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Changes in Skin Disaccharide Components Correlate with the Severity of Sclerotic Skin in Systemic Sclerosis

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The disaccharide contents of chondroitinase-digestible glycosaminoglycans extracted from a 6-mm punch biopsy of the forearm skin were determined using high-performance liquid chromatography after 1-phenyl-3-methyl-5-pyrasolone labelling. In 9 patients with systemic sclerosis, the amounts of both the main disaccharide unit of dermatan sulfate and chondroitin sulfate C increased significantly, as compared with 7 site-matched controls. Furthermore, the increase in dermatan sulfate was significantly correlated with both the clinical severity and the extent of skin sclerosis, while the main disaccharide unit of hyaluronic acid tended to decrease. These results confirm that changes in skin glycosaminoglycans are closely related to fibrotic processes and suggest that the alterations of disaccharide components may play a role in the collagen deposition in systemic sclerosis. ***Key word: fibrosis.***

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Systemic sclerosis (SSc) is a multi-systemic disorder characterized by sclerotic fibrosis of the skin and other organs, though the pathogenesis remains to be elucidated (1, 2). With respect to the changes of the skin glycosaminoglycans (GAGs) in patients with SSc, an increased amount of dermatan sulfate (DS) or chondroitin sulfate (CS) has been established by the use of conventional methods (3–7). We have previously demonstrated that the distribution pattern of the main disaccharides, DS and CS, in SSc differed from that in scars as well as normal controls, using pyridylamination (PA) methods (8). These results suggest that GAGs as a major component in the dermis may be involved in the fibrosis of SSc.

In the present study, we examined the disaccharide composition of a small skin specimen (6-mm punch biopsy) to investigate the relationship between the changes of skin disaccharide composition and the extent and severity of skin sclerosis in patients with SSc, using the precolumn labelling with 1-phenyl-3-methyl-5-pyrasolone (PMP) reported by Honda et al. (9).

MATERIALS AND METHODS

Subjects

The patients studied in the present investigation are listed in Table I. A 6-mm punch biopsy specimen using Dispopunch (Stiefel Laboratory, Main, Germany) was obtained from the extensor surface of the forearm of 9 patients with SSc (1 male and 8 females, aged 61 ± 6 years; mean ± SD, ranged: 48–69). All patients with SSc met the criteria of the American Rheumatism Association (10), and 7 patients showed proximal scleroderma on the forearm when the biopsy was taken. Five patients also showed truncal skin sclerosis, classified as diffuse cutaneous SSc (11). The remaining 4 patients were classified as limited cutaneous SSc. Histological examination of skin specimens next to the punch biopsy was simultaneously performed. The clinical severity of skin sclerosis was classified as unaffected (–), moderately sclerotic (+) or severely sclerotic (++). In 7 patients, biopsies were taken before treatment; however, 2 patients received oral pronase treatment without corticosteroid or penicillamine. Seven punch biopsies of clinically and histologically normal skin from healthy volunteers (all females, aged: 59 ± 10, ranged: 42–69) were studied as controls.

Preparation of GAG samples

Crude GAG was isolated using a modification of the previously described method (8, 12). Briefly, after removal of the subcutaneous fat and epidermis, the dermis was minced into pieces, defatted with acetone, dried and weighed. Samples were treated with 2% NaOH overnight, neutralized with HCl, digested with pronase, deproteinized with 10% trichloroacetic acid and centrifuged. The supernates were dialyzed

Table I. *Clinical characteristics of the patients studied*

Patient No.	Age/Sex	Duration[a] (months)	Clinical classification	Skin sclerosis Forearm[b]/Trunk	Digital pitting scar	Digital contracture	Diffuse pigmentation	Raynaud's phenomenon	Pulmonary fibrosis	Esophageal dysmotility	Autoantibody
1	61/F	24	limited	–/–	+	–	–	+	–	–	ACA[c]
2	60/F	120	limited	–/–	–	+	+	+	+	–	ANA[d], anti-RNP
3	69/F	16	limited	+/–	+	–	–	+	+	+	ANA, anti-mitochondria
4	66/F	24	limited	+/–	–	–	+	+	–	+	ANA
5	61/F	6	diffuse	+/+	–	–	–	–	–	+	ACA
6	63/F	120	diffuse	++/+	+	+	+	+	+	–	ANA, anti-Scl-70
7	63/F	60	diffuse	++/+	+	+	+	+	+	+	ANA, anti-Scl-70
8	48/F	48	diffuse	++/+	–	+	+	+	+	+	ANA
9	56/M	72	diffuse	++/+	+	+	+	+	+	+	ANA, anti-Scl-70

[a] Disease duration after onset. [b] The degree of skin sclerosis was classified as unaffected (–), moderately sclerotic (+) and severely sclerotic (++). [c] Anti-centromere antibody. [d] Anti-nuclear antibody.

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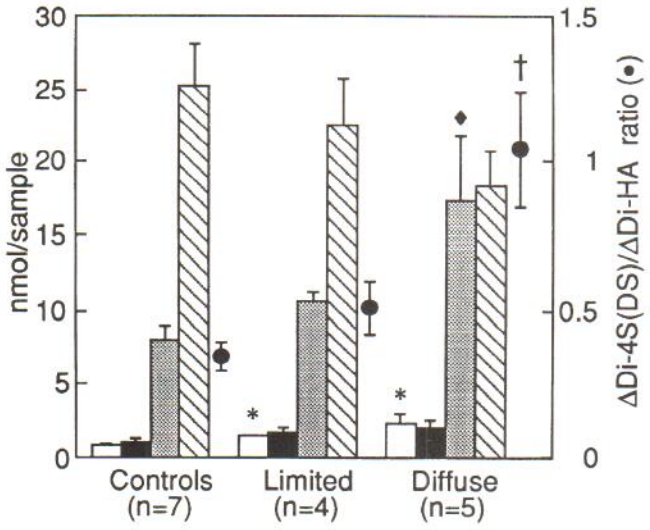

Fig. 1. Main disaccharide contents in the total number of 6-mm punch-biopsied skin specimens and the ratio of ΔDi-4S (DS) to ΔDi-HA in controls, limited and diffuse cutaneous SSc. *Bars* indicate mean ± SE. *: $p < 0.02$ vs controls. ◆: $p < 0.001$ vs controls and $p < 0.01$ vs limited. †: $p < 0.01$ vs controls. □: ΔDi-6S (CS), ■: ΔDi-4S (CS), ▨: ΔDi-4S (DS), ●: ΔDi-HA.

against water and GAGs were precipitated with 0.1% cetylpyridinium chloride in the presence of 0.012 N sodium sulfate. After centrifugation, the precipitates were washed with 95% ethanol saturated with NaCl twice and then with pure ethanol, and dried. The crude GAGs were suspended in water at the concentration of 1 mg/100 μl and used for further analysis.

Disaccharide analysis

Fifty μl of the sample solution was evaporated and digested with chondroitinase-ABC or with chondroitinase-AC II (Seikagaku Kogyo, Tokyo, Japan) as described elsewhere (8). The precolunm labelling with PMP was carried out using a modification of the method described by Honda et al. (9). The samples or commercial CS/DS and hyaluronic acid (HA)-derived disaccharides (Seikagaku Kogyo, Tokyo, Japan) were dissolved in 0.3 M NaOH (20 μl). An equal volume of 0.5 M PMP in methanol was added to this solution and the mixture was kept at 70°C for 30 min. Xylose was added as an internal standard. After PMP labelling, 60 μl of 0.1 M HCl was added to neutralize and the mixture was extracted with 50 μl of chloroform twice. The aqueous layer was evaporated to dryness, then the residue was dissolved in 100 μl of water, and an aliquot was applied to a high-performance liquid chromatography (Model L-6200, Hitachi, Japan) equipped with a CHEMCO 3C18 column (6×100 mm). Elution was performed with a linear-gradient of acetonitrile/water (1:3, v/v) in water and 10% of 200 mM phosphate buffer, pH 7.5 containing 5% acetonitrile at a flow rate of 1 ml/min at 50°C. Peaks were detected at 245 nm.

ΔDi-6S (CS), ΔDi-4S (CS), the main disaccharide unit of CS A. and ΔDi-HA were determined by unsaturated disaccharides liberated with chondroitinase AC digestion. ΔDi-4S (DS) was calculated by deducting the above ΔDi-4S (CS) from total ΔDi-4S liberated with chondroitinase ABC digestion. The values obtained were expressed as per 6-mm punch-biopsied skin or per 1 mg dry skin.

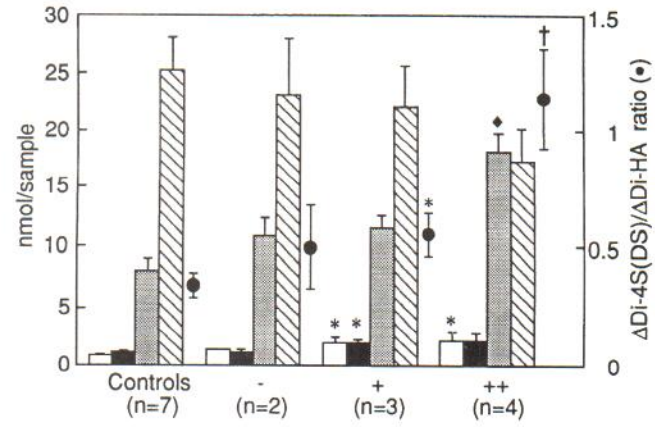

Fig. 2. Main disaccharide contents in the total number of 6-mm punch-biopsied skin specimens and the ratio of ΔDi-4S (DS) to ΔDi-HA with special reference to the degree of skin sclerosis. −: not affected, +: moderately sclerotic and ++: severely sclerotic. *Bars* indicate mean ± SE. *: $p < 0.05$ vs controls. ◆: $p < 0.001$ vs controls and $p < 0.05$ vs + (moderately sclerotic). †: $p < 0.01$ vs controls. □: ΔDi-6S (CS), ■: ΔDi-4S (CS), ▨: ΔDi-4S (DS), ●: ΔDi-HA.

Glucosamine and galactosamine contents of the sample were determined with an amino-acid analyzer after hydrolysis with 3 M HCl at 100°C for 15 h.

Student's *t*-test was used for statistical analysis.

RESULTS

Recovery rates of disaccharides calculated from commercial CS A, DS, CS C and HA were 82.0 ± 2.9% (mean ± SE), 82.3 ± 2.3%, 103.0 ± 9.2% and 118.0 ± 5.2%, respectively, in three analyses on different days. Recovery rates of disaccharide (ΔDi-HA) to the glucosamine content of GAG from controls and SSc skins after chondroitinase digestion were 106.3 ± 9.6% and 103.3 ± 7.0%, respectively, while those of disaccharides (ΔDi-6S (CS), ΔDi-4S (CS) and ΔD-4S (DS)) to galactosamine content of GAG from controls and SSc skins were 76.1 ± 5.0% and 78.8 ± 4.6%, respectively.

The main disaccharide contents in 6-mm punch biopsy skin are presented in Table II. All disaccharides but ΔDi-HA increased in SSc patients and, especially the increases in ΔDi-6S (CS) and ΔDi-4S (DS) were significant, as compared with normal controls. The same results were also obtained by the calculation that each main disaccharide was divided by dry weight (data not shown). As for the total main disaccharide contents, amounts in 6-mm punch biopsy skin were almost equal in SSc patients (38.37 ± 2.34 nmol) and controls (35.33 ± 3.64 nmol). However, the total main disaccharide contents expressed as per mg tended to decrease in SSc patients (3.46 ± 0.68 nmol/mg), when compared with those in controls (5.09 ± 0.48 nmol/mg). This is probably because dry skin weight of 6-mm

Table II. *Main disaccharide contents in 6-mm punch-biopsied skin, nmol (mean ± SE)*

		Di-6S (CS)	ΔDi-4S (CS)	ΔDi-4S (DS)	ΔDi-HA	Total	Dry weight (mg)
SSc	($n=9$)	1.99±0.34*	1.91±0.32	14.31±1.44**	20.16±2.02	38.37±2.34	6.9±0.3
Controls	($n=7$)	0.84±0.16	1.13±0.16	8.05±0.96	25.31±2.80	35.33±3.64	5.0±0.8

* $p < 0.05$ vs controls. ** $p < 0.01$ vs controls.

punch biopsy skin in SSc patients (6.9 ± 0.3 mg) exceeded that of controls (5.0 ± 0.8 mg). These data indicate that 1 mg dry skin of SSc patients contains more substances other than GAGs when compared with controls. Thus, the total disaccharide contents in a definite area seemed to reflect clinical sclerotic findings more correctly. Therefore, subsequent analyses were performed with respect to total contents in 6-mm punch biopsy skin. Fig. 1 shows the main disaccharide contents in limited and diffuse cutaneous SSc, and controls. Significantly increased ΔDi-6S (CS) was observed in both limited and diffuse cutaneous SSc as compared with controls. ΔDi-4S (CS) tended to increase in both types of SSc. ΔDi-4S (DS) tended to increase in limited cutaneous SSc. This increase of ΔDi-4S (DS) reached a statistical significance in diffuse cutaneous SSc as compared with both controls ($p < 0.001$) and limited cutaneous SSc ($p < 0.01$). The ratio of ΔDi-4S (DS) to ΔDi-HA tended to increase in limited cutaneous SSc, and a significant increase was found in diffuse cutaneous SSc as compared with controls.

We also compared the disaccharide contents according to the clinical severity of skin sclerosis (Fig. 2). All disaccharides but ΔDi-HA tended to increase in accordance with the degree of skin sclerosis in SSc. The increase of ΔDi-6S (CS) was significant in both SSc groups as compared with controls. The increase of ΔDi-4S (DS) was also significant in severely sclerotic skin when compared with other controls or moderately sclerotic skin. The increase of the ΔDi-4S (DS)/ΔDi-HA ratio was significant in SSc with sclerotic skin as compared with controls. There was no association between this ratio and disease duration (data not shown).

DISCUSSION

Increased GAG amounts both in vivo and in vitro in SSc have been reported in previous publications (3, 13, 14). The content of each main disaccharide in 1 mg normal dry skin liberated with chondroitinases in this study was similar to a previous report using the PA method (8). The altered composition of main disaccharides in SSc skin became more prominent when total content of main disaccharides in punch-biopsied skin was used instead of 1 mg dry skin. This difference may be due to the fact that the dermis of SSc is heavier than normal skin.

The increase in ΔDi-4S (DS), ΔDi-6S (CS) and the decrease in ΔDi-HA and HA/DS in SSc agree with previous reports (3–7). In the present study, we found that the changes of skin GAGs in SSc correlated with both clinical subtypes (limited and diffuse) and the severity of skin sclerosis. However, we consider that the changes of skin GAGs depend more critically on the severity of sclerotic skin changes than clinical subtypes of SSc. We could not find any relationship between the changes of GAGs and disease duration. So, the above findings may correspond to the observation that skin changes progress more rapidly in the diffuse type of SSc (15).

Although the mechanisms of pathological fibrosis in SSc are still unknown, the deposition of collagen has been considered as a key finding. The collagens in SSc skin are distinct from normal collagens light microscopically in that they are swollen or hyalinized. Immunoelectron microscopically, SSc skin contained a large amount of thin immature collagen fibrils, which were confirmed to be mainly type III collagen fibrils (16). These observations suggest an abnormal maturation of collagen fibrils. Electron microscopic study revealed that collagen fibrils in normal skin were bridged or anchored by proteoglycans (glycosaminoglycan-protein complex) (17). Vogel et al. demonstrated that small dermatan sulfate proteoglycans of bovine tendon specifically inhibit fibrillogenesis of both type I and II collagens (18). These findings suggest that the interaction of collagen fibrils and proteoglycans may be important in the metabolism of collagen fibrils. Our results that the increase of ΔDi-4S (DS) and ΔDi-6S (CS) and possible decrease of HA correlated with the degree of skin sclerosis may support this concept.

It remains to be elucidated whether the change of GAG composition in the SSc skin is the primary or the secondary event for collagen deposition. Akimoto reported that the GAG composition of the SSc skin was distinct from that of hypertrophic scar (8). A recent paper by Vuorio et al. demonstrated that mRNA levels of decorin (core protein of dermatan sulfate proteoglycan) were variable in fibroblasts originated from SSc patients and not correlated with increased mRNA levels of type I collagen (19). This finding may indicate, at least, that mRNA expressions of type I collagen and decorin are not coordinated in SSc fibroblasts.

In conclusion changes of skin disaccharides are closely related to pathological fibrosis in the skin of SSc. It would be of clinical importance to investigate which substances could normalize the abnormal disaccharide metabolism of fibroblasts.

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