Acta Dermatovener (Stockholm) 60: 39–43, 1980

VARIATION OF URINARY ACID GLYCOSAMINOGLYCAN AND COLLAGEN METABOLITE EXCRETION WITH DISEASE ACTIVITY IN GENERALIZED SCLERODERMA

Nelly Blumenkrantz and Gustav Asboe-Hansen

Department of Dermatology (with Connective Tissue Research Laboratories), University of Copenhagen, Rigshospital, Copenhagen, Denmark

Abstract. Follow-up of patients with generalized scleroderma improving during treatment with collagen biosynthesis inhibitors showed a decrease towards normalization of the elevated urinary values for acid glycosaminoglycans and collagen catabolites. The determinations supply objective evidence of the disease activity as well as of the efficacy of treatment. In the control group, relatively high urinary values for the two macromolecules were found in the second decade of life in comparison with older age groups.

Key words: Activity of scleroderma; Urinary acid glycosaminoglycans, collagen

In generalized scleroderma, periods of activity and progression may alternate with static periods. There is clinical and theoretical evidence that drugs used for treatment of scleroderma are preferentially or exclusively given a chance to act favourably during the phases of progression. An increased urinary excretion of high molecular weight peptides derived from newly formed collagen takes place in active scleroderma (2). In order to be able to select the most suitable period(s) for treatment and to ascertain whether a drug is effective or not or to what extent, we determined the characteristic breakdown products, not only of collagen, but also of acid glycosaminoglycans (AGG) present in dermal connective tissue ground substance. The imino acid hydroxyproline (Hyp) characterizes and indicates collagen quite specifically, while uronic acid (UA) indicates AGG, which, with the exception of keratan sulphate, contain uronic acid in their repeating disaccharide unit.

MATERIAL AND METHODS

Controls

Twenty non-hospitalized youngsters and adults (Table II).

Patients with active generalized scleroderma of the acrosclerosis type

Ninety-five hospitalized patients were grouped according to clinical disease activity. The urinary excretion of hydroxyproline was determined.

Twenty-four hospitalized youngsters and adults undergoing treatment with the drugs mentioned below were followed-up with repeated determinations of AGG and collagen metabolites in urine. These patients were divided into sub-groups according to their response to treatment. The grades 0–4 signified the following disease states.

0: Unrestrained progression. No effect of treatment.

1: Progression arrested. No regression of cutaneous sclerosis

2: Moderate, but distinct regression

3: Sclerosis no longer traceable with the exception of persistent finger sclerosis

4: Complete regression of the sclerosis

The grading was based on clinical criteria (1). The patients were beingt treated with inhibitors of collagen synthesis, i.e. benzylpenicillin-diethylaminoethylester hydroiodide 500 000 units daily for 10–14 days, D-penicillamine (dimethylcysteine) 450–900 mg, glutamine 300–600 mg, and/or hydralazine 75 mg daily (1, 3, 4, 5). The drugs were given separately, consecutively or concurrently.

Urine collection

A 24-hour urine sample was collected from each control subject. From the hospitalized patients, one sample was collected on the day before and another on the day after they had been submitted to a collagen-free diet.

Preparation of urine samples

Uronic acid. The urinary AGG were precipitated with cetyltrimethylammonium bromide (CTAB) according to Blumenkrantz & Asboe-Hansen (6). When the specific gravity of the urine was 1.020 or higher, 3 ml of urine plus 3 ml of water was precipitated with 0.2 ml of 5% CTAB. In the case of lower specific gravity, 6 ml of urine was used. Before the addition of the precipitating agent the corresponding volumes of filtered urine were brought to pH 5.0 by addition of 2 N HCl to an aliquot of the urine containing one drop of methyl red as internal indicator. This aliquot, which was only used to determine the volume of HCl required to adjust the pH, was discarded. After the addition of the CTAB solution, the samples were

Table 1. *Urinary excretion of collagen and acid glycosaminoglycan metabolites by patients suffering from generalized scleroderma as related to age and efficacy of treatment with inhibitors of collagen biosynthesis*

Number of determinations within parentheses
UA = Uronic acid; Hyp = Hydroxyproline in 1+5 fraction as % of total Hyp. All absolute values were expressed as mg/24 h

Pat.	Sex	De-cade of life	Treatment efficacy grade[a]	Meta-bolite	1971	1972	1973	1974
D. D.	♀	2	4	UA				
				Hyp				
M. W.	♂	3		UA			8.38± 0.9 (2)	4.20± 0.7 (2)
				Hyp	28.0±2.0 (2)	30.2 ± 6.0 (4)	35.0 ± 1.0 (2)	
F. M.	♀	3		UA				5.56± 2.0 (9)
				Hyp				32.1 ± 2.5 (8)
S. I.	♀	3		UA				5.41± 1.3 (5)
				Hyp	35.0±2.0 (2)	34.6 ± 2.3 (3)	32.4 ± 1.6 (9)	38.0 ± 1.0 (2)
A. J.	♀	4		UA				
				Hyp				
J. P.	♂	5		UA				
				Hyp				
G. C.	♀	6		UA			5.32± 0.4 (2)	2.89± 0.2 (3)
				Hyp			23.4 ± 9.1 (2)	22.6 ± 1.2 (8)
J. H.	♀	6		UA				3.93± 1.2 (4)
				Hyp				18.0 ± 2.1 (4)
E. P.	♂	6		UA				3.95± 1.0 (7)
				Hyp				15.4 ± 3.8 (4)
P. A.	♂	7		UA		3.26 (1)	2.65± 1.2 (2)	1.31± 0.3 (2)
				Hyp			16.0 ± 2.0 (2)	17.0 ± 2.4 (2)
R. B.	♀	7		UA			3.73± 0.8 (2)	2.74± 0.8 (5)
				Hyp			38.0 ± 7.0 (2)	26.2 ±10.0 (5)
S. M.	♀	2	3	UA		8.75 (1)	12.89 (1)	4.02 (1)
				Hyp		42.8 ±12.0 (6)	35.1 ± 7.6 (4)	15.0 (1)
R. M.	♀	4		UA		4.55± 0.8 (2)	4.52± 1.5 (4)	4.45± 0.7 (4)
				Hyp		16.4 ± 3.5 (2)	28.7 ± 5.9 (5)	18.0 ± 2.8 (2)
L. I.	♀	5		UA				2.21± 0.5 (4)
				Hyp	29.0±7.0 (2)	24.6 ± 5.2 (6)	19.4 ± 4.0 (4)	16.7 ± 2.0 (4)
H. M.	♀	7		UA				
				Hyp				
C. A.	♂	4	2	UA			6.37 (1)	5.07± 0.8 (4)
				Hyp				
W. E.	♀	5		UA				
				Hyp	26.3±8.0 (3)	36.6 ± 9.1 (6)	35.0 ±12.6 (5)	31.7 ± 2.2 (4)
N. R.	♂	6		UA				
				Hyp				
P. A.	♂	6		UA				
				Hyp				
R. O.	♂	7		UA		8.91± 0.6 (2)		4.06 (1)
				Hyp		51.7 ± 4.7 (4)	43.4 ± 4.9 (2)	24.4 ± 4.8 (2)
H. P.	♂	6	1	UA			12.64 (1)	
				Hyp			21.4 ± 0.7 (2)	17.6 ± 2.8 (3)
P. S.	♀	8		UA				
				Hyp				
H. C.	♀	5	0	UA				
				Hyp				
K. G.	♀	6		UA				5.53± 0.3 (4)
				Hyp				21.4 ± 3.5 (2)

[a] Fisher's omnibus test for the three well-responding groups:

Group 4 UA $\chi^2=81.17$ $f=20$ $p<0.0001$
Hyp $\chi^2=41.5$ $f=20$ $p<0.005$

Group 3: UA $\chi^2=12.88$ $f=6$ $p<0.05$
Hyp $\chi^2=13.26$ $f=8$ $0.10<p<0.20$

Group 2: UA $\chi^2=14.28$ $f=8$ $0.05<p<0.10$
Hyp $\chi^2=29.6$ $f=10$ $p<0.005$

1975	1976	1977	1978	t-test $p\{t>t_{obs}\}$
4.25± 1.1 (4)	3.71±1.9 (5)	3.91± 0.8 (4)		0.320
34.8 ±10.0 (6)	28.7 ±6.6 (6)	33.0 ± 6.0 (4)	28.4 ± 3.5 (2)	0.214
4.30± 0.2 (2)	3.40±1.0 (2)	3.86± 0.53 (2)	4.67± 0.4 (2)	0.017
25.0 ± 5.0 (2)	15.4 ±2.0 (2)	31.4 ± 2.0 (2)	28.4 ± 5.0 (3)	0.538
3.16± 0.38 (6)	3.21±0.6 (8)	2.15± 0.02 (2)		0.0005
32.6 ± 6.0 (6)	20.4 ±2.6 (4)	22.4 ± 0.7 (2)		0.0004
5.68± 1.6 (4)	4.6 ± 0.8 (5)	2.02± 0.5 (2)	3.99± 0.3 (2)	0.103
29.6 ± 1.1 (3)	22.0 ±2.8 (2)	24.4 ± 5.5 (2)		0.062
	4.87±0.1 (4)	2.64± 0.8 (3)	3.65± 0.14 (2)	0.0001
	26.4 ±2.6 (4)	31.2 ± 9.2 (4)	22.7 ± 5.3 (4)	0.128
3.75± 1.3 (8)	4.36±0.7 (3)	3.50± 1.2 (3)	2.86± 0.13 (2)	0.191
	20.4 ±1.0 (4)	24.6 ± 5.1 (3)	30.4 ± 2.1 (2)	0.999
2.6 ± 0.4 (3)	1.74±0.2 (2)	3.04± 0.3 (3)	2.18± 0.63 (2)	0.0135
19.7 ± 1.8 (4)	16.0 ±1.4 (2)	19.0 ± 0.8 (4)	24.5 ± 2.2 (2)	0.558
3.65± 1.4 (6)	2.22 (1)	1.58± 0.4 (4)	2.03± 0.2 (3)	0.023
17.4 ± 3.1 (4)	17.7 ±2.8 (2)	18.4 ± 3.1 (4)	17.2 ± 3.7 (4)	0.360
3.27± 0.3 (2)	3.05±0.7 (2)	2.06± 0.18 (2)		0.019
17.3 ± 0.02 (2)	18.3 ±4.0 (3)	15.4 ± 3.5 (2)		0.500
2.40± 0.1 (2)	2.19±0.8 (2)	2.09± 0.2 (4)		
21.2 ± 0.25 (2)	18.5 ±2.1 (2)	20.7 ± 7.6 (4)	15.0 (1)	
4.07± 0.5 (4)	2.44±0.6 (4)	2.01± 0.7 (2)		0.075
24.1 ± 2.8 (2)	23.0 ±7.6 (6)	16.4 ± 2.1 (2)		0.026
4.72± 0.3 (3)				
24.0 ± 2.8 (2)				0.041
2.91± 1.3 (4)	4.02±2.5 (6)	7.43± 2.0 (6)	2.72± 0.3 (2)	0.047
20.0 ± 1.0 (3)	20.0 ±4.8 (6)	30.1 ± 8.0 (6)	19.3 ± 1.1 (3)	0.877
2.97± 0.6 (2)	1.94±0.6 (2)			0.293
21.7 ± 3.7 (4)	16.4 ±0.7 (2)		17.0 ± 1.7 (2)	0.071
	3.00±0.7 (6)	1.58± 0.5 (4)	2.22± 0.8 (2)	0.116
	25.8 ±6.0 (6)	34.0 ± 7.9 (4)	26.0 ± 1.4 (2)	0.517
5.46± 0.4 (4)	4.83±0.9 (2)	4.39± 0.5 (4)		
	36.0 ±7.1 (2)	25.4 ± 8.0 (6)	24.3 ± 9.1 (5)	0.085
	4.31±1.3 (2)	6.32± 2.1 (6)	3.56± 0.9 (4)	0.221
33.8 ±4.1 (6)	33.6 ±4.9 (3)	29.2 ± 7.4 (4)	32.4 ±12.0 (2)	0.733
	5.68±1.2 (3)	5.85± 0.5 (2)		0.567
	29.7 ±6.1 (4)	23.6 ± 5.8 (3)	16.2 ± 0.05 (2)	0.011
	6.55±1.7 (5)	5.53± 2.1 (2)		0.263
		40.4 ± 3.8 (6)	44.0 ± 4.5 (4)	0.896
4.30± 0.05 (2)	2.63 (1)	3.88± 0.8 (5)	3.29± 0.02 (2)	0.024
24.0 ± 9.0 (2)	25.8 ±9.0 (2)	33.8 ±10.0 (6)	33.4 ± 6.5 (6)	0.0006
4.64± 0.15 (2)	5.13±0.7 (2)	5.26± 1.5 (6)	5.12± 0.8 (2)	
28.6 ± 7.5 (4)	16.3 ±1.3 (6)	20.0 ± 2.8 (2)		0.282
	3.32±1.1 (6)	2.70± 0.3 (4)	3.20± 0.28 (2)	0.445
	29.6 ±7.2 (6)	26.2 ± 6.1 (4)	27.0 ± 4.2 (2)	0.328
	2.57±0.1 (3)	2.28± 0.18 (2)		0.048
	57.3 ±6.1 (3)	51.0 ± 1.4 (2)		0.132
2.95± 0.8 (2)	5.47±0.5 (2)	2.87± 0.38 (2)		0.0003
29.0 ± 1.4 (2)	32.4 ±3.5 (2)	26.0 ± 2.8 (2)	22.7 ± 3.5 (4)	0.655

Table II. *Urinary excretion of acid glycosaminoglycan and collagen metabolites by normal subjects in relation to age*

Decade of life	No. of subs.	Uronic acid (mg/24 h)	1+5 fraction Hyp (% of total Hyp)
2. (non-pubertal)	5	8.70±2.7	43.67±4.0
2. (pubertal)	7	8.10±2.6	47.87±6.7
3.	3	3.06±1.1	24.75±1.5
4.	2	2.08±0.30	23.00±1.2
5.	2	2.49±0.05	21.12±0.9
6.	1	1.55	20.0

cooled in a water and ice bath for 30 min. The samples were then centrifuged at 2000 rpm for 15 min. The supernatant was discarded and the pellet washed with 98% ethanol, saturated with NaCl and re-centrifuged at 2000 rpm. The alcoholic supernatant was discarded, and the final pellet obtained was dissolved in 1 ml of distilled water. Some 100–200 ml of this solution was used for analysis of the uronic acid content according to the m-hydroxydiphenyl procedure of Blumenkrantz & Asboe-Hansen (6, 8). Results were expressed as mg uronic acid (UA) excreted per 24 hours (Table II).

Total hydroxyproline. One ml of urine was hydrolysed with 1 ml of 12 N HCl at 118°C for 16 hours. The HCl was then evaporated under vacuum at 65°C. The dry residue was dissolved in phosphate-citrate buffer and used for the assay of total hydroxyproline (Hyp) according to the method of Blumenkrantz & Asboe-Hansen (7, 8). The Hyp excretion was expressed as mg/24 h.

1+5 fraction hydroxyproline. One ml of urine was precipitated with 5 ml of acetone (2). The sample was centrifuged and the supernatant discarded. The pellet was hydrolysed with 1 ml of 6 N HCl at 118°C for 16 hours. Evaporation of HCl and analysis of Hyp were performed as indicated above. The Hyp contained in this "1+5 fraction" was expressed as mg per 24 hours and calculated as a percentage of the total Hyp. In Table III, the percentage 1+5 fraction Hyp values of urine from patients with scleroderma of varying degrees of activity are listed.

The figures in Table I represent the yearly averages of the urine excretion of uronic acid-containing AGG, and of the percentage 1+5 fraction Hyp by scleroderma patients on treatment with inhibitors of collagen synthesis.

Statistical evaluation. The statistical significance of the difference between the three groups in Table III was evaluated by the Kruskall-Wallis test.

The change in each of the 24 patients' values listed in Table I was evaluated by the *t*-test.

$t = \frac{\bar{x}_1 - \bar{x}_2}{s(\bar{x}_1 - \bar{x}_2)}$ was calculated

$\bar{x}_1$ being the average of first values, $\bar{x}_2$ the average of last values, while s represents the standard deviation of the difference between the two averages. Under the null hypothesis the probability of getting a t-value higher than that observed was evaluated:

$p = p\{t > t_{obs}\}$.

The combined probability of the fractiles observed in each group was evaluated by Fisher's omnibus test.

RESULTS

Table II shows the age variation in the urinary excretion of AGG and collagen metabolites by a group of normal controls. High values were observed in the second decade of life with no difference between pubertal and non-pubertal subjects. A decrease was noticed from the 3rd decade on.

Patients suffering from generalized scleroderma in a static, non-progressive phase excreted amounts of 1+5 fraction Hyp, % of total Hyp, which were

Table III. *1+5 fraction Hyp, % of total Hyp, in 95 patients with generalized scleroderma of different degrees of clinical activity*

1. Static	23.9		28.6	29.4	20.5	20.0	20.1
2. Chronic progressive	32.3	30.6	43.9	35.8	37.5	44.3	33.8
	29.6	33.0	32.4	47.6	28.6	37.4	28.9
	33.8	49.2	40.2	33.7	27.4	33.5	39.5
	34.0	30.1	33.3	28.9	30.2	49.0	21.1
	48.7	33.0	31.7	28.9	30.7	30.1	25.8
	33.8	25.0	38.3	38.7	36.1	28.9	34.0
	29.2	29.9	32.1	29.2	34.5	35.1	42.5
	34.5	28.6	50.3	33.1	33.0	33.1	49.8
	34.5	31.5	29.4	42.3	30.2	26.5	36.1
	36.0	47.3	31.5	20.3	42.6	29.1	29.6
	37.3	33.0	34.7	32.8	22.4	42.0	32.1
	29.8						
3. Fulminant	64.4	39.8	43.0	40.9	47.4	56.1	51.4
	49.1	43.9	40.9	52.9			

Kruskall-Wallis test: $\chi^2 = 31.6$. $f = 2$. $p < 0.001$.

normal or close to normal, while the patients with chronic progressive scleroderma had pathologically elevated (the fulminant cases even very high) values. The differences between the three groups were statistically highly significant. (Table III.)

An increased urinary excretion of uronic acid in parallel with an increase of the percentage 1+5 fraction Hyp was found in most cases of active scleroderma undergoing treatment with collagen synthesis inhibitors (first value; Table I). A decrease towards normalization of the urinary excretion of AGG and, with a few exceptions, the percentage 1+5 fraction Hyp, was found in the groups reacting favourably to the treatment with inhibitors of collagen synthesis, i.e. by total, subtotal and moderate regression of dermal sclerosis. The variation of the same parameters in the patients responding less favourably, viz. groups 0 and 1, was insignificant. A decrease was even evident in most urines in which the initial value was within normal limits.

DISCUSSION

The parallelism of the high urinary values for metabolites of the main macromolecular components of connective tissue in active scleroderma and the decrease during successful treatment confirm that both macromolecules are involved in the pathology of scleroderma. The percentage 1+5 fraction Hyp is a reliable indicator of disease activity in scleroderma. The urinary uronic acid values seem to be an even more sensitive indicator of the efficacy of treatment. The laboratory results adduce useful objective evidence to the clinical evaluation of the disease activity as well as of the efficacy of the treatment.

ACKNOWLEDGEMENT

The statistical evaluations were made by Mr Jørgen Nyboe, whose expert assistance is gratefully acknowledged.

REFERENCES

1. Asboe-Hansen, G.: Treatment of generalized scleroderma with inhibitors of connective-tissue formation. Acta Dermatovener (Stockholm *55:* 461–465, 1975.
2. Blumenkrantz, N. & Asboe-Hansen, G.: High molecular collagen peptide fraction in urine indicates disease activity in generalized scleroderma. Acta Dermatovener (Stockholm) *56:* 415–421, 1976.
3. Blumenkrantz, N. & Asboe-Hansen, G: Effect of chelating agents on the biosynthesis of collagen. Acta Dermatovener (Stockholm) *54:* 94–98, 1973.
4. Blumenkrantz, N. & Asboe-Hansen, G.: Effect of amino acids on collagen biosynthesis. In Vitro *8:* 342–346, 1973.
5. Blumenkrantz, N., Hein Christiansen, Aa. & Asboe-Hansen, G.: Hydralazine and dihydralazine effect on connective tissue and binding to serum protein. Scand J Rheumatol (in press).
6. Blumenkrantz, N. & Asboe-Hansen, G.: New method for quantitative determination of uronic acids. Anal Biochem *54:* 484–489, 1973.
7. Blumenkrantz, N. & Asboe-Hansen, G.: An automated procedure for quantitative determination of hydroxyproline. Clin Biochem *7:* 251–257, 1974.
8. Blumenkrantz, N. & Asboe-Hansen, G.: Methods for analysis of connective tissue macromolecules by determination of certain constituents. *In* Methods of Biochemical Analysis (ed. D. Glick), vol. 24, p. 4●. John Wiley & Sons, Inc. New York, 1977.

Received May 25, 1979

G. Asboe-Hansen, M.D.
Department of Dermatology
Rigshospital
Blegdamsvej 9
DK-2100 Copenhagen
Denmark