Acta Dermatovener (Stockholm) 55: 97–107, 1975

AN APPROACH TO EXPERIMENTAL SCLERODERMA, USING URINARY GLYCOSAMINOGLYCANS FROM PATIENTS WITH SYSTEMIC SCLERODERMA

Hidekazu Ishikawa, Shigeoki Suzuki, Ryuya Horiuchi and Hideya Sato

From the Department of Dermatology, Gunma University, School of Medicine, Maebashi, Japan

Abstract. As a result of intraperitoneal injections in mice of crude glycosaminoglycans obtained from the urine of patients with systemic scleroderma, a fibrotic process similar to the initial changes of human scleroderma was frequently observed, especially in the skin and esophagus when the glycosaminoglycans contained a probable variant of heparan sulfate. Furthermore, the scleroderma-like change was also observed, to some extent, in the ultrastructure of collagen fibers and glycosaminoglycans of the skin.

Key words: Scleroderma, systemic; Glycosaminoglycans; Urine; Scleroderma-like change in mouse; Scleroderma-influencing factor

Systemic scleroderma (scleroderma) is a disease in which abnormal fibrosis develops not only in the skin but also in the connective tissue of the lungs, heart, digestive organs and other sites. However, the details of its etiology and patho-mechanism remain to be elucidated. Klemperer et al. (11) reported in 1942 that this disease as well as SLE was demonstrated to be diffuse collagenosis, indicating fibrinoid degeneration to be a characteristic manifestation of these diseases and attaching especially great importance to the changes in ground substance. In this connection, changes in skin glycosaminoglycans (GAG) in scleroderma have been studied by a few investigators (5, 15, 21). On the other hand, increase in thin collagen fibrils and their irregular arrangement were disclosed by electron microscopy (1, 8, 12, 13, 17). It is a known fact that collagen fibers and glycosaminoglycans mutually exert closely-related influence. The problem is —which is the more primary change in the development of scleroderma.

In recent studies, we conducted one-dimensional cellulose acetate electrophoresis of GAG excreted in the urine of patients with scleroderma to observe the presence of carbazol reaction-positive GAG, which were subjected to changes apparently connected with the skin alteration of scleroderma (10). In view of the facts that the GAG showed a lower mobility than did chondroitin sulfate, and were only slightly digested by chondroitinase ABC or testicular hyaluronidase, heparan sulfate-like GAG might be considered. The GAG were obtained by elution mainly with 0.5 M NaCl and 1.25 M NaCl from a Dowex 1-X2 column. Especially GAG of 0.5 M NaCl fraction were mostly heparan sulfate-like. Assuming that these GAG, related to skin alterations of scleroderma, might have an influence on the occurrence of pathological changes in scleroderma, urinary GAG from patients were administered to mice, and the skin and visceral organs were histologically examined and the skin also with electron microscope. This paper is the first report on the series of experiments.

MATERIALS AND METHODS

Isolation of crude GAG from the urine was performed after the method of DiFerrante & Rich (3): The urine from untreated patients was adjusted to pH 5.0, and 5% cetyltrimethylammonium bromide (CTAB) was added to it in an amount of 1 ml per 30 ml. Subsequently, before the procedure, thimerosal was added to the urine in order to avoid possible bacterial growth, although the experimental results were nearly the same in both types. Then, after allowing the urine to stand in a refrigerator over 2 nights in order to yield complete precipitation of the CTAB-GAG complex, it was centrifuged at 3 000 rpm, and the precipitate was first washed 3 times with NaCl-saturated 96% ethanol, then washed and dried with ethanol and ethyl ether to obtain crude GAG. Next, after confirming that all the electrophoretic patterns of GAG obtained from the urines of individual cases were the same, the samples were pooled. As a result, crude GAG obtained from 52 l of the urine collected from 5 patients (25–64 years of age, females) (Sample 1) and from 43 l of the urine from 2 female patients (25 and 54 years of age) (Sample 2) were used for the experiments. To the pooled GAG were added 10 volumes of 0.05 M Tris-HCl buffer (pH 7.5) and 15 mg Pronase E per gram GAG (from Kaken Kagaku, Tokyo) to carry out digestion at 50°C for 24 hours. Then, after readjusting the pH, the same quantity of Pronase E was further added to perform digestion

Table I. *Urinary glycosaminoglycans (GAG) used for experimental scleroderma*

ChS = chondroitin sulfate, HS = heparan sulfate, Ch = chondroitin, HA = hyaluronic acid, ( ) = faintly observed, NI = not identified, ND = not done

Sample no.	Dowex-fraction (M NaCl)	GAG Volume[a]	GAG Fraction	Protein (volume)[a]
Scleroderma				
1	0.5	100	Unknown	19
	1.25	280	Unknown, HS, ChS	—
	1.5	340	ChS	—
	2.0	120	ChS	—
2	0.5	180	Unknown, Ch	230
	1.25	1 400	Unknown, ChS, HS	120
	1.5	990	ChS	50
	2.0	840	ChS	50
Normal				
3	0.5	80	HA, Ch, (HS)	ND
	1.25	360	Ch, ChS, HS	ND
	1.5	240	ChS, (HS)	ND
4	0.5	60	HA, HS	210
	1.25	1 000	Ch, HS, (ChS)	70
	1.5	1 040	ChS	20
	2.0	380	ChS	30
Scleredema				
5	0.5	470	NI	ND

[a] μg uronic acid or protein per 3 mg Dowex-fraction, which is dissolved in 1 ml water.

for another 24 hours. Deproteinization was carried out by adding trichloroacetic acid (final: 10%) and by centrifugation at 4°C, and then the supernatant was dialysed at 4°C for 2 days, in seamless cellulose tubing (27/32) from Visking. Next, 1% sodium acetate and 4 volumes of ethanol were added, to be allowed to stand in a refrigerator for 2 days, and centrifugation was conducted to obtain precipitate. This was applied to a Dowex 1-X2 column (Cl^-) (0.9 × 10 cm) (19). After passing a sufficient amount of water and 0.03 M NaCl, GAG were eluted successively with 0.5 M NaCl, 1.25 M NaCl, 1.5 M NaCl and 2.0 M NaCl. After the elution, each fraction was desalted by dialysis, and lyophilized to determine the weight. As controls, 2 samples of normal urine (Sample 3 from 10.8 l of the urine from 7 normal persons (22–66 years of age, females) and Sample 4 from 20 l from 4 subjects (31–43 years of age, males)) and also 2.8 l from a 7-year-old girl with scleredema (Sample 5) were used to fractionate GAG in a similar manner.

In animal experiments, 3 mg amounts of the above-mentioned samples were dissolved in 1 ml of 0.9% NaCl solution as thoroughly as possible, and after the uronic acid-containing GAG were confirmed to be completely dissolved in the solution, the supernatant was sterilized through a Millipore filter. Then the original liquid or diluted samples thereof was intraperitoneally administered to male mice of the DDD strain weighing about 20 g, at a dosage of 0.1 ml/day for 12–13 consecutive days (in some samples, for fewer days). As controls, 3 mg each of chondroitin sulfate (chondroitin 4-sulfate and chondroitin 6-sulfate), dermatan sulfate, potassium hyaluronate, chondroitin, heparin and heparan sulfate was similarly dissolved in 1 ml of 0.9% NaCl solution for injection into mice under the same conditions as in the urinary GAG. Heparan sulfate was prepared by our laboratory from 1.25 M NaCl fraction of normal urine through testicular hyaluronidase digestion, and was identified by two-dimensional electrophoresis.

Potassium hyaluronate was purchased from Sigma and other GAG from Seikagaku Kogyo, Tokyo. Two days after completion of the administration, the animals were sacrificed with ether or by carotid dissection, and the dorsal skin near the root of forelimb, as well as visceral organs, was cut out for histological examination: the skin was also examined electronmicroscopically. Preparation of specimens for electron microscopy: fixed in 2.5% glutaraldehyde, 2% OsO_4 in 0.14 M Veronal acetate buffer (pH 7.4) at 4°C for 2 hours. Dehydrated with ethanol, and embedded in Epon 812. Then fine-sectioned and double-stained with uranyl acetate–lead citrate. Observed with JEOL JEM-7 electron microscope at 60 kV. In addition, for electron microscopic demonstration of GAG, other specimens were fixed in 1.2% glutaraldehyde with 500 ppm Ruthenium red in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 1 hour. Then, fixed again in 2% OsO_4 with 500 ppm Ruthenium red in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 3 hours (14). Determination of GAG: Total amount was estimated as hexuronic acid by Dische's method (4). Qualitative analysis was carried out by two-dimensional electrophoresis on cellulose acetate (7).

RESULTS

(*A*) *Administered Urinary GAG*

GAG volumes in 3 mg samples are shown in Table I. The 0.5 M NaCl fraction from scleroderma or from normal controls showed considerable protein reaction by the Lowry method, whereas protein amounts in other fractions were small or negligible, as compared with GAG volume. The quantities of injected GAG were 10–140 μg for each injection, totalling 120–1 820 μg in the case of GAG-fractions obtained from the urine of scleroderma patients (Table II).

Qualitative analysis. In the case of normal urine, two-dimensional electrophoresis detected, as shown in Table I and Fig. 1, hyaluronic acid, chondroitin and heparan sulfate (faint) from 0.5 M NaCl fraction of Sample 3 and hyaluronic acid and heparan sulfate from that of Sample 4; chondroitin, heparan sulfate and chondroitin sulfate from 1.25 M NaCl fraction of both samples; from 1.5 M NaCl fraction of Sample 3 chondroitin sulfate and heparan sulfate (faint) and from Sample 4 chondroitin sulfate only; and from 2.0 M NaCl fraction of Sample 4, chon-

Table II. *Skin change in mice injected with urinary glycosaminoglycans*

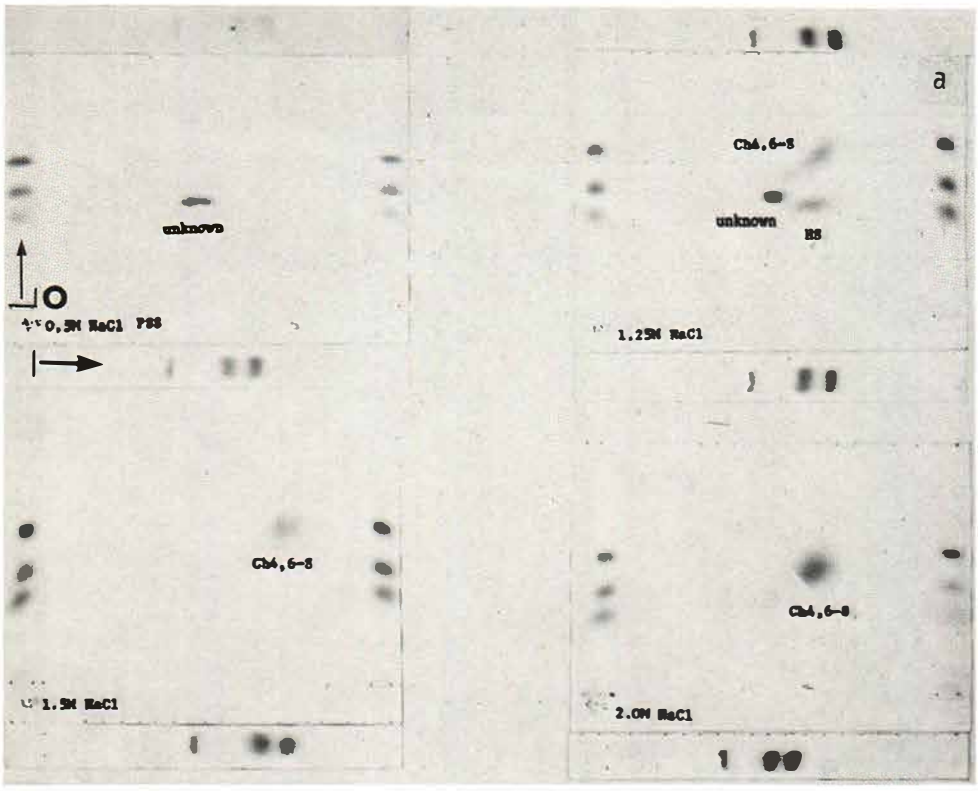
++ showing marked fibrosis, + slight fibrosis. HA = hyaluronic acid, DS = dermatan sulfate, HS = heparan sulfate, Ch4-S = chondroitin 4-sulfate, Ch6-S = chondroitin 6-sulfate, Ch = chondroitin, HP = heparin

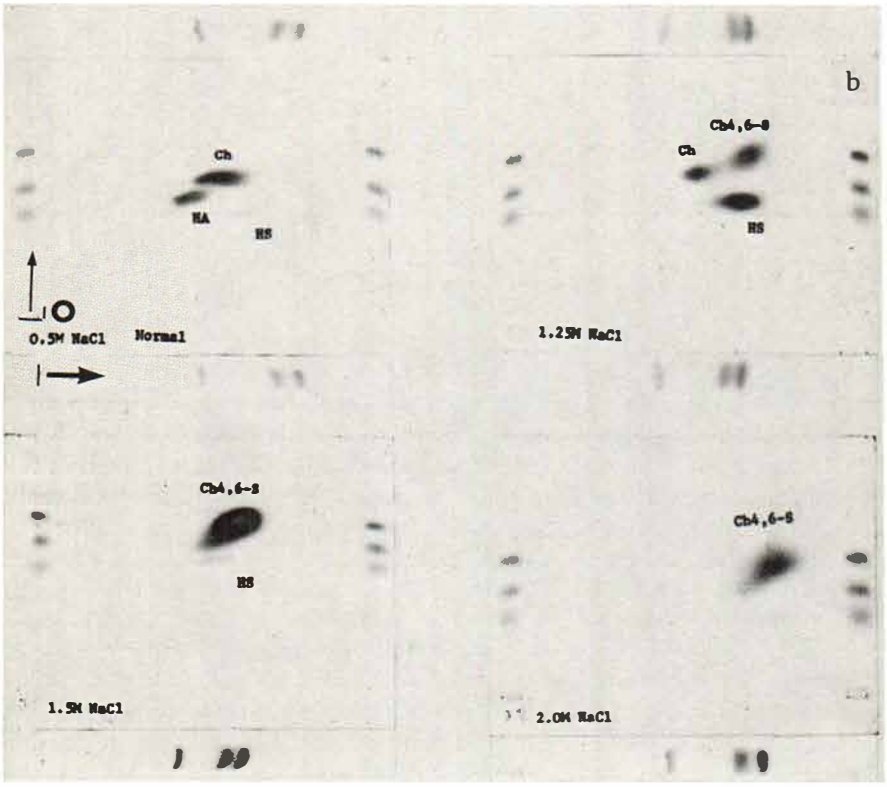
	Scleroderma-urine					Normal urine				
Dowex-fraction	Sample no.	Injected GAG ($\mu g^a \times$ day)	Mice no.	++	+	Sample no.	Injected GAG ($\mu g \times$ day)	Mice no.	++	+
0.5 M NaCl	1	10 × 12	4	3	1	3	8 × 12	3	0	1
	2	18 × 13	5	2	1	4	30 × 12	4	0	0
						4	60 × 12	4	0	0
Total			9	5	2			11	0	1
1.25 M NaCl	1	28 × 12	3	1	1	3	36 × 12	3	0	2
	2	140 × 8–13	5[b]	4[b]	0	3	72 × 13	1	0	0
						4	100 × 12	3	0	0
Total			8	5	1			7	0	2
1.5 M NaCl	1	34 × 12	3	0	1	3	24 × 12	1	0	0
	2	99 × 13	1	0	0	4	100 × 12	2	0	0
Total			4	0	1			3	0	0
2.0 M NaCl	1	12 × 12	1	0	0	4	38 × 12	1	0	0
	2	84 × 13	1	0	0					
Total			2	0	0			1	0	0
Scleredema (0.5 M NaCl)	5	47 × 12	2	0	2					
Standard GAG										
HA		82 × 12	7	0	1					
DS		58 × 12	7	0	0					
HS		100 × 12	3	0	0					
Ch4-S		130 × 13	4	0	0					
Ch6-S		80 × 11	7	0	0					
Ch		32 × 12	6	0	0					
HP		124 × 12	5	0	0					
Saline		× 12–13	11	0	0					

[a] Expressed as uronic acid.
[b] 2 mice dead during the experiment.

droitin sulfate. On the other hand a 0.5 M NaCl fraction of the urine from scleroderma patients, showed in both Samples 1 and 2 an unknown spot, which was detected neighbouring, but non-corresponding to either chondroitin, heparan sulfate, or hyaluronic acid. In addition to the unknown spot, Sample 2 showed chondroitin. In 1.25 M NaCl fraction of both samples, this unknown spot was also detected in addition to chondroitin sulfate and heparan sulfate. In 1.5 M NaCl and 2.0 M NaCl fractions, chondroitin sulfate was detected in both samples, not distinct from the normal urine.

The unknown glycosaminoglycan in scleroderma-urine, apparently characteristic of scleroderma, was extracted from the sheets after electrophoresis. It showed carbazol-positivity and the glucosamine galactosamine molar ratio (2.47) in Sample 2 was quite close to that (1.64) of heparan sulfate in this sample (determined with the JLC-6AH automatic amino acid analyzer). Amino acids content was negligible. From the above results and the facts that the glycosaminoglycan was digested neither by testicular hyaluronidase nor by chondroitinase ABC and that it was found in a position relatively close to heparan sulfate on electrophoresis, the unknown glycosaminoglycan may be considered to be a variant of heparan sulfate of differing polymerization and/or sulfation.


a
unknown
0.5M NaCl PBS
Ch4,6-S
1.5M NaCl
Ch4,6-S
unknown
HS
1.25M NaCl
Ch4,6-S
2.0M NaCl


b
Ch
HA
HS
0.5M NaCl Normal
Ch4,6-S
HS
1.5M NaCl
Ch4,6-S
Ch
HS
1.25M NaCl
Ch4,6-S
2.0M NaCl

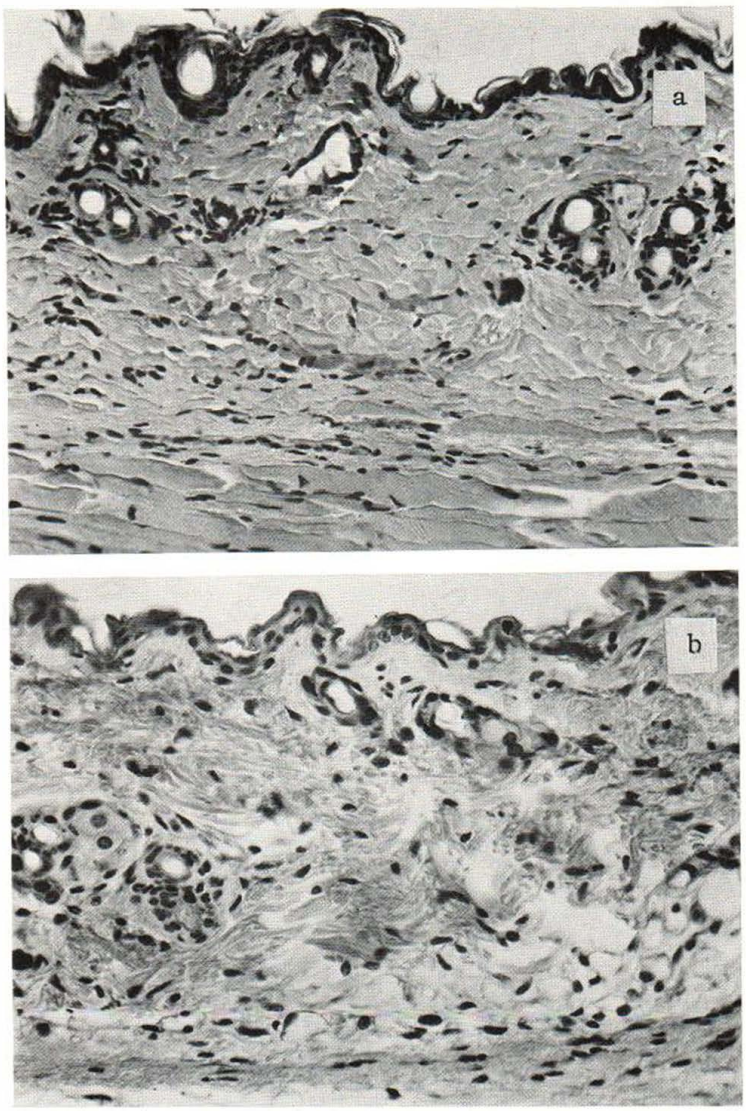


Fig. 2. Skin change in the mouse injected with urinary GAG (1.25 M NaCl fraction) from scleroderma, showing homogenization of collagen fibres and fibrosis from the dermis till the subcutaneous muscle layer (*a*). (*b*) Control skin injected with saline. Hematoxylin-eosin stain, × 260.

(*B*) *Results of Animal Experiments*

In a hyaluronic acid-injected group, body weight was decreased after the injection and in the group injected with a large dosage of 1.25 M NaCl-GAG from Sample 2, 2 of 5 animals died during the experiment. However, in other groups there was no significant difference from saline-injected controls in body weight before and after injection.

(1) *Findings in skin*

The results are summarized in Table II.

(*a*) *Histological findings*. Of the groups given GAG

Fig. 1. Two-dimensional electrophoresis of urinary glycosaminoglycans (GAG) fractions, used for experimental scleroderma (*a*) from scleroderma (Sample 1), (*b*) from normal (Sample 3). A thick arrow shows migrating direction of the first electrophoresis in 0.1 M pyridine, 0.47 M formic acid (pH 3.0) at 0.5 mA/cm for 1 hour (Reference GAG from the origin (○) side; potassium hyaluronate, dermatan sulfate and chondroitin sulfate). A thin arrow shows the second dimension in 0.1 M barium acetate (pH 8.0) at 0.5 mA/cm for 3 hours (standard GAG from the bottom; heparin, dermatan sulfate and chondroitin sulfate). Ch4, 6-S: chondroitin sulfate, HA: hyaluronic acid, Ch: chondroitin, HS: heparan sulfate. Unknown spot in scleroderma probably represents a variant of heparan sulfate (see text).

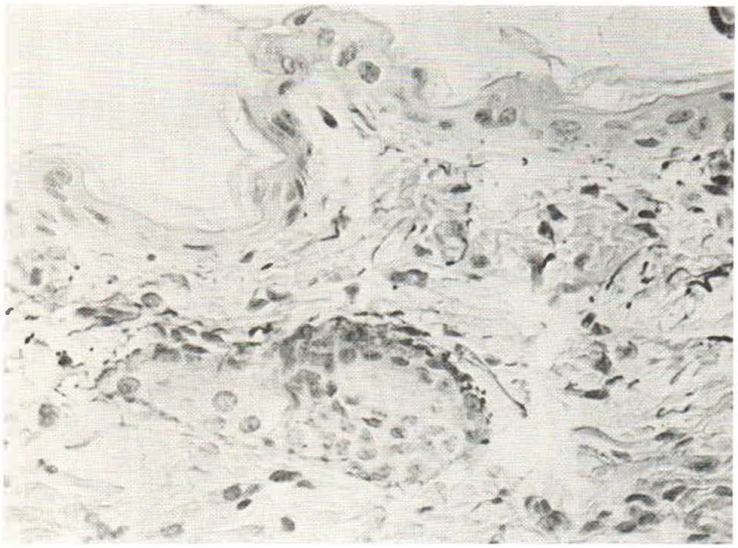


Fig. 3. Increase of fragmented elastic fibres in the mouse injected with urinary GAG (1.25 M NaCl fraction) from scleroderma. Resorcinfuchsin stain, × 260.

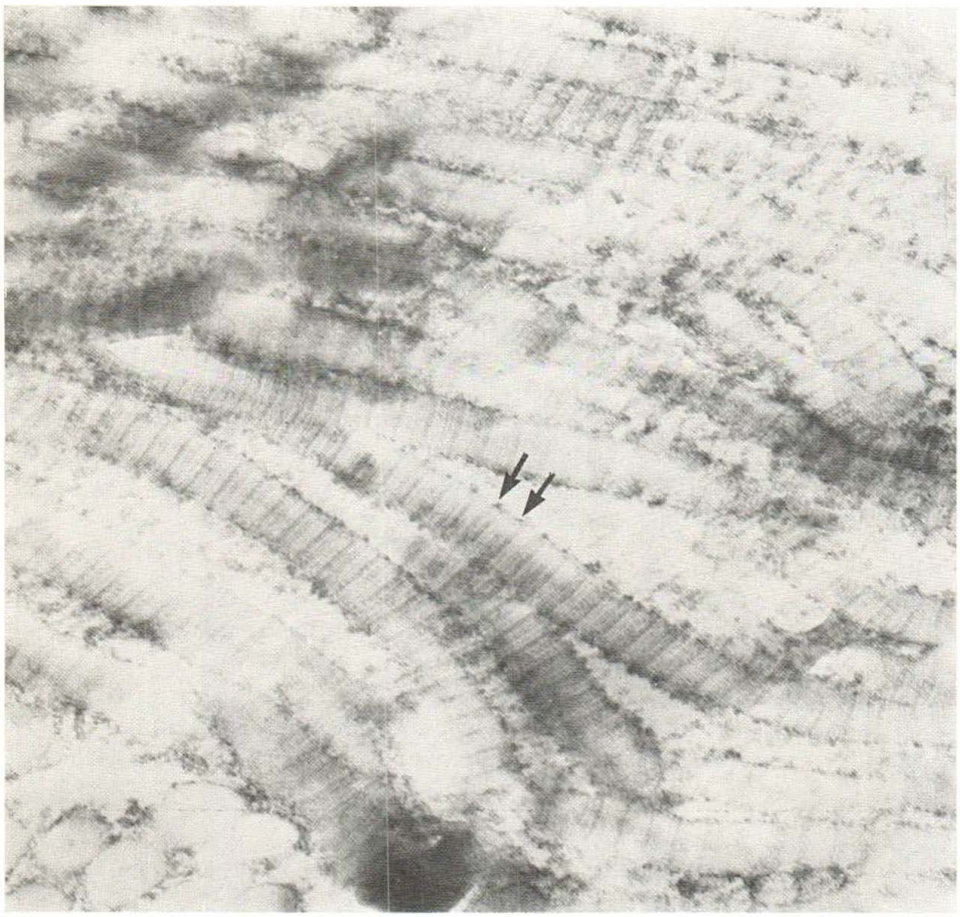


Fig. 4. Ultrastructure of the skin in the mouse injected with urinary GAG (1.25 M NaCl fraction) from scleroderma, showing irregularly arranged collagen fibrils. Arrows indicate increased Ruthenium red-positive particles close to cross bandings of fibrils. × 71 800.

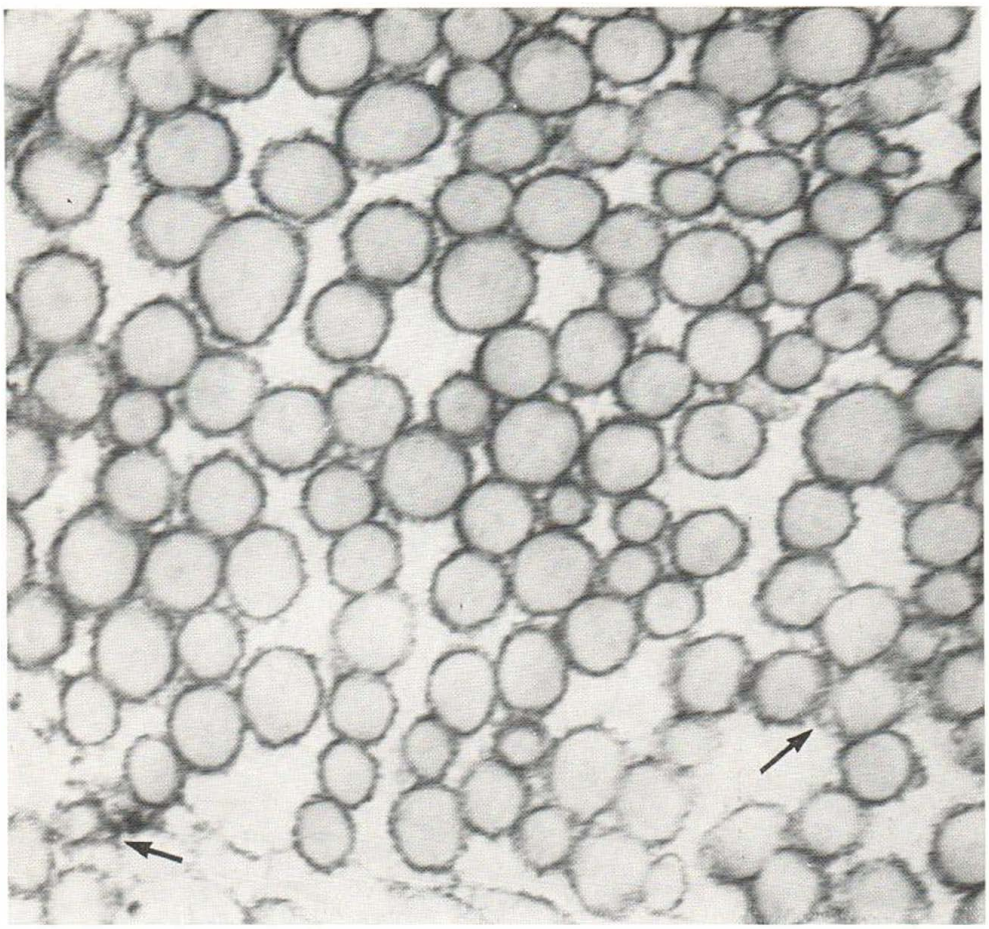


Fig. 5. Both thin and thick collagen fibrils within one bundle in the skin of mouse injected with urinary GAG (0.5 M NaCl fraction) from scleroderma. Furthermore, Ruthenium red-positive filaments or particles are visible around the collagen fibrils. Arrows indicate lattice-like filaments (Kobayasi & Asboe-Hansen). × 96 400.

from the urine of patients with scleroderma, 0.5 NaCl and 1.25 M NaCl fraction groups (5 of 9 mice of 0.5 M NaCl fraction group; 5 of 8 mice of 1.25 M NaCl fraction group) were observed, as shown in Fig. 2, to develop swelling of collagen fibers and considerable fibrosis from the middle layer of the corium to the subcutis. In some sections a perivascular fibrosis of the subcutaneous tissue was seen. Fragmented elastic fibers were increased in number in the upper corium (Fig. 3). Furthermore, with Hale-PAS stain, an intensified PAS reaction was seen in addition to many mast cells. In other words, in the groups given the fraction containing a variant of heparan sulfate, alteration reminiscent of early scleroderma was observed. In other groups, no definite change was seen, apart from slight subcutaneous fibrosis in some cases.

(*b*) *Electron microscopic findings.* It was noted that the preparations showing the above changes demonstrated irregular arrangement and mutual diastasis of collagen fibrils (Fig. 4) and also significant occurrence of collagen fibrils of small diameter (ca. 300–400 Å) intermingled with 650–1 000 Å thick collagen fibrils (Fig. 5). No abnormalities were found regarding banding pattern. Further, Ruthenium red-positive filaments or particles were increased in number around collagen fibrils, intermingled with Ruthenium red-negative indistinct fibrils (Fig. 6); these findings were also observed in the group injected with urinary GAG from scleredema, however. Moreover, with Ruthenium red stain the matrix was observed to be rich in fine network of filaments with aggregated particles. There was no remarkable change in elastic fibers but the

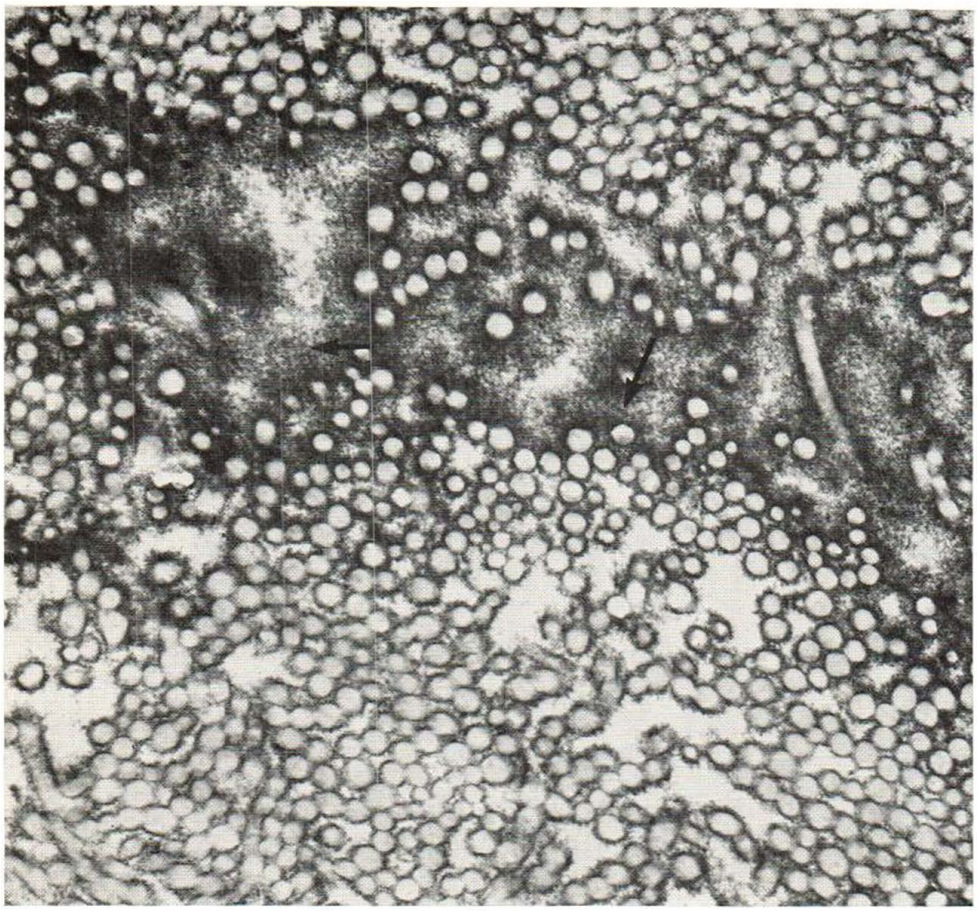


Fig. 6. The skin of the mouse injected with urinary GAG (0.5 M NaCl fraction) from scleroderma, showing increased number of Ruthenium red-positive filaments or particles around collagen fibrils and in the periphery of collagen bundles, intermingled with Ruthenium red-negative indistinct fibrils (arrows). ×32 800.

Table III. *Visceral changes in mice injected with urinary glycosaminoglycans*

	M NaCl															
	Scleroderma-urine				Normal Urine				Standard GAG							
	0.5	1.25	1.5	2.0	0.5	1.25	1.5	2.0	HA	HS	DS	Ch4-S	Ch6-S	HP	Ch	Saline
Lungs																
Perivascular fibrosis	4/8	6/8	1/4	1/1	1/9	0/7	0/3	0/1	0/4	0/3	0/4	0/4	0/4	0/5	0/4	0/8
Interstitial edema and cell infiltration	3/8	7/8	0/4	0/1	1/9	1/7	0/3	0/1	0/4	0/3	0/4	1/4	0/4	0/5	1/4	0/8
Esophagus																
Edema of mucosa	6/7	5/6	0/4	0/1	0/9	0/7	0/3	0/1	0/4	0/3	0/4	0/4	0/4	0/5	0/4	0/8
Muscle atrophy and/or fibrosis	4/7	5/6	2/4	0/1	1/9	2/7	1/3	0/1	0/4	0/3	0/4	0/4	0/4	0/5	0/4	0/8
Heart																
Myocardial fibrosis and/or welling of muscle fibres	2/8	3/8	0/4	0/1	0/9	0/7	0/3	0/1	0/4	0/3	0/4	0/4	0/4	0/5	0/4	0/8

presence of parallel-arranged elastic fibrils as described by Kobayashi & Asboe-Hansen (12) among collagen fibrils was observed.

2. *Visceral findings*

The results are shown in Table III.

In many of mice injected with the 0.5 M NaCl fraction and the 1.25 M NaCl fraction from scleroderma, pathological changes were observed in the lungs, esophagus and heart as well as in the skin, while the liver, spleen or kidney showed scarcely any noticeable change. The lungs showed perivascular fibrosis, interstitial edema and cell-infiltration composed of histiocytes, lymphocytes and/or plasma cells (Fig. 7). The lower end of the esophagus showed subepithelial homogenization of the connective tissue, and partial atrophy and fibrous replacement of the muscularis (Fig. 8). Also, in the heart, a slight fibrosis of the myocardium was observed. These changes were more conspicuous in the groups given a large dosage of 0.5 M NaCl or 1.25 M NaCl–GAG. Similar changes were seen, through rarely, in mice given other fractions from scleroderma and in mice given 0.5–1.5 M fractions of normal urine.

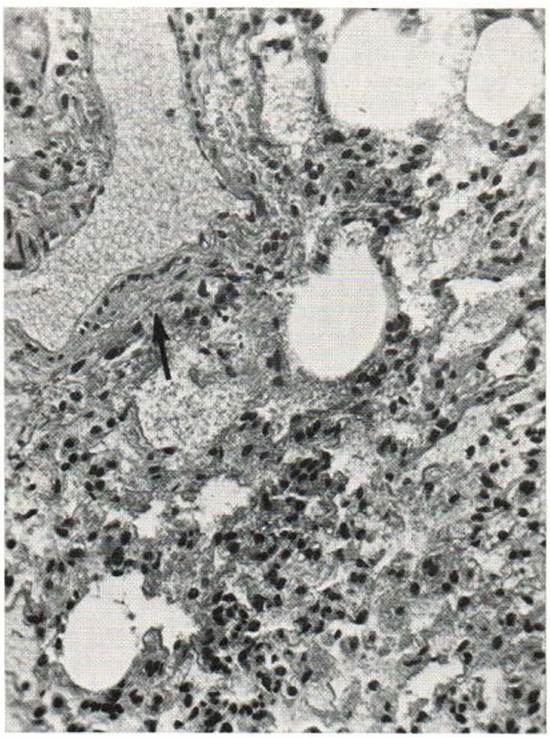


Fig. 7. Lung of the mouse injected with urinary GAG (1.25 M NaCl fraction) from scleroderma, showing interstitial edema with cellular infiltration and perivascular fibrosis (arrow). Hematoxylin-eosin stain, × 260.

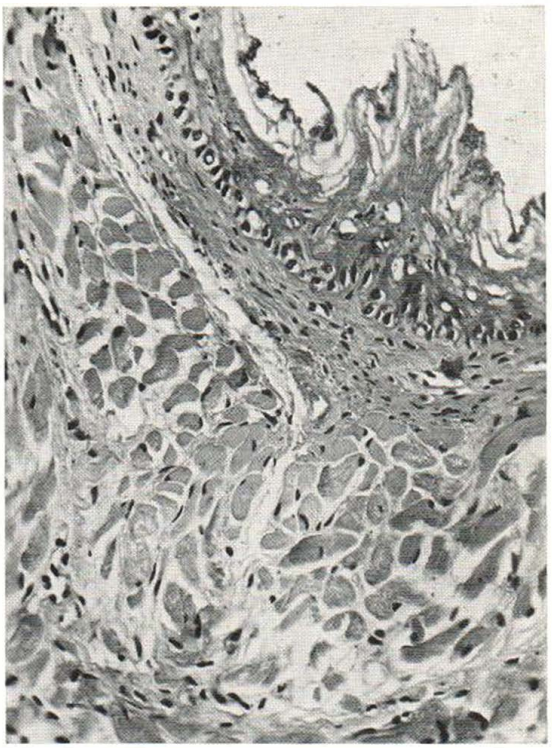


Fig. 8. Lower end of esophagus in the mouse injected with urinary GAG (1.25 M NaCl fraction) from scleroderma. There are atrophy of the muscularis, replaced by fibrosis and subepithelial homogenization of the connective tissue. Hematoxylin-eosin stain, × 260.

DISCUSSIONS

Previously, we reported that histological observations of the skin in systemic scleroderma demonstrated the sclerosis not only in collagen but also on the blood vessels (9). The vascular sclerosis appeared to occur first in the subendothelial area and later to spread to the circumference. This indicates, as already suggested by Szodoray (20), the possibility that a certain substance (scleroderma-influencing factor) induces the pathological changes in the connective tissue through the blood circulation. We have observed, as already described, that some GAG subjected to changes in close connection with scleroderma were excreted in the urine of patients (10). It is well-known that glycosaminoglycans (especially sulfated glycosaminoglycans) play an important role in maturation of collagen fibers or the fibrotic process. Noda (15) and Fleischmajer &

Perlish (5) described an increase of dermatan sulfate in the sclerotic skin. Uitto (21) stated that in the grossly normal skin of scleroderma, there was more correlationship between collagen biosynthesis and synthesis of chondroitin sulfate. In the present experiment, assuming that the above-described characteristic GAG, when excreted in the urine of patients with scleroderma might induce abnormal fibrosis in this disease, we injected mice with urinary and standard GAG to examine the skin and visceral changes. As a result, the groups injected with fractions containing an unknown glycosaminoglycan-spot (probably a variant of heparan sulfate) were observed to demonstrate interstitial edema, fibrosis, or inflammatory reaction, not only in the skin but also in the lungs, esophagus and heart. These changes seem, to some extent, to be similar to those of scleroderma (2, 6, 13, 16, 18) especially in alterations of the skin and esophagus. However, fibrosis or sclerosis is less conspicuous than scleroderma, and elastosis in late scleroderma was not observed at all. On the other hand, regarding electron microscopic skin changes, in the preparations showing histological change, an increase in thin collagen fibrils was observed among thick collagen fibrils, and in the distribution pattern an appearance of fibrils finer than 400 Å was impressive, when compared with specimens showing no histological change.

In human scleroderma it has been reported that fine collagen fibrils increase markedly with bimodal distribution (1, 13, 17) or with unimodal distribution (8). Kobayashi & Asboe-Hansen (12) noticed 3 kinds of collagen fibrils in scleroderma. They were (i) clusters of thin and thick collagen fibrils, diameter 200–900 Å, found among collagen bundles, (ii) 700 Å thick collagen fibrils, and (iii) compact bundles of 1 000 Å thick fibrils. The former finding may be compatible with that seen in our experiment.

In addition, in the present study, Ruthenium red-positive filaments or particles were increased in number around collagen fibrils. Moreover, Ruthenium red-negative fibrils were observed to be intermingled with the filaments. The former probably represent GAG, while the latter are similar to the "indistinct elastic fibrils" reported by Kobayashi. "Parallel-arranged elastic fibrils" were likewise seen in our preparations. However, degeneration of elastic fibers was not observed. Furthermore, in interfibrillar matrix there was a fine network of Ruthenium red-positive filaments with aggregated particles, probably representing hyaluronic acid.

To summarize, it is considered that from the histological and electron microscopic aspects the changes seen in mice injected with urinary GAG resemble to some extent those of early scleroderma. Thus, this study has indicated the presence of a "scleroderma-influencing factor" in the urine of scleroderma and it may well be a variant of heparan sulfate that acts as this factor, although heparan sulfate itself isolated from normal urine induced no change. Nevertheless, further long-term studies under various conditions are still required in order to induce a perfect experimental scleroderma: Neither marked fibrosis in the organs of necropsied patients nor marked elastosis of the skin in late scleroderma was observed in this experiment. Furthermore, the kidney, though one of the most affected organs at autopsy of scleroderma cases, exhibited no change in mice. Mice injected with other urinary GAG fractions including fractions from normal urine or with chondroitin, and chondroitin 4-sulfate also demonstrated a similar change in lungs or esophagus, though in few cases.

ACKNOWLEDGEMENTS

This work was supported by Research Grants from the Ministry of Education, Japan.

The authors express sincere thanks to Miss Masayo Kurihara and Miss Takako Tuihiji for skilful technical assistance.

REFERENCES

1. Bahr, G. F.: Elektronenmikroskopische Untersuchungen bei Sklerodermie. Aerztl Forsch *10:* 255, 1956.
2. D'Angelo, W. A., Fries, J. F., Masi, A. T. & Shulman, L. E.: Pathologic observations in systemic sclerosis (scleroderma). A study of fifty-eight autopsy cases and fifty-eight matched controls. Amer J Med *46:* 428, 1969.
3. DiFerrante, N. & Rich, C.: The mucopolysaccharide of normal human urine. Clin Chim Acta *1:* 519, 1956.
4. Dische, Z.: A new specific color reaction of hexuronic acid. J Biol Chem *167:* 189, 1947.
5. Fleischmajer, R. & Perlish, J. S.: Glycosaminoglycans in scleroderma and scleredema. J Invest Dermatol *58:* 129, 1972.
6. Goldgraber, M. B. & Kirsner, J. B.: Scleroderma of the gastrointestinal tract. AMA Arch Pathol *64:* 255, 1957.
7. Hata, R. & Nagai, Y.: A rapid and micro method for separation of acidic glycosaminoglycans by two-dimensional electrophoresis. Anal Biochem *45:* 462, 1972.
8. Hayes, R. L. & Rodnan, G. P.: The ultrastructure of skin in progressive systemic sclerosis (scleroderma). II. Dermal collagen fibres. Am J Pathol *63:* 433, 1971.
9. Ishikawa, H. & Mori, S.: Ein Beitrag zum histologischen Unterschied zwischen progressiver und circumscripter Sklerodermie. Hautarzt *23:* 404, 1972.

10. Ishikawa, H., Mori, S. & Kamakura, K.: Urinary acid mucopolysaccharides in patients with diffuse scleroderma. Connective Tissue *4*: 17, 1972.
11. Klemperer, P., Pollack, A. D. & Baehr, G.: Diffuse collagen disease: acute disseminated lupus erythematosus and diffuse scleroderma. JAMA *119*: 331, 1942.
12. Kobayasi, T. & Asboe-Hansen, G.: Ultrastructure of generalized scleroderma. Acta Dermatovener (Stockholm) *52*: 81, 1972.
13. Korting, G. W. & Holzmann, H.: Die Sklerodermie und ihr nahestehende Bindegewebsprobleme, 41 pp. Georg Thieme Verlag. Stuttgart, 1967.
14. Luft, J. H.: Electron microscopy of cell extraneous coats as revealed by ruthenium red staining. J Cell Biol *23*: 54A, 1964.
15. Noda, M.: Studies on fractional determination of the acid mucopolysaccharides in the cutaneous tissues. Jap J Dermat Ser B *75*: 396, 1965.
16. Piper, W. N. & Helwig, E. B.: Progressive systemic sclerosis. Visceral manifestations in generalized scleroderma. AMA Arch Dermatol *72*: 535, 1955.
17. Rupec, M. & Braun-Falco, O.: Elektronenmikroskopische Untersuchungen über das Verhalten der Kollagenfibrillen der Haut bei Sklerodermie. Arch Klin Exp Derm *218*: 543, 1964.
18. Sackner, M. A.: Scleroderma, 22 pp. Grune & Stratton, New York and London, 1966.
19. Schiller, S., Slover, G. A. & Dorfman, A.: Method for the separation of acid mucopolysaccharides: Its application to the isolation of heparin from the skin of rats. J Biol Chem *236*: 983, 1961.
20. Szodoray, L. & Tuza, C.: Über die Histochemie der Sklerodermie. Hautarzt *11*: 63, 1960.
21. Uitto, J., Helin, G., Helin, P. & Lorenzen, I.: Connective tissue in scleroderma. Acta Dermatovener (Stockholm) *51*: 401, 1971.

Received July 25, 1974

H. Ishikawa, M.D.
Department of Dermatology
Gunma University
School of Medicine
Maebashi, Showa-Cho
Japan