Acta Dermatovener (Stockholm) 51: 401–406, 1971

CONNECTIVE TISSUE IN SCLERODERMA

A Biochemical Study on the Correlation of Fractionated Glycosaminoglycans and Collagen in Human Skin

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Abstract. An attempt was made to characterize the connective tissue disorder of the skin in patients with scleroderma by quantifying the fractionated glycosaminoglycans; the fractions were then correlated with collagen. Nine patients with the generalised form of the disease were included in the study, and the values obtained were compared with those from age-matched control subjects. The following glycosaminoglycans were recovered in the skin: hyaluronic acid, heparan sulphate, chondroitin-4,6-sulphate and dermatan sulphate. The percentages of these fractions in normal skin were 53, 6, 6 and 35%, respectively. In scleroderma, the concentration of total glycosaminoglycans was increased, the change being mostly due to a significant increase in chondroitin-4,6-sulphate. Furthermore, as shown by the assays from the same skin specimens, the concentration of hydroxyproline, an amino acid characteristic of collagen, was unchanged in scleroderma. However, the rate of collagen biosynthesis, measured as ^{14}C-hydroxyproline formation in vitro, was increased in 4 patients with scleroderma as compared with controls. In scleroderma, a statistically significant correlation was found between the values for total glycosaminoglycans, those for hyaluronic acid, chondroitin-4,6-sulphate and heparan sulphate on the one hand, and the rate of collagen biosynthesis on the other, whereas this could not be seen in the controls. The present results may be interpreted as evidence that in scleroderma the unspecific repair process is activated and that this is reflected as increased total glycosaminoglycan and chondroitin-4,6-sulphate concentration and enhanced collagen biosynthesis in the skin.

Scleroderma is a complex clinical disorder of unknown etiology. The connective tissue is obviously involved in this disease, but the exact nature of the pathological process or the primary pathogenetic mechanism is so far not understood. Recently, there has been an increasing number of reports on sclerodermatous skin suggesting disturbances in the metabolism of collagen, the main fibrillar component of the connective tissue (21, 22, 23). This biochemical evidence is mainly consistent with an increase in collagen biosynthesis during the active stage of the disease, whereas in later phases, characterized mainly by fibrosis, a decrease in the rate of collagen formation has been observed (for review see ref. 18).

In the present study our aim was to look for any changes in the glycosaminoglycan fraction, another main component of the intercellular matrix. For this purpose we separated and quantified the individual glycosaminoglycans (hyaluronic acid, heparan sulphate, chondroitin-4,6-sulphate and dermatan sulphate) in skin removed from patients suffering from generalized scleroderma. For comparison, changes in the biosynthesis of skin collagen were measured from the same specimens as radioactive hydroxyproline formation in vitro.

MATERIAL AND METHODS

Patients and control subjects. The series consisted of 9 patients with untreated scleroderma and of 9 age-matched control subjects. All the patients were hospitalized at the University of Copenhagen Department of Dermatology, Rigshospital, Denmark. The diagnosis of scleroderma was based on clinical evaluation and the histological picture of the skin biopsy. All patients had the generalized type of the disease, but the tissue specimens were taken from a site that was grossly unaffected. The controls were patients hospitalized due to local venereological or dermatological disorders. Neither their disease nor their medication could possibly be thought to influence the metabolism of the connective tissue.

All the subjects, both patients and controls, were sub-

Table I. *Concentrations of glycosaminoglycans and hydroxyproline in skin of patients with scleroderma and of control subjects*

Patient no.	Total glycosaminoglycans[a]	Hydroxyproline[b]
Scleroderma		
1	1.00	65.2
2	0.87	49.2
3	1.19	56.0
4	2.20	55.8
5	2.33	47.5
6	2.46	75.0
7	0.87	50.9
8	0.79	42.7
9	0.34	50.7
Mean	1.34	54.8
Controls		
10	0.59	35.2
11	0.80	69.8
12	0.44	51.5
13	0.95	39.1
14	0.75	58.6
15	0.86	81.0
16	0.39	63.0
17	0.92	74.9
18	0.46	64.4
Mean	0.68	59.7
p[c]	<0.05	NS

[a] The values are expressed as μg hexosamine in the isolated glycosaminoglycan fraction per mg dry weight of skin.
[b] The values are expressed as μg hydroxyproline per mg dry weight of skin.
[c] Calculated using Wilcoxon's test; NS = statistically not significant.

mitted to the study on the same day and the biopsy specimens were taken by the same person.

Preparation of skin samples. Skin biopsies, weighing about 250–400 mg wet weight, were removed by scalpel from the interscapular region under local anaesthesia. The biopsy specimens were carefully trimmed of subcutaneous tissue and, thereafter, divided into three samples. One of them was weighed and homogenized with a VirTis homogenizer in distilled water. The homogenate was lyophilized and dried to a constant weight. Thereafter, the tissue was defatted with acetone and ether and further used for isolation and separation of the individual glycosaminoglycans. Another skin specimen was treated as above and then used for determination of hydroxyproline in the skin. The third specimen of the skin biopsy was sliced to a maximum thickness of 0.5 mm with a Stadie-Riggs microtome. The slices were weighed and then immediately used for incubation with radioactive proline.

Assay of glycosaminoglycans. The dried, defatted tissue specimens were used for isolation and separation of glycosaminoglycans, following the method described by Thunell (17). The specimens were first digested with papain (Worthington; 14.8 U/mg), 1 mg/ml at 68°C for 8 h. The glycosaminoglycans were then precipitated with cetylpyridinium chloride, dissolved in *n*-propanol, and then reprecipitated with absolute ethyl alcohol as sodium salts. The precipitate was digested overnight with 0.075 mg/ml hyaluronidase (AB Leo, Helsingborg, Sweden; 15 000–20 000 IU/mg) and then divided into depolymerized and polymerized fractions with cellulose acetate columns saturated with cetylpyridinium chloride. The individual fractions were measured after separation and recovery of glucosamine and galactosamine on a Dowex-50 microcolumn (1). The hexosamine content was determined using the method of Elson & Morgan (5).

Determination of collagen biosynthesis in vitro. The sliced tissue specimens were incubated under conditions previously shown to be optimal for the incorporation of radioactive proline into skin collagen hydroxyproline (19). The incubation mixture contained 20 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid buffer (pH 7.4), 20 mM glucose, 50 μg/ml ampicillin (Pentrexyl®—H. Lundbeck & Co., Copenhagen, Denmark) and 1 μCi ^{14}C-proline (New England Nuclear Corporation; 180 mCi/

Table II. *Concentrations of hyaluronic acid, heparan sulphate, chondroitin-4,6-sulphate and dermatan sulphate in skin of patients with scleroderma and of control subjects*

For details on the fractionation of individual glycosaminoglycans, see Material and Methods

Patient no.	Hyaluronic acid[a]	Heparan sulphate[a]	Chondroitin-4,6-sulphate[a]	Dermatan sulphate[a]
Scleroderma				
1	0.40	0.12	0.17	0.31
2	0.49	0.04	0.08	0.26
3	0.48	0.08	0.22	0.41
4	0.82	0.31	0.99	0.08
5	0.82	0.40	0.95	0.16
6	0.66	0.50	1.02	0.28
7	0.20	0.04	0.17	0.46
8	0.30	0.06	0.13	0.30
9	0.14	0.02	0.01	0.17
Mean	0.48	0.17	0.42	0.27
Controls				
10	0.27	0.06	0.05	0.21
11	0.43	0.07	0.14	0.13
12	0.25	0.02	0.04	0.32
13	0.54	0.04	0.05	0.32
14	0.48	0.04	0.05	0.18
15	0.39	0.06	0.11	0.30
16	0.11	0.06	0.05	0.17
17	0.53	0.04	0.05	0.30
18	0.20	0.02	0.01	0.23
Mean	0.36	0.05	0.06	0.24
p[b]	NS	NS	<0.05	NS

[a] The values are expressed as μg hexosamine in the corresponding glycosaminoglycan fraction per mg dry defatted weight of skin.
[b] Tested using Wilcoxon's test; NS = statistically not significant.

mmole), all in 3.0 ml of phosphate-free Krebs-Ringer solution. After 10 hours' incubation at 37°C, the specimens were homogenized with a VirTis homogenizer at 0°C for 15 min, the homogenates dialysed against running tap water for 24 hours, and finally hydrolysed in 6 M HCl at 136°C for 6 h. Thereafter, the amount (11) and radioactivity (10) of hydroxyproline were assayed in the hydrolysate.

Assay of hydroxyproline. The dried skin specimens were used for determination of total hydroxyproline after the tissue samples had been hydrolysed in 6 M HCl at 136°C for 6 h. After hydrolysis, the samples were evaporated to dryness and then eluted with distilled water. An aliquot of the eluate was used for assay of hydroxyproline, following the method described by Kivirikko et al. (11).

RESULTS

The concentration of the total glycosaminoglycan fraction in the skin was significantly increased in scleroderma as compared with the control subjects (Table I). When the concentrations of the individual glycosaminoglycans in the skin were determined, the chondroitin-4,6-sulphate fraction was significantly increased in scleroderma, whereas the values for hyaluronic acid, heparan sulphate or dermatan sulphate did not differ from those of the controls (Table II).

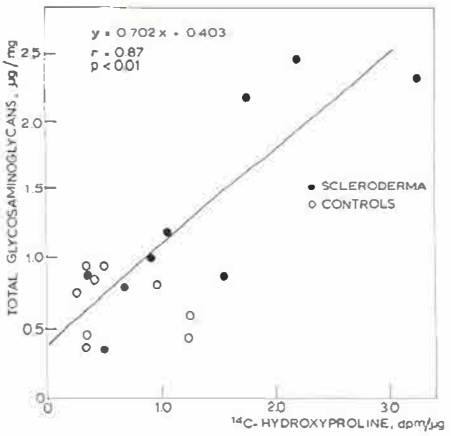

Fig. 1. Correlation between total glycosaminoglycans and the rate of collagen biosynthesis in skin of patients with scleroderma (●) and of controls (○). The regression equation, the correlation coefficient (r) and the statistical significance of the correlation (p) of the values in scleroderma are indicated in the figure. The correlation coefficient in the skin of control subjects was -0.26 (p = statistically not significant).

Table III. *Collagen biosynthesis in skin of patients with scleroderma and of control subjects*

The skin biopsies were sliced into maximum thickness of 0.5 mm and the slices were incubated in a medium containing 1 μCi ^{14}C-proline, as described in Material and Methods

Patient no.	^{14}C-hydroxyproline: dpm/mg wet weight of skin	^{14}C-hydroxyproline: dpm/μg hydroxyproline
Scleroderma		
1	22.2	0.90
2	36.4	1.55
3	22.8	1.05
4	55.0	1.76
5	72.8	3.24
6	68.5	2.21
7	9.8	0.36
8	15.3	0.68
9	8.1	0.24
Mean	34.5	1.33
Controls		
10	27.9	1.25
11	25.6	0.97
12	26.0	1.23
13	8.3	0.50
14	4.2	0.25
15	10.9	0.38
16	7.8	0.31
17	11.2	0.34
18	10.1	0.36
Mean	14.7	0.62
p[a]	NS	NS

[a] Tested using Wilcoxon's test; NS = statistically not significant.

The concentration of hydroxyproline in the skin taken from patients with scleroderma did not differ from that in the skin from control subjects (Table I). When the rate of ^{14}C-hydroxyproline formation in skin was measured by incubating tissue specimens in vitro in a medium containing radioactive proline, 4 out of the 9 patients with scleroderma (nos. 2, 4, 5 and 6) showed values exceeding the limit of mean + 2 S.D., 1.44 dpm/μg hydroxyproline, of the control values (Table III). However, when evaluated as one group, scleroderma did not significantly differ from the control group.

When the statistical correlation between the concentrations of total glycosaminoglycans and hydroxyproline or that between the individual glycosaminoglycans (i.e. hyaluronic acid, heparan sulphate, chondroitin-4,6-sulphate and dermatan sulphate) on the one hand, and hydroxyproline on the other was calculated, no significant correlations between these values could be observed.

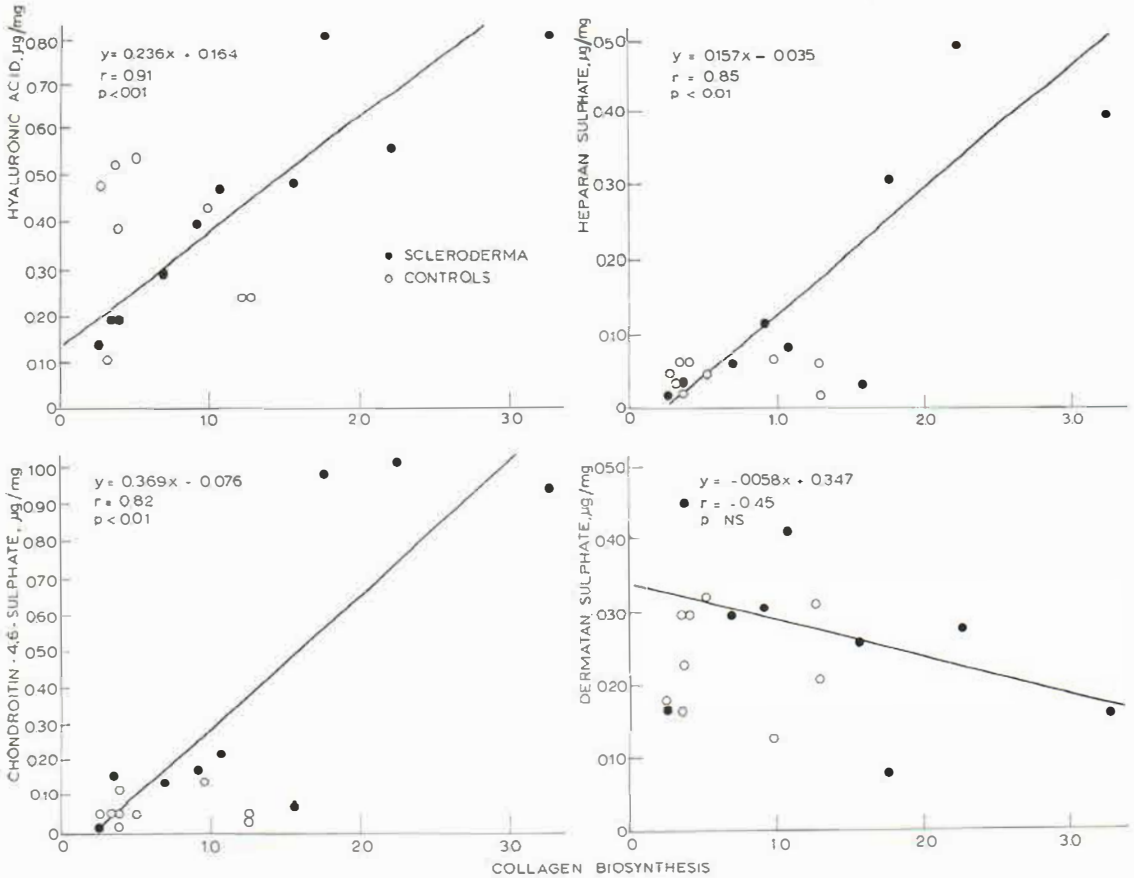

Fig. 2. Correlation between hyaluronic acid, chondroitin-4,6-sulphate, heparan sulphate or dermatan sulphate on the one hand and the rate of collagen biosynthesis, expressed as ^{14}C-hydroxyproline formation (dpm/µg hydroxyproline) on the other, in skin of patients with scleroderma (●) and of controls (○). Regression equations, correlation coefficients (r) and statistical significance of the correlations in scleroderma are indicated in the figures. The regression of hyaluronic acid ($r = -0.20$), chondroitin-4,6-sulphate ($r = 0.17$), heparan sulphate ($r = 0.09$) or dermatan sulphate ($r = -0.01$) in relation to the rate of collagen biosynthesis in the skin of control subjects was statistically not significant.

However, when the corresponding values for glycosaminoglycans were compared with the values for ^{14}C-hydroxyproline formation in vitro, a significant correlation between total glycosaminoglycan concentration and the rate of collagen biosynthesis could be observed in the skin of patients with scleroderma (Fig. 1). By contrast, there was no similar correlation between corresponding values in control tissue specimens ($r = -0.26$). Furthermore, in patients with scleroderma there was a significant correlation between the values for hyaluronic acid, chondroitin-4,6-sulphate or heparan sulphate on one hand and the values for ^{14}C-hydroxyproline formation on the other (Fig. 2). However, in control tissue specimens no significant correlation between the corresponding values could be observed (Fig. 2). The correlation between dermatan sulphate and the rate of collagen biosynthesis was significant neither in sclerodermatous skin ($r = -0.47$) nor in the control specimens ($r = -0.01$).

DISCUSSION

Despite the rapid advance made in the field of connective tissue chemistry, few studies have as yet been published on collagen metabolism in human tissues. Consequently, knowledge of

changes in collagen metabolism in various clinical disorders is relatively scanty. Recently, however, there have been reports indicating definite changes in the biosynthesis of collagen in various connective tissue disorders; among those, scleroderma (18). The data presented are consistent with increased biosynthesis of collagen in the active stages of the disease process in scleroderma. This conclusion is based on the finding that in sclerodermatous skin the conversion of radioactive proline to radioactive hydroxyproline in vitro is increased. Accordingly, the activity of protocollagen proline hydroxylase, the enzyme catalysing the hydroxylation of peptide-bound proline to collagen hydroxyproline, is increased in skin biopsies taken from patients with scleroderma (20, 21, 22). Furthermore, the solubility of skin collagen in neutral salt solutions is increased in such patients (12, 13, 23).

In agreement with several authors (2, 6, 7, 13, 14), in the present study we found no change in the concentration of skin collagen in scleroderma as compared with control subjects. Furthermore, enhanced collagen biosynthesis in vitro observed in some of the patients with scleroderma is in agreement with previous reports indicating that collagen formation is increased in about one-third of patients suffering from scleroderma (14, 21).

At present even less information is available about glycosaminoglycans in human skin than about collagen. A recent study reported the presence of hyaluronic acid, dermatan sulphate and chondroitin-4,6-sulphate in adult skin, whereas in young fetuses only hyaluronic acid and chondroitin-4,6-sulphate could be detected (3). In the same study the concentration of the acid glycosaminoglycans in human skin was reported to decrease during development, being twenty times as high in the 3-month-old fetus and twice as high in the 9-month-old fetus as in the adult (3). The latter findings are in agreement with the report by Clausen (4) indicating a decrease of acid mucopolysaccharides in the skin with age.

The object of the present study was the quantitative determination of glycosaminoglycans and collagen, two major components of the connective tissue, in human skin taken from the same individual. The poor correlation of glycosaminoglycans and hydroxyproline concentration both in sclerodermatous and control skin may be explained by the comparatively low turn-over rate of insoluble collagen fibers. On the other hand, in scleroderma glycosaminoglycans that have a relatively short biological half-life (9) showed a significant correlation with the ^{14}C-hydroxyproline formation, an index of the rate of collagen biosynthesis in the skin. The lack of significant correlation between dermatan sulphate and the rate of collagen biosynthesis is somewhat astonishing since dermatan sulphate has been suggested to play a role in the maturation of collagen fibers (16). In addition, the concentration of dermatan sulphate has been shown to increase in unspecific repair processes in aorta (8) and granulation tissue (15). Therefore, based upon these findings, a possible defect in the formation of the dermatan sulphate-collagen complex may be suggested for scleroderma.

The findings observed in scleroderma are not specific for this disorder. Increased collagen biosynthesis has been reported in the skin of patients with other connective tissue disorders and in various other dermatological diseases (18). Thus the present results may be interpreted as a sign of an unspecific general repair process initiated by an unknown agent and manifested in the skin as a disease called scleroderma.

ACKNOWLEDGEMENT

This study has been supported by grants from the National Research Council for Medical Sciences, Finland, and the Nordiska Samarbetsdelegationen för Medicinsk Forskning.

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Received May 24, 1971

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