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Biochemical Characterization of Scleroderma-inducing Glycosaminoglycan

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The scleroderma-inducing *N*-sulfated glycosaminoglycan previously isolated by us from the urine of patients with systemic scleroderma was further purified: it was biochemically characterized by low *O*-sulfation and relative high *N*-sulfation. Consistent with this finding, desulfated and *N*-resulfated heparin, which had a similar composition to the urine-derived scleroderma-inducing glycosaminoglycan, induced a significant degree of sclerotic fibrosis in the skin of mice which had received intraperitoneal injections of it, whereas *N*-desulfated heparin with contrasting sulfation to it failed to cause any significant change in the skin. (Received February 11, 1988.)

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Systemic scleroderma (SS) is characterized by sclerotic fibrosis of the skin, which is preceded by an interstitial edema (1). The etiology of SS remains uncertain. Although collagen and fibronectin may play an important role in the formation of altered fibrosis in this disease, recent studies on glycosaminoglycan (GAG), another connective tissue component, revealed an increase in GAG synthesis by scleroderma fibroblasts (2–5) particularly in response to mitogen-activated T lymphocytes (6) or growth factors (7), as well as increased GAGs in the tissue, serum or urine of SS patients (8–14). As evidence of a direct effect of GAG on the pathogenesis of scleroderma, we previously isolated, from the urine of patients with SS, a particular glycosaminoglycan which induced a scleroderma-tous change in mice (15, 16). Biochemical analysis of the GAG revealed a heparan sulfate-like structure (16, 17).

In the present study, the GAG was further purified in order to elucidate the chemical characteristics. In addition, GAG with a similar structure to the scleroderma-inducing one was synthesized from commercial heparin, and its scleroderma-inducing ability was examined.

MATERIALS AND METHODS

Purification of scleroderma-inducing GAG from the urine of patients with SS

Crude GAG isolated from pooled urine samples (15) was purified by a four-stage procedure in the present study: initially it was thoroughly digested with pronase, testis-hyaluronidase and chondroitinase ABC in succession (16). After precipitation with ethanol, the sample was subjected to Dowex 1-X2 column chromatography (16). The 0.8 M NaCl eluate was further applied to a Sephadex G-50 column, as previously described (17). In the present study, the 0.8 M NaCl fraction was directly applied to a Sephadex G-50 column (2.5×90 cm), and the first peak fraction (fraction A) was further separated on a Superose™ 12 column by FPLC (Pharmacia) into three fractions (A-1, -2 and -3), as depicted in Fig. 1.

Production of chemically modified heparins

Desulfated heparin: Solvolytic desulfation was carried out according to a modified procedure (18) of Usov et al. (19). 200 mg of heparin sodium (Sigma) dissolved in 2 ml of water was applied to a Dowex 50 W-X8 column (200–400 mesh, H^+), eluted with 20 vol of water, then adjusted to pH 6.8 with

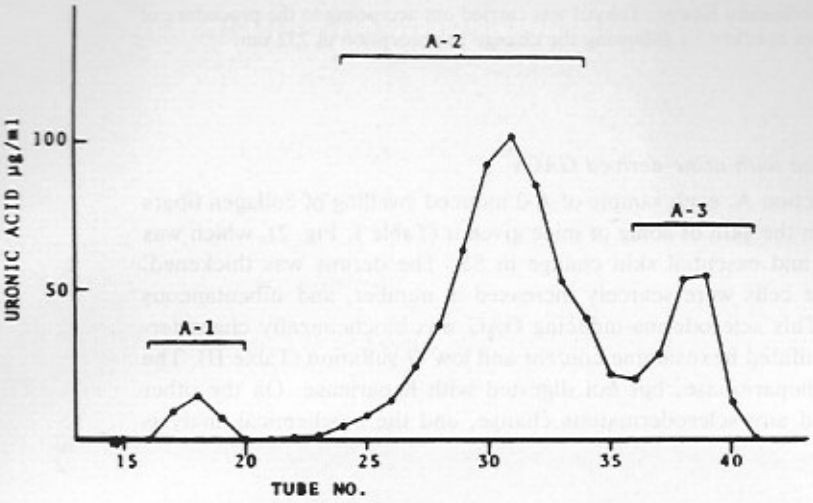

Fig. 1. Fractionation of urine-derived *N*-sulfated glycosaminoglycan by FPLC. After Sephadex G-50 column chromatography, the fraction A was applied to a Superose™ 12 column (1.0×30 cm) and eluted with 200 mM NaCl at a speed of 0.2 ml/min. 0.5 ml-fractions were collected. Fraction A sample applied was 400 µg as uronic acid volume.

pyridine (special grade) and lyophilized. 100 mg of the sample in 7 ml dimethyl sulfoxide with 140 µl pyridine was heated at 100°C for 9 h, then cooled in 3.5 ml of ice-cold water, dialysed against distilled water at 4°C for 2 days, and lyophilized.

Desulfated and N-acetylated heparin: For *N*-acetylation (20), 22 mg of the desulfated heparin was dissolved in 0.4 ml water with 40 µl methanol and 20 µl acetic anhydride, and to it was added 0.5 ml suspension of Dowex 1-X2 (CO_3^-) in water. Then the sample was allowed to stand at 4°C for 3 h. After centrifugation at 3000 rpm for 10 min, the supernatant was dialysed, and lyophilized for use.

Desulfated and N-resulfated heparin: According to the method described by Kosakai & Yosizawa (21), 23 mg of desulfated heparin in 0.4 ml water was adjusted to pH 9.5 with sodium hydroxide and heated at 55°C for a few minutes. After adding 19 mg sodium bicarbonate and 23 mg trimethylamine-sulfur trioxide (22) to it, the sample, initially stirred vigorously, was allowed to stand at 55°C for 24 h under constant shaking, then dialysed and lyophilized for use.

N-desulfated heparin: According to the procedure of Kosakai & Yosizawa (23), 100 mg of heparin sodium in 4 ml of 0.1 N hydrochloride was heated at 100°C for 1 h, neutralized with 0.1 N sodium hydroxide, then dialysed and lyophilized for use.

Induction of experimental scleroderma

In experiments with fraction A or chemically modified heparins, 0.1 ml of 100 µg (uronic acid)/ml 0.9% saline was injected intraperitoneally on 10 successive days into a dd-strain mouse, weighing 20 g at 4–6 weeks of age, and 2 days later the animal was decapitated under anesthesia. In experiments with fraction A-1, -2 or -3, the uronic acid volume of the subfraction per 100 µg fraction A was determined after Superose™ 12 column chromatography, and in animal experiments, the above volume was dissolved in 1 ml of 0.9% saline and its 0.1 ml was injected into a mouse for 10 days in a similar manner to fraction A. The mice examined were males. Induction of experimental scleroderma was histologically determined using the back skin near the forelimb (16). In the study with urine-derived GAGs, the assessment was made in a double-blind trial according to the histological criteria for scleroderma previously described (16), i.e. there is collagen swelling and/or marked fibrosis. In experiments with chemically modified heparins, the histological determination was done in an open trial, since the present study was the first.

Chemical characterization of GAG

Chemical composition was determined by measuring uronic acid, hexosamine, *N*-sulfated hexosamine, and total sulfate with rhodizonate, as described in previous reports (16, 17). Digestion with

heparitinase and heparinase (Seikagaku Kogyo, Tokyo) was carried out according to the procedure of Hovingh & Linker (24) and was checked by following the change in absorption at 232 nm.

RESULTS

1. *Experimental scleroderma with urine-derived GAGs*

Of major subfraction of fraction A, each sample of A-2 induced swelling of collagen fibers with an interstitial edema in the skin of some of mice given it (Table I, Fig. 2), which was consistent with the initial and essential skin change in SS. The dermis was thickened. However, connective tissue cells were scarcely increased in number, and subcutaneous fibrosis was not marked. This scleroderma-inducing GAG was biochemically characterized by a relative high *N*-sulfated hexosamine content and low *O*-sulfation (Table II). The GAG was digestible with heparitinase, but not digested with heparinase. On the other hand, A-3 scarcely induced any sclerodermatous change, and the biochemical analysis

Table I. *Scleroderma-inducing ability of scleroderma urine-derived GAG (Fraction A) and its subfractions (A 1, 2, 3) in mouse*

Expt	Fraction A	A-1	A-2	A-3	Saline
1	ND	2[a]/5[b]	2/5	0/5	1/5
2	1/5	0/5	2/5	0/5	0/5
3	2/5	0/5	1/5	0/5	0/5
4	0/5	1/5	2/5	1/5	0/4
5	0/4	1/5	2/5	0/5	0/5
Total	3/19	4/25	9/25[c]	1/25	1/24
Yield of subfractions as UA	100%	7±3[d]	44±10	52±11	

ND = not done; UA = uronic acid; [a] positive sclerotic change; [b] examined. [c]Significant vs saline at $p<0.02$ (χ^2-determination with Yates' correction). Each experiment was done with different fraction A samples. [d] M±SE.

Tabel II. *Chemical characteristics of urine-derived GAG and chemically modified heparins examined*

	Urine GAG		Chemically modified heparin			Standard GAG	
	A-2 (n=3)	A-3 (n=3)	Desulfated, *N*-resulfated HP	*N*-desulfated HP	Desulfated, *N*-acetylated HP	HS	HP
UA	0.95[a] ±0.16	0.79 ±0.11	1.35	1.30	1.10	1.00	1.30
HexN	1.00	1.00	1.00	1.00	1.00	1.00	1.00
N-S	0.61[a] ±0.16	0.14 ±0.02	0.68	0.00	0.00	0.45	0.92
TS	0.67[a] ±0.18	0.98 ±0.06	0.78	0.79	0.00	0.94	2.40

[a]molar ratio to hexosamine taken as 1.00 (M±SE). UA = uronic acid; HexN = hexosamine; N−S = N-sulfated hexosamine; TS = total sulfate; HP = heparin (Sigma); HS = heparan sulfate (Seikagaku Kogyo).

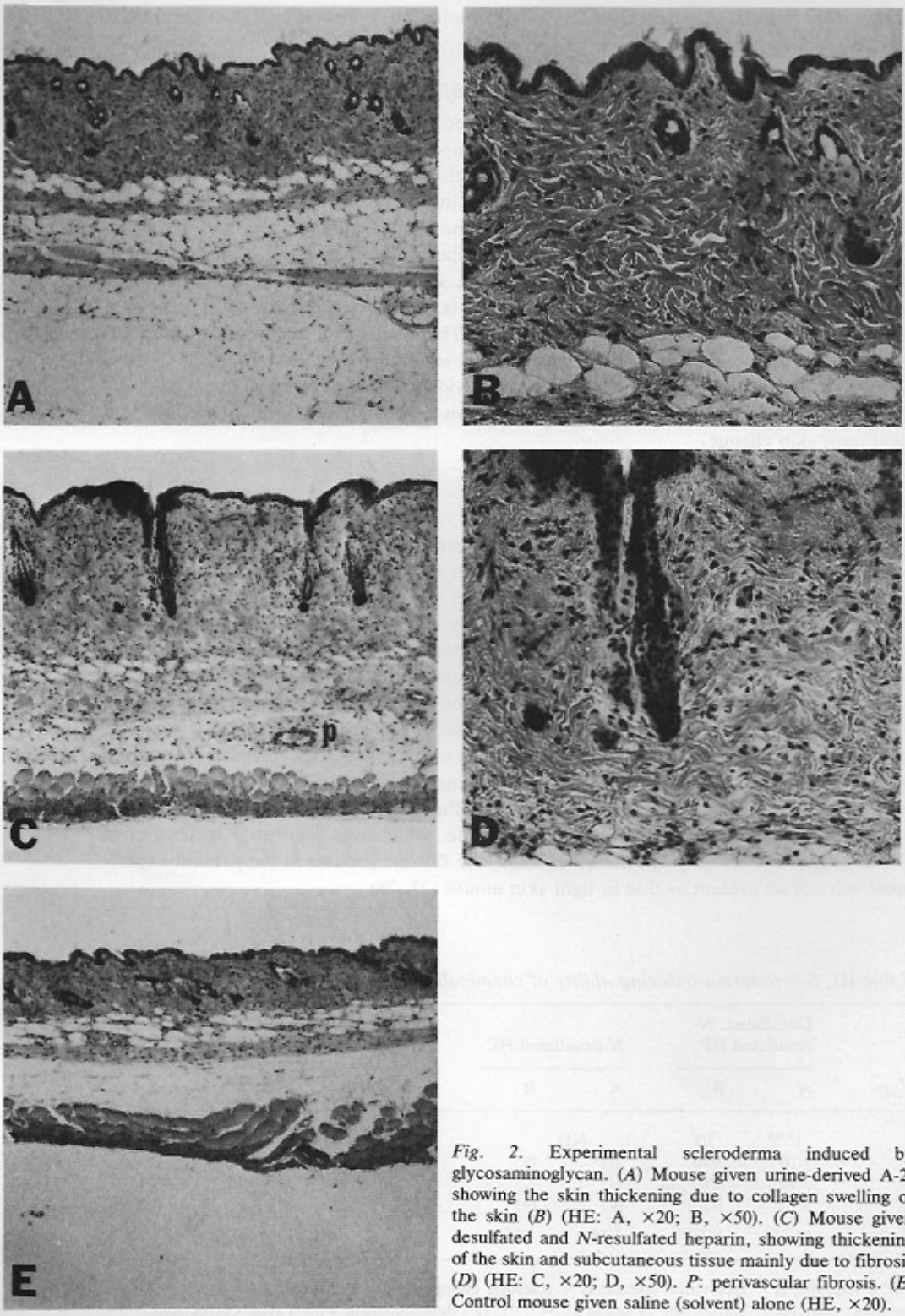

Fig. 2. Experimental scleroderma induced by glycosaminoglycan. (*A*) Mouse given urine-derived A-2, showing the skin thickening due to collagen swelling of the skin (*B*) (HE: A, ×20; B, ×50). (*C*) Mouse given desulfated and *N*-resulfated heparin, showing thickening of the skin and subcutaneous tissue mainly due to fibrosis (*D*) (HE: C, ×20; D, ×50). *P*: perivascular fibrosis. (*E*) Control mouse given saline (solvent) alone (HE, ×20).

showed low *N*-sulfated hexosamine and high *O*-sulfation, which in sulfation contrasted with the composition of fraction A-2.

2. *Experimental scleroderma with chemically modified heparins*

The results are summarized in Table III. Desulfated and *N*-resulfated heparin used in the present study, which had a similar chemical composition to urine-derived fraction A-2 (Table II) and showed slightly lower sulfation than that in the work of Kosakai & Yosizawa (21), induced sclerodermatous changes in the murine skin with a significantly high frequency, as compared with mice given saline alone (Table III, Fig. 2).

Of sclerodermatous changes, an increase in collagen fibres arranged in parallel to the skin surface was particularly noted. In addition, in some mice there was swelling of collagen fibres and evident subcutaneous fibrosis. Connective tissue cells composed mainly of fibroblasts were increased in number. There were few mononuclear cell infiltrates. Desulfated and *N*-acetylated heparin also induced the sclerodermatous change, although collagen swelling and fibrosis were less evident and less frequent. In contrast, *N*-desulfated heparin which had a similar composition to A-3 (Table II), did not induce any significant skin change.

DISCUSSION

The present study clearly demonstrated that a variant of heparan sulfate or chemically modified heparin could induce an early change in scleroderma, as seen under the light microscope, although macroscopically there was no evident manifestation. The skin change induced by urine-derived GAG was mainly collagen swelling with moderate skin thickening, whereas desulfated and *N*-resulfated heparin-treated mice often showed conspicuous dermal and/or subcutaneous fibrosis, suggesting that the chemically modified heparin-induced experimental scleroderma might be analogous to the skin change caused by SS and changing from interstitial edema to sclerotic fibrosis, although there were still individual differences in the results from the mice examined. Replacement of subcutaneous fat by abnormal fibrosis is the striking skin change seen in SS (25). In comparison with other animal models for scleroderma, the skin change in the present experiment was deficient in remarkable mononuclear cell infiltrate, which was seen prior to fibrosis in White Leghorn fowls (26). On the other hand, the fibrotic process in the present experiment was not so evident as that in tight skin mouse (27, 28).

Table III. *Scleroderma-inducing ability of chemically modified heparin*

	Desulfated, *N*-resulfated HP		*N*-desulfated HP		Desulfated, *N*-acetylated HP		Saline	
Expt	A	B	A	B	A	B	A	B
1	1[a]/5[b]	2/5	ND		1/5	2/5	0/5	0/5
2	7/10	3/10	1/5	0/5	2/5	0/5	0/5	0/5
3	0/10	8/10	1/5	1/5	1/5	2/5	0/5	1/5
4	3/10	1/10	0/5	0/5	0/5	2/5	1/5	0/5
Total	11/35	14/35[c]	2/15	1/15	4/20	6/20	1/20	1/20

HP = heparin; A = interstitial edema; B = sclerotic change; ND = not done.
[a]number examined. [c]significant vs saline at $p<0.02$. (χ^2- determination with Yates' correction).

Biochemically, the scleroderma-inducing GAG was characterized by low *O*-sulfation in both urine-derived and synthetic GAG. Furthermore, GAG with high *N*-sulfation in addition to low *O*-sulfation, such as fraction A-2 and desulfated and *N*-resulfated heparin, induced the most marked change in the murine skin. Consequently, low *O*-sulfation may be the key structure in the scleroderma-inducing ability of the GAG, while high *N*-sulfation could enhance the activity. The mechanism occurring in fraction A-2, that is, which of the synthetic or degraded *N*-sulfated GAG processes is disturbed in patients with SS in addition to the possible production of the final stage of scleroderma in animals by chemically modified heparins, remains to be elucidated.

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