution was
adjusted to pH 1.8 with 5 N HCl, digested with pepsin for 48 hr at 38°, 10 N NaOH was
added till pH 8.5, and digested with trypsin for 24 hr. During incubation the suspension
was covered with a layer of toluene. It was cooled to 5° and 0.25 volume of 40% trichlo-
roacetic acid was added. The precipitated material was removed by centrifugation, and
calcium acetate was added to the supernatant to make a concentration of 2.5% and
acetic acid to make 0.25 N. Crude acid mucopolysaccharides were precipitated with 1.25
volumes of ethanol, allowed to stand for 24 hr at 4°, and centrifuged. The precipitate
was washed with 80% ethanol, dissolved in a mixture of 10% sodium acetate and 1 N
acetic acid, and stirred with a chloroform-amyl alcohol mixture. To the clear aqueous
layer obtained by centrifugation Lloyd’s reagent and kaolin were added. The solution
was stirred, filtered, and treated with 1.5 volumes of ethanol. The precipitate was washed
with 80% ethanol and redissolved in water. The solution was dialyzed against running
water for 72 hr, and subsequent addition of ethanol yielded whole acid mucopolysaccha-
rides. The whole acid mucopolysaccharides collected by centrifugation were dissolved in
0.035 M NaCl, and a 1% solution of cetylpyridinium chloride was added until precipita-
tion was complete. After the precipitate was allowed to incubate at 37° for 1 hr, 20 mg
of Celite-555 per mg of acid mucopolysaccharides was added. The mixture was centrifuged
for 20 min at 700 g, and the precipitate was washed with 0.03 M NaCl. The acid
mucopolysaccharides were then extracted from the precipitate successively with 0.4 M
NaCl containing 0.1% cetylpyridinium chloride, 1.2 M NaCl containing 0.1% cetyl-
pyridinium chloride, and 2.1 M NaCl. They were precipitated from the respective
solutions by adding 2 volumes of ethanol. The precipitates were collected, redissolved
in water, and dialyzed against running water for 24 hr. Subsequent addition of ethanol
yielded purified acid mucopolysaccharides. The precipitate was collected by centrifuga-
tion, washed several times with ethanol, then with ether, and dried.

**Analytical Procedures** Uronic acid was determined by Bitter-Muir’s method of
carbazole reaction. For hexosamine and sulfur analyses, samples were hydrolyzed for
16 hr with 4 N HCl at 100°. Determinations were made with an aliquot of the hydrolysate
following a modified Belcher’s method of Elson-Morgan reaction and the method of
Egami and Takahashi, respectively.

**Electrophoresis** Electrophoresis was performed on a cellulose acetate strip (Selecta,
Carl Schleicher & Schüll, Germany) in 0.2 M calcium acetate at a constant current of
1 mA/cm for 3 hr, or in formic acid-pyridine buffer (pH 3.0) at 0.5 mA/cm for 50 min. The
strip was stained by 0.5% Alcian Blue 8GS or 0.5% Toluidine Blue in 3% acetic
acid, and the background was discolored with tap water for 10 min.

**Quantitative Analysis of Isomeric Chondroitinsulfates** A quantitative analysis
of isomeric chondroitinsulfates in the 1.2 M NaCl fraction was carried out using electros-
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phoresis on cellulose acetate strips following the method of Seno et al. The dried electropherogram was cleared in decalin, and the relative density of stained chondroitinsulfates was scanned at 570 nm with a densitometer, Densitron model SP-3 (Jokō Sangyo Co., Ltd., Japan) with a slit 7×0.5 mm wide. Chondroitinsulfates A and B used for calibration curves were obtained from Seikagaku Kogyo Co., Ltd., Japan (super-special grade).

RESULTS

Table I shows the amount of acid mucopolysaccharides obtained in each fraction from silicone-induced tumors. When the fractionation procedure involving the extraction of acid mucopolysaccharide-cetylpyridinium chloride complex with increasing concentration of NaCl is carried out, hyaluronic acid is extracted with 0.4M NaCl, chondroitinsulfate with 1.2M, and heparin with 2.1M.

According to the results of a chemical analysis (Table II) and the electropherogram (Fig. 1) of each fraction of the four induced tumors, 0.4M NaCl fractions of four induced tumors proved to be hyaluronic acid, and 1.2M fractions of fibroma and fibroadenoma to be chondroitinsulfate B. On the other hand, the 1.2M fractions of fibrosarcoma and fibrosarcoma transformed or being transformed from fibroma proved to be chondroitinsulfate B and A. Unlike other tumors, fibroadenoma also contained acid mucopolysaccharide in the 2.1M NaCl fraction. Although the amount was too small for chemical analysis, the electropherogram in which formic acid-pyridine buffer (pH 3.0) was employed suggests that fibroadenoma contains a small amount of heparin or a closely related compound. On the electropherogram of the 1.2M NaCl fraction, the spots migrating more slowly than chondroitinsulfate B may be heparitin sulfate which is extracted in the 1.2M NaCl fraction containing cetylpyridinium chloride. However, no spots were detected because heparitin sulfate was not obtained as a standard material.

Since the 1.2M NaCl fraction contained unconfirmed material in a minimal amount, this fraction was separated quantitatively following the method of Seno et al. The results are shown in Table III. No difference was found in the amount of acid mucopolysaccharides contained in tumor tissue among four tumors. However, the amount of chondroitinsulfate contained in fibroadenoma was 2.5 times as much as that of hyaluronic acid.

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>No. of tumors</th>
<th>Tissue weight (g)</th>
<th>Fraction (M NaCl)</th>
<th>Amount(a) (mg/g tumor)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wet</td>
<td>Dry</td>
<td></td>
</tr>
<tr>
<td>Fibroma</td>
<td>3</td>
<td>42.2±5.8</td>
<td>6.1±0.7</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>3</td>
<td>45.2±9.2</td>
<td>7.2±1.2</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.1</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>3</td>
<td>53.1±11.7</td>
<td>5.8±1.0</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>Fibrosarcoma transformed or being transformed from fibroma</td>
<td>2</td>
<td>62.0±10.3</td>
<td>7.8±1.0</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.2</td>
</tr>
</tbody>
</table>

(a) Based on the dry weight of tumor. Each value is the mean±standard deviation.
Fig. 1. Electropherogram of each fraction by the CPC method

Buffer solution: 0.2M calcium acetate (A,B)
formic acid-pyridine (C)
Staining: 0.5% Alcian Bule (A,B)
0.5% Toluidine Bule (C)

A-1: Hyaluronic acid
2: 0.4M NaCl fraction of fibrosarcoma
3: 0.4M NaCl fraction of fibroma
4: 0.4M NaCl fraction of fibroadenoma
5: 0.4M NaCl fraction of fibrosarcoma transformed or being transformed from fibroma

B-1: Chondroitin sulfate C, A, B
2: 1.2M NaCl fraction of fibrosarcoma
3: 1.2M NaCl fraction of fibroma
4: 1.2M NaCl fraction of fibroadenoma
5: 1.2M NaCl fraction of fibrosarcoma transformed or being transformed from fibroma

C-1: Chondroitin sulfate A, B
2: Heparin
3: 2.1M NaCl fraction of fibroadenoma
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DISCUSSION

In spite of many reports3,5,6,9-12,15,17,18) so far made on acid mucopolysaccharides isolated from various animal tumors by biochemical procedures, no evidence seems to have been established as to the relation between the presence of acid mucopolysaccharides and tumor growth. The present study shows that fibroma and fibroadenoma contain hyaluronic acid and chondroitinsulfate B, and that fibroadenoma also contains heparin. On the other hand, both fibrosarcoma and fibrosarcoma transformed or being transformed from fibroma contain hyaluronic acid, chondroitinsulfate B, and chondroitinsulfate A. The findings on fibrosarcoma are not in agreement with those of Danishefsky et al.3) who showed that fibrosarcoma induced by the imbedding of polystyrene into Wistar rats contained hyaluronic acid and chondroitinsulfate, and that the amount of the former was twice that of the latter and chondroitinsulfate might be chondroitinsulfate C. It is considered that such a difference may depend on the difference in the strain of rats used or in the imbedded material.

Kuroda et al.6) extracted acid mucopolysaccharides from induced tumor and the skin of Donryu rats injected with methylcholanthrene. They showed that the induced

Table II. Analytical Data of Each Fraction

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Fraction (M NaCl)</th>
<th>Composiition of each fraction (%)</th>
<th>Sulfur</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroma</td>
<td>0.4</td>
<td>35.3</td>
<td>30.9</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>26.5</td>
<td>29.0</td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>0.4</td>
<td>28.2</td>
<td>25.6</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>23.5</td>
<td>24.3</td>
</tr>
<tr>
<td></td>
<td>2.1</td>
<td>26.5</td>
<td>26.2</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>0.4</td>
<td>33.7</td>
<td>25.9</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>23.9</td>
<td>23.8</td>
</tr>
<tr>
<td>Fibrosarcoma transformed or</td>
<td>0.4</td>
<td>31.2</td>
<td>26.7</td>
</tr>
<tr>
<td>being transformed from fibroma</td>
<td>1.2</td>
<td>28.3</td>
<td>28.0</td>
</tr>
</tbody>
</table>

Table III. Acid Mucopolysaccharide Subfractions from 1.2M NaCl Fraction

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Amount of chondroitinsulfate (ng/g tumor)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Fibroma</td>
<td>1.98±0.05</td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>2.53±0.19</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>1.23±0.15</td>
</tr>
<tr>
<td>Fibrosarcoma transformed or</td>
<td>1.29±0.17</td>
</tr>
<tr>
<td>being transformed from fibroma</td>
<td></td>
</tr>
</tbody>
</table>

a) Based on the dry weight of tumor.
Each value is the mean±standard deviation.

acid, though the amounts of these substances in fibroma, fibrosarcoma, and fibrosarcoma transformed or being transformed from fibroma were similar.

Kuroda et al.6) extracted acid mucopolysaccharides from induced tumor and the skin of Donryu rats injected with methylcholanthrene. They showed that the induced
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tumor as well as its adjacent skin contained hyaluronic acid and chondroitinsulfates B and A. On the other hand, the normal skin of a rat contained hyaluronic acid and chondroitinsulfate B. In the previous report\textsuperscript{12} we showed that chondroitinsulfate A accelerated tumor growth, but not hyaluronic acid or chondroitinsulfate B. The present study shows that acid mucopolysaccharides in fibroma and fibroadenoma, which were found to be benign, proved to be hyaluronic acid and chondroitinsulfate B, while fibrosarcoma and fibrosarcoma transformed or being transformed from fibroma, which were found to be malignant, contained chondroitinsulfate A in addition to these substances.

From these results chondroitinsulfate A contained specifically in fibrosarcoma and fibrosarcoma transformed or being transformed from fibroma, which are histopathologically proved as malignant, seems somewhat to be related to the neoplastic growth of a tumor tissue.

The authors express their sincere thanks to Prof. Toshikazu Tawa for his helpful guidance and valuable suggestions.

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REFERENCES


EXPLANATION OF PLATE XXI

Photo 1. Fibroma induced by silicone implantation, 508 days after imbedding. H–E. \times 130.

Photo 2. Fibroadenoma induced by silicone implantation, 616 days after imbedding. H–E. \times 130.

Photo 3. Fibrosarcoma induced by silicone implantation, 417 days after imbedding. H–E. \times 130.

Photo 4. Fibrosarcoma transformed from fibroma induced by silicone implantation, 665 days after imbedding. H–E. \times 130.

H–E= Hematoxylin-Eosin stain.