Serum Xylosyltransferase: a New Biochemical Marker of the Sclerotic Process in Systemic Sclerosis

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UDP-D-xylose:proteoglycan core protein β-D-xylosyltransferase (EC 2.4.2.26) is the initial enzyme in the biosynthesis of chondroitin sulfate and dermatan sulfate proteoglycans in fibroblasts and chondrocytes. Secretion of xylosyltransferase into the extracellular space was determined in cultured human dermal fibroblasts. A more than 6-fold accumulation of xylosyltransferase activity in cell culture supernatant was observed (day 1, 0.6 μU per 10^6 cells; day 9, 4.1 μU per 10^6 cells); however, intracellular xylosyltransferase activity remained at a constant level (0.4 μU per 10^6 cells). Exposure of human chondrocytes to colchicine led to a 3-fold decreased level of xylosyltransferase and chondroitin-6-sulfate concentration in cell culture. Specific xylosyltransferase activity and chondroitin-6-sulfate concentration decreased in a concentration-dependent manner and in parallel in culture medium and accumulated 5-fold in cell lysates indicating that xylosyltransferase is secreted simultaneously into the extracellular space with chondroitin sulfate proteoglycans. Xylosyltransferase activities were determined in serum samples of 30 patients with systemic sclerosis. Xylosyltransferase activities in female (mean value 1.28 mU per liter, 90% range 1.10–1.55 mU per liter) and male patients (mean 1.39 mU per liter, 90% range 1.16–1.57 mU per liter) with systemic sclerosis were significantly increased in comparison with blood donors of a corresponding age. Furthermore, xylosyltransferase activity was correlated with the clinical classification of systemic sclerosis. Female patients with diffuse cutaneous systemic sclerosis showed higher serum xylosyltransferase activities than patients with limited systemic sclerosis. These results confirm that the increase of proteoglycan biosynthesis in sclerotic processes of scleroderma is closely related to an elevated xylosyltransferase activity in blood and demonstrate the validity of xylosyltransferase as an additional diagnostic marker for determination of sclerotic activity in systemic sclerosis. ***Key words: chondroitin sulfate proteoglycan/systemic sclerosis/xylosyltransferase. J Invest Dermatol 112:919–924, 1999***

The term scleroderma summarizes a heterogeneous group of diseases, which comprise systemic sclerosis (SSc; scleroderma), localized scleroderma, and the so-called overlap syndromes. SSc is a chronic inflammatory disease of the connective tissue characterized by: (i) excessive accumulation of extracellular matrix in skin and various internal organs; (ii) severe alterations in the microvasculature; and (iii) prominent inflammatory and immunologic alterations (for review see Krieg and Meurer, 1988; Perez and Kohn, 1993). Severity of the sclerotic processes and other clinical features are the basis for the clinical classification of patients with SSc (LeRoy *et al*, 1988; Perez and Kohn, 1993).

Sclerotic processes led to a massive deposition of collagen fibrils in skin and other organs and to an elevation of proteoglycan metabolism in sclerotic organs (Kitabatake *et al*, 1983). Cultured dermal fibroblasts from patients with SSc have been shown to synthesize collagen at an increased rate and to accumulate multiple amounts of glycosaminoglycans in comparison with normal skin fibroblasts (LeRoy, 1974; Bashey *et al*, 1984). Electron microscopy investigations revealed that collagen fibrils in normal skin are anchored by proteoglycans (Maeda *et al*, 1981). Decorin and biglycan are small proteoglycans containing chondroitin and dermatan sulfate chains. They have been shown to interact specifically with type I collagen fibrils and other components of the extracellular matrix on the surface of cultured human fibroblasts (Schmidt *et al*, 1987; Kresse *et al*, 1994). *In vitro*, recombinant biglycan and decorin are potential inhibitors of fibrillogenesis of types I and II collagen (Vogel *et al*, 1984; Vogel and Trotter, 1987).

These findings indicate the importance of proteoglycan and glycosaminoglycan synthesis for the metabolism of collagen fibrils. Increased amounts of glycosaminoglycans have been found in patients with SSc (Ishikawa and Horiuchi, 1975; Tajima *et al*, 1982) and in cultured fibroblasts isolated from scleroderma patients (Cabral and Castor, 1983; Bashey *et al*, 1984). In skin biopsies of patients suffering from SSc an elevated chondroitin sulfate and dermatan sulfate concentration was found, indicating their role in fibrosis (Tajima *et al*, 1982; Akimoto *et al*, 1992). Further investigations showed that the increased chondroitin sulfate and dermatan sulfate glycosaminoglycan content in the affected skin correlates with the severity of sclerotic skin (Higushi *et al*, 1994).

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Abbreviations: SSc, systemic sclerosis; XT, xylosyltransferase.

Chondroitin sulfate and dermatan sulfate are bound to the proteoglycan core protein by a xylose–galactose–galactose binding region (Kjellen and Lindahl, 1991). UDP-D-xylose:proteoglycan core protein β-D-xylosyltransferase (EC 2.4.2.26, XT) is the chain-initiating enzyme involved in the biosynthesis of glycosaminoglycan containing proteoglycans (Schwartz, 1977; Kearns *et al*, 1991). The enzyme catalyzes the transfer of D-xylose from UDP-D-xylose to specific serine residues of the core protein and is a regulatory factor in chondroitin sulfate biosynthesis (Roden, 1980). The XT is present in the cisternae of the rough endoplasmatic reticulum of various species (Hoffmann *et al*, 1984). XT from bovine chondrocytes was shown to be secreted simultaneously from the endoplasmatic reticulum into the extracellular space with chondroitin sulfate proteoglycans (Kahnert *et al*, 1991). Therefore, the increased biosynthesis and secretion of dermatan sulfate and chondroitin sulfate proteoglycans during sclerosis is suspected to result in elevated levels of XT in the affected organs and in blood.

XT activities in the synovial fluid were found to be significantly increased in chronic inflammatory joint diseases (Kleesiek *et al*, 1987). The determination of XT activity in serum, however, showed no significant differences due to the insensitivity of the used XT assay with silk fibroin as acceptor. Meanwhile we developed a sensitive XT assay using recombinant bikunin, the inhibitory component of the inter-α-trypsin-inhibitor, as substrate (Weilke *et al*, 1997). With this assay we are able to determine small changes of XT activity in blood reliably.

In our study we investigated XT activities in cultured human dermal fibroblasts and the secretion of XT and chondroitin-6-sulfate into the extracellular space by human chondrocytes. Furthermore, we determined XT activities in serum of patients with SSc in order to evaluate the validity of XT as a diagnostic marker for fibrotic alterations of skin and internal organs during these diseases.

MATERIALS AND METHODS

Materials UDP-[^{14}C]-D-xylose (9.88 kBq per nmol) was purchased from DuPont (Bad Homburg, Germany) and 25 mm diameter nitrocellulose discs from Sartorius (Gottingen, Germany). Scintillation mixture and the liquid scintillation counter LS500TD were supplied by Beckman (Fullerton, CA) and Microcon 3000 ultrafiltration tubes by Amicon (Beverly, MA). Serum monovettes were purchased from Sarstedt (Numbrecht, Germany).

Heat-inactivated fetal calf serum, trypsin/ethylenediamine tetraacetic acid solution, antibiotic/antimycotic solution, L-glutamine, Dulbecco's phosphate-buffered saline, Trypan Blue, 425–600 μm glass beads, colchicine, RPMI1640 cell culture medium, and the Bicinchoninic Acid Protein Assay Kit were obtained from Sigma (Deisenhofen, Germany). Cell culture flasks, serological pipettes, and sterile tubes were provided by Becton Dickinson (Heidelberg, Germany) and human albumin solution by Centeon Pharma (Marburg, Germany). Twenty-four well plates were purchased from Nunc (Wiesbaden, Germany). Human adult dermal fibroblasts and fibroblast growth medium FGM-2 were obtained from Biowhittaker (Walkersville, MD). The Super G analyzer was supplied by RLT (Mohnesee, Germany) and the ACA analyzer by Dade Behring Diagnostica (Munchen, Germany).

The ANA Profil ELISA was purchased from Elias (Freiburg, Germany) and anti-dsDNA radioimmunoassay from DPC Biermann (Bad Nauheim, Germany). The Quantafluor HEp2-cell line substrate immunofluorescence assay and mouse stomach/kidney slides were provided by Sanofi Diagnostics Pasteur (Chaska, MN). Freka-Fluor *Borrelia* IgG IFT/IFA assay was obtained from Fresenius (Bad Homburg, Germany) and *Borrelia burgdorferi* IgG/IgM western blot from Autoimmun Diagnostika (Straβberg, Germany).

All other chemicals in *pro analysi* quality were obtained from Merck (Darmstadt, Germany).

Synthesis of recombinant bikunin Recombinant bikunin was expressed in *Escherichia coli* strain BL21(DE3) as described previously (Brinkmann *et al*, 1997).

Determination of XT activity The method for determination of XT activity is based on the incorporation of [^{14}C]-D-xylose with recombinant bikunin as acceptor and is published elsewhere in detail (Weilke *et al*, 1997). The reaction mixture for the assay contained in a total volume of 100 μl/50 μl of XT solution, 25 mM 4-morpholine-ethanesulfonic acid (pH 6.5), 25 mM KCl, 5 mM KF, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1.0 μM UDP-[^{14}C]-D-xylose, and 1.5 μM recombinant bikunin. Following incubation for 1 h at 37°C, the reaction mixtures were placed on nitrocellulose discs. After drying, the discs were washed for 10 min with 100 g per liter trichloroacetic acid and three times with 50 g per liter trichloroacetic acid solution. Incorporated radioactivity was quantitated by liquid scintillation counting. The enzyme activity was expressed in units (1 unit = 1 μmol of incorporated xylose per min).

Quantitation of chondroitin-6-sulfate Chondroitin-6-sulfate concentrations in cell lysates and cell culture medium were determined with a competitive enzyme immunoassay using biotinylated chondroitin-6-sulfate antigen as described before (Kahnert *et al*, 1994).

Cell culture Human chondrocytes from sternal cartilage obtained during open heart surgery were prepared as described previously (Brinkmann *et al*, 1997). Cells were grown in 24 well plates with RPMI1640 medium supplemented with antibiotic/antimycotic solution, 2 mM L-glutamine and 10% heat-inactivated fetal calf serum at 37°C in a humidified atmosphere (95% air and 5% CO_2) using standard procedures (Butler and Dawson, 1992). Glucose and lactate concentrations of the cell culture media were monitored using the Super G and the ACA analyzers, respectively. After the cells reached confluence the medium was changed to RPMI1640 without fetal calf serum for 2 d. The cell culture medium was then changed to serum-free RPMI1640 supplemented with colchicine. After 24 h the medium was harvested and stored at –20°C after centrifugation at 1000 × *g* for 10 min. For quantification of intracellular XT activities cells were detached and mechanically lysed as described. All experiments were performed in triplicates.

Human adult dermal fibroblasts were cultivated in FGM-2 according to the supplier's instructions. After the cells reached 80% of confluence the medium was changed and intracellular and secreted XT activities were measured for a period of 9 d. XT activity in the spent medium was determined after harvesting and centrifugation at 1000 × *g* for 10 min. Intracellular XT activity was determined after detachment and lysis of the cells.

Preparation of cell lysates Cells were mechanically lysed in order to quantify intracellular XT activity. Cells were detached using trypsin/ethylenediamine tetraacetic acid solution, centrifuged at 1000 × *g* for 10 min and then gently washed twice with Dulbecco's phosphate-buffered saline supplemented with 1% human albumin and 0.1% Tween20. The number of viable cells was determined using Trypan Blue exclusion. Glass beads (425–600 μm) were added and the samples were vigorously shaken on a vortex mixer for 2 min followed by 10 min incubation on ice. This procedure was repeated twice and the degree of lysis was optically controlled. Samples were then centrifuged at 1000 × *g* for 10 min and the supernatant was stored at –20°C.

Determination of total protein concentration Total protein concentrations in cell culture supernatant and cell lysates were determined using the Bicinchoninic Acid Protein Assay Kit. Free amino acids in the samples were removed prior to protein determination by ultrafiltration with Microcon 3000 tubes according to the manufacturer's instructions.

Collection of serum samples Venous blood samples were collected in serum monovettes. After clotting and centrifugation at 4000 × *g* for 15 min the serum was stored at –70°C until assayed. Samples were collected from male (n = 150) and female (n = 165) blood donors, aged 20–65 y. To determinate XT activities in serum of patients with scleroderma specimens were collected from 24 female and six male patients with SSc.

Detection of auto-antibodies Anti-nuclear antibodies in serum of patients with scleroderma were quantitated using the immunofluorescence assay Quantafluor HEp2-cell line substrate. Identification of different auto-antibodies in patient serum samples was performed with commercially available assays. The specimens were investigated for presence of the following auto-antibodies: anti-U-1-RNP, anti-U1-ribonucleoprotein; anti-RNP/SM, anti-ribonucleoprotein particle; anti-Sm, anti-RNAse, and Sm-antigen protein complex; anti-SS-A (Ro), anti-Sjogren-syndrome-A antigen; anti-SS-B (La), anti-Sjogren-syndrome-B; anti-Scl70, anti-DNA topoisomerase I; anti-CENP, anti-centromer antigen; anti-PmScl, anti-PmScl protein complex; anti-JO1, anti-histidyl-tRNA-synthetase; AMA, anti-mitochondrial antibodies; ASMA, anti-smooth muscle antibodies.

Differentiation of auto-antibodies against extractable nuclear antigens was performed with the ANA Profil ELISA. Anti-mitochondrial and anti-smooth muscle antibodies were determined by immunofluorescence with

Table I. Clinical characteristics of the SSc patients studied

Patient no.	Sex	Age	Clinical classification	Raynaud's phenomenon	Organs affected[a]	Skin score[b]	ANA[c] (IFT)	ENA[d]	XT activity (mU per liter)
1	M	59	limited	+	S	5	1280	anti-Scl-70	1.07
2	M	61	limited	+	S, L, GI, A		2560	anti-Scl-70	1.76
3	M	69	limited	–	S, L, GI, K		2560	negative	1.55
4	M	57	limited	+	S, L	4	320	negative	1.35
5	M	66	limited	–	S, L, A	0	160	anti-PmScl	1.09
6	M	58	diffuse	+	S, L, A	9	1280	negative	1.53
7	F	46	limited	+	S, L		1280	anti-SS-A (Ro); anti-SS-B (La)	1.15
8	F	55	limited	+	S, L, GI		640	anti-Scl-70	1.39
9	F	56	limited	+	S, L		320	negative	1.40
10	F	59	limited	+	S, K		640	anti-Scl-70	1.31
11	F	64	limited	+	S, K, GI		1280	anti-Scl-70	1.20
12	F	79	limited	+	S, L		640	anti-SS-A (Ro); anti-Scl-70	1.53
13	F	72	limited	+	S, L, GI		1280	negative	1.34
14	F	56	limited	+	S, GI		640	anti-SS-A (Ro); anti-SS-B (La); anti-CENP	1.14
15	F	31	limited	+	S		80	anti-PmScl	1.06
16	F	44	limited	+	S		5120	anti-CENP	0.96
17	F	55	limited	+	S		320	anti-CENP	1.37
18	F	59	limited	+	S, L		640	anti-U1-RNP	1.14
19	F	65	limited	+	S	4	5120	anti-CENP	1.51
20	F	68	limited	+	S	4	2560	negative	1.12
21	F	72	limited	+	S, L	3	5120	anti-CENP	1.55
22	F	72	limited	–	S	2	5120	negative	1.12
23	F	78	limited	+	S, GI	1	640	anti-CENP	1.10
24	F	80	limited	+	S, K, L		5120	anti-CENP	1.16
25	F	82	limited	+	S, K		2560	anti-PmScl	1.05
26	F	32	diffuse	+	S, L	10	320	negative	1.24
27	F	46	diffuse	+	S, L, H, A, GI	10	5120	anti-Scl-70	1.31
28	F	57	diffuse	+	S	6	5120	negative	1.46
29	F	57	diffuse	+	S, A	6	5120	anti-Scl-70	1.57
30	F	68	diffuse	+	S		<40	negative	1.52

[a]S, skin; L, lung; K, kidneys; A, joints; H, heart; GI, gastrointestinum.
[b]Skin scores were determined using a modified Rodnan skin thickness score.
[c]Anti-nuclear antibodies (ANA) were quantitated as immunofluorescence titer (IFT; normal range, < 40).
[d]The following auto-antibodies against extractable nuclear antigens (ENA) were detected in the investigated sera: anti-CENP, anti-centromer antigen; anti-Scl70, anti-DNA topoisomerase I; anti-SS-A (Ro), anti-Sjogren syndrome A antigen; anti-SS-B (La), anti-Sjogren syndrome B antigen; anti-PmScl, anti-PMScl protein complex; anti-U1-RNP, anti-U1 ribonucleoprotein particle.

mouse stomach/kidney slides and antibodies directed against double-stranded DNA by anti-dsDNA radioimmunoassay. IgG antibodies against *Borrelia burgdorferi* were detected using the Freka-Fluor *Borrelia* IgG IFT/IFA assay and the *Borrelia burgdorferi* IgG/IgM western blot. All tests were performed according to the manufacturers' instructions.

Determination of skin score values in scleroderma patients The skin score representing the numerical severity grade at a specific number of skin sites was determined using a modified Rodnan skin thickness score (Silman *et al*, 1995). The modified Rodnan skin score was determined using the 0–3 scale (0, normal skin; 1, thickened; 2, unable to pinch; 3, unable to move) at eight distinct sites: upper arms, forearms, dorsum of hands, fingers, thighs, lower legs, dorsum of feet, and regio pectoralis. The evaluation of the skin score was always performed by the same investigator (C.H.).

Characteristics of patients studied In our study we investigated 24 female and six male patients with SSc (**Table I**). All fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for the classification of SSc (Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee 1980). Five women, aged 32–57, were suffering from the diffuse form of SSc and 19 female patients, aged 31–82, from limited SSc. One male patient with the diffuse form and five men with limited SSc were investigated. The classification of systemic scleroderma in the two subtypes (limited and diffuse disease) was done according to LeRoy *et al* (1988). In all patients studied the skin was affected by sclerotic processes. Elevated immunofluorescence titers against anti-nuclear antibodies were observed in 96% of women and in 100% of men. The predominant autoantibodies were found to be anti-DNA topoisomerase I (anti-Scl70) and anti-centromer antigen (anti-CENP). Skin score values were determined in 13 of the patients studied using a modified Rodnan skin thickness score (Silman *et al*, 1995) (**Table I**).

Statistical analysis Statistical analysis was performed using the t-test and Kolmogoroff–Smirnoff test. Correlation and regression analysis was performed using Pearsson's correlation coefficient and analysis of variance (ANOVA) including the F-test. $p \leq 0.05$ were considered significant.

RESULTS

Human dermal fibroblasts secrete XT into the extracellular space XT activities in cell lysates and in cell culture supernatant obtained from cultured human dermal fibroblasts were analyzed over a period of 9 d (**Fig 1**). Mean XT activities in cell lysates representing the intracellular amount of XT were 0.36 μU per 10^6 cells (SD 0.23). No increase of intracellular XT activity was observed during the 9 d incubation period; however, we found an accumulation of XT activities in cell culture supernatant. After incubation for 7–9 d XT activities in harvested culture medium were 11-fold higher than in cell lysates. The mean values of secreted XT in cell culture supernatant after 7–9 d were calculated as 3.89–4.06 μU per 10^6 cells (SD 0.35–0.51) (**Fig 1**).

Colchicine inhibits secretion of XT in human chondrocytes In order to clarify whether the release of XT into the extracellular space is due to a secretion process and whether it is linked to the secretion of chondroitin sulfate proteoglycans we exposed cultured human sternal cartilage chondrocytes to the secretion blocking agent colchicine. After 24 h XT activities and chondroitin-6-sulfate concentrations in cell culture medium and cell lysates were measured (**Fig 2**). Exposure of cultured chondrocytes to colchicine led to a 3–5-fold decreased level of total XT activity and total chondroitin-6-sulfate concentration in the cell culture. XT activity and chondroitin-6-sulfate concentration decreased in a concentration-dependent manner and in parallel in the spent medium and

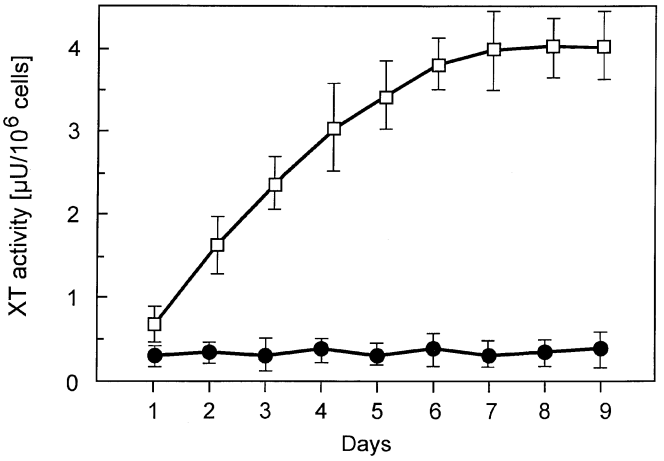

Figure 1. XT is secreted by human adult dermal fibroblasts. XT activities in cell culture supernatant and cell lysates of cultured human dermal fibroblasts are shown. Cells were grown until they reached 80% of confluence and XT activities in culture medium and in cell lysates were determined for a period of 9 d. XT secreted into the extracellular space is represented by open boxes whereas intracellular XT is indicated by filled circles. Mean values and the corresponding SD are shown.

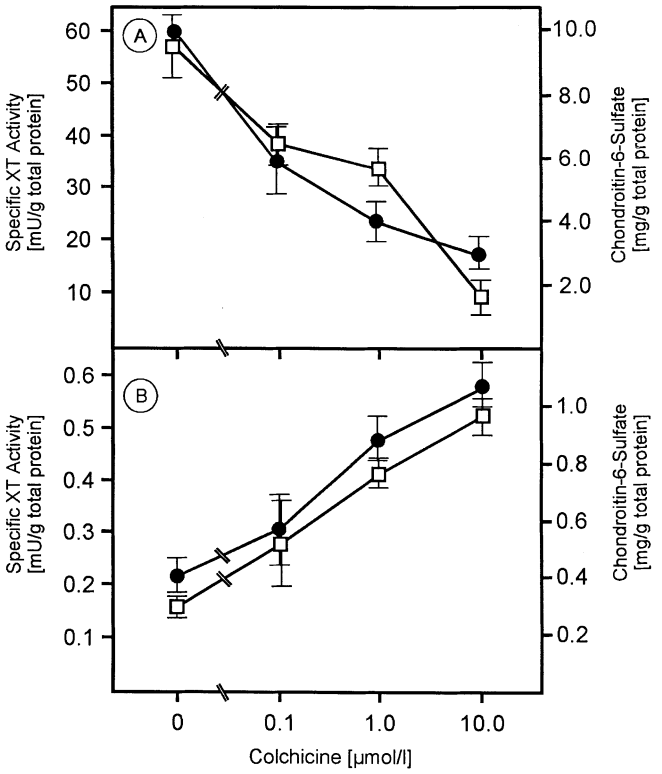

Figure 2. Colchicine inhibits secretion of XT and chondroitin-6-sulfate proteoglycans in cultured human chondrocytes. Chondroitin-6-sulfate concentrations and specific XT activities were determined in cultured human sternal cartilage chondrocytes after exposure to colchicine for 24 h. (*A*) XT activity (□) and chondroitin-6-sulfate concentration (●) in the harvested cell culture supernatant representing the amount of the enzyme and the polysaccharide secreted into the extracellular space. (*B*) Corresponding enzyme activity and chondroitin-6-sulfate concentration in cell lysates representing the intracellular amount of XT and of the measured glycosaminoglycan. Mean values and SD are shown. Values are averages of the analysis of three cultures.

accumulated 4–5-fold in cell lysates. The ratio of extracellular to intracellular XT activity changed from 350:1 in the control group to 20:1 after exposure to 10 µmol per liter colchicine for 24 h.

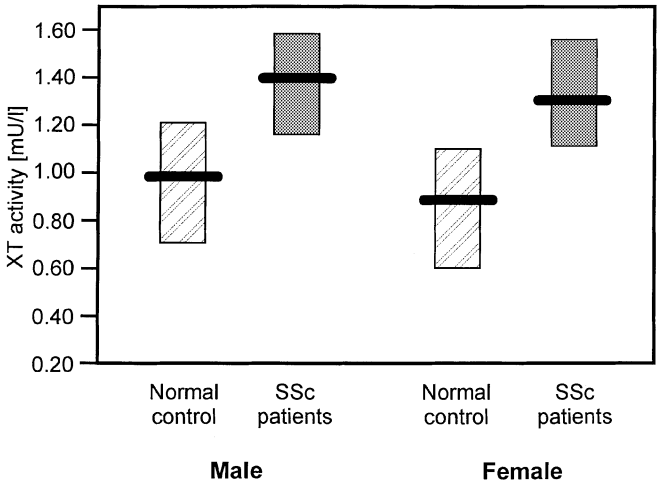

Figure 3. Elevated serum XT activities in patients with SSc. XT activities in serum of male patients with SSc (n = 6), aged 57–69, and of healthy blood donors (n = 150), 55–65 y old. In women with SSc (n = 24), mean XT activities were 1.5-fold increased in comparison with 20–65 y old female blood donors (n = 165). Mean values and 90% ranges are shown.

XT activity is elevated in serum of scleroderma patients We determined XT activities in serum samples of 30 patients suffering from SSc. XT activities in female patients with SSc (n = 24), aged 31–82 y, were significantly elevated in comparison with female blood donors (n = 165), 20–65 y old (**Fig 3**). In serum specimens of women with SSc the mean value and 90% range were 1.28 mU per liter (SD 0.18) and 1.10–1.55 mU per liter. In female blood donors the mean value and 90% range were 0.89 mU per liter (SD 0.20) and 0.6–1.1 mU per liter. XT activities in serum of male patients with SSc (n = 6), 57–69 y old, were calculated as 1.39 mU per liter and 1.16–1.57 mU per liter. In the corresponding blood donor control group (n = 150), aged 55–65, mean XT activities of 0.98 mU per liter were measured. The 90% range was determined as 0.72–1.21 mU per liter.

Serum XT activities in diffuse and limited forms of SSc We analyzed the determined XT activities referring to the clinical classification of the sclerotic disease. In women with diffuse forms of SSc (n = 5) mean serum XT activities were increased in comparison with patients with limited SSc (n = 19). Mean values and 90% ranges were 1.42 mU per liter (SD 0.14) and 1.31–1.55 mU per liter in diffuse forms and 1.24 mU per liter (SD 0.18) and 1.05–1.53 mU per liter in limited SSc (**Fig 4**). In men with limited cutaneous SSc (n = 5) serum XT activities were 1.07–1.76 mU per liter (mean value 1.36 mU per liter; SD 0.29) and in the male patient with diffuse SSc (n = 1) XT activity was determined as 1.53 mU per liter. A significant correlation of XT activities and clinical classification could not be proven due to the limited number of patients investigated in our study.

Correlation of serum XT activity with skin scores XT activities in serum of five female patients with limited SSc and in four women with diffuse SSc were compared with the clinical skin scores (**Table I**). No significant correlation of serum XT activities and skin score values was found.

Serum XT activities in SSc patients remain elevated in longitudinal studies We investigated the longitudinal alterations of serum XT activities in 12 SSc patients. Three male and nine female patients suffering from SSc were re-examined after 1 y. The skin score remained constant during this period as well as the XT activity. Neither a major increase nor decrease of serum XT activity values was observed. Serum XT activities were differing less than ± 11% in comparison with the enzyme activities determined 1 y before (**Table II**).

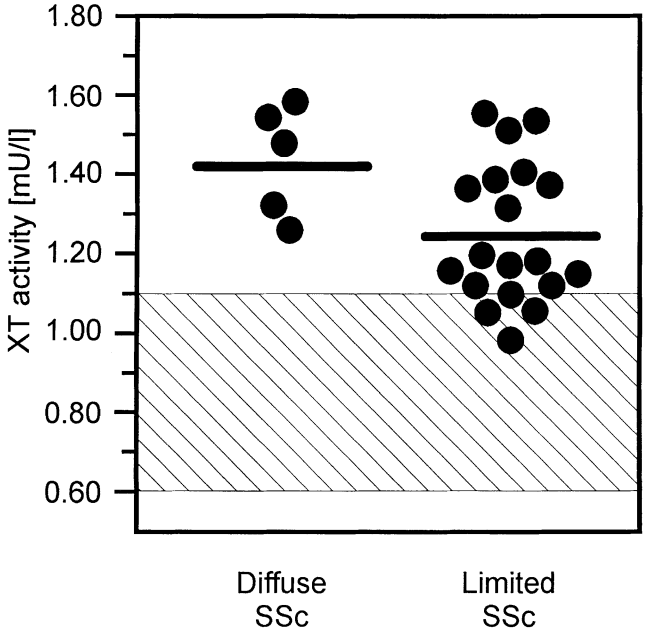

Figure 4. XT activities in serum of female patients with SSc. Mean values in serum of women with diffuse SSc and of female patients with limited SSc and XT activities of each patient are given. The striped box indicates the 90% range of serum XT activities of normal blood donors.

DISCUSSION

The degree and activity of sclerosis are important parameters for the clinical classification of SSc (LeRoy *et al*, 1988). An elevation of dermatan sulfate and chondroitin sulfate concentrations in skin biopsies was shown to correlate with the severity of skin involved (Higushi *et al*, 1994). Biosynthesis of these glycosaminoglycans is initiated by XT, which is secreted into extracellular space by cultured dermal fibroblasts (**Fig 1**). The observed effects of the exposure of human chondrocytes to colchicine demonstrate that XT and chondroitin-6-sulfate proteoglycans are simultaneously secreted into the culture medium (**Fig 2**). Therefore, an increase in dermatan sulfate and chondroitin sulfate biosynthesis in SSc should result in an elevation of XT activity. Using our new sensitive XT assay with recombinant bikunin as substrate (Weilke *et al*, 1997) we found increased levels of XT activity in the serum of patients suffering from diseases associated with fibrotic processes, e.g., hepatitis C virus induced liver fibrosis and lung fibrosis (unpublished observations). This XT assay is suitable for the determination of low XT activities in blood (lower detection limit 0.02 mU per liter) and therefore, enables the detection of small changes in the enzyme activity (Weilke *et al*, 1997).

Determination of XT activities in dermal fibroblast culture revealed a secretion of XT into extracellular space shown by the accumulation of XT activity in the cell culture supernatant during the incubation period (**Fig 1**). After 7 d more than 90% of the total XT activity was found to be located in the culture medium whereas the intracellular amount of XT remained at a constant level. Similar results were observed in cultured human chondrocytes (**Fig 2**). In order to elucidate whether XT is released autonomously into culture medium or whether it is attached to proteoglycans we investigated the effect of the secretion blocking agent colchicine on human chondrocytes. Experiments were performed with chondrocytes as they secrete large amounts of chondroitin sulfate proteoglycans, whereas the major secretory products of human skin fibroblasts is a 110 kDa proteodermatan sulfate (Glossl *et al*, 1984). Colchicine inhibits the microtubule-mediated release of chondroitin sulfate into the extracellular matrix (Kim and Conrad, 1980). Exposure of sternal cartilage chondrocytes to colchicine resulted in a concentration-dependent decrease in XT activity and chondroitin-6-sulfate concentration in the extracellular space (**Fig 2**); however, a 5-fold accumulation of enzyme activity and chondroitin sulfate concentration in the cells was observed. Colchicine was shown not to influence total protein synthesis (Rennison *et al*, 1992; Evangelisti *et al*, 1995). Therefore, our results show that XT is secreted simultaneously into the extracellular matrix with chondroitin sulfate containing proteoglycans. Evangelisti *et al* (1995) have demonstrated that the effect of colchicine on the secretion of sulfated glycosaminoglycans in cultured skin fibroblasts is comparable with that in human chondrocytes. This lets us conclude that an elevation of dermatan and chondroitin sulfate biosynthesis during the sclerotic process in scleroderma may result in an increased secretion of XT. Future experiments on XT secretion in fibroblasts from SSc patients may reveal differences in XT expression in comparison with normal control fibroblasts. It remains to be elucidated, however, whether *in vitro* studies using cultured SSc fibroblasts are suitable to simulate complex inflammatory and fibrotic processes. Further detailed studies using immunologic staining may yield information on the colocalization and secretion of proteoglycans and XT as soon as anti-XT antibodies are available.

Table II. Serum XT activities in SSc patients remain elevated in longitudinal studies

Patient[a]	Serum XT activity (mU per liter)		Alteration
	Initial value	Value after 1 y	
Men	1.07	1.18	+9%
	1.53	1.56	+2%
	1.09	1.06	−3%
Women	1.31	1.47	+11%
	1.46	1.34	−9%
	1.57	1.75	+10%
	1.31	1.22	−7%
	1.20	1.19	−1%
	1.14	1.13	−1%
	1.51	1.37	−10%
	1.12	1.26	+11%
	1.55	1.43	−8%

[a]Patients were re-examined after 12 mo. The serum XT activity was determined and compared with the initial value. The differences between both values are given in percentage. No significant alterations of skin score values were observed during the 1 y period.

Significantly elevated XT activities were observed in the serum of patients with SSc. Mean values were increased 1.4–1.5 times in comparison with blood donors of corresponding age groups (**Fig 3**). Analysis of serum XT activities in blood donors revealed an age and sex dependence (Weilke *et al*, 1997). Therefore, in our experiment the control groups were of the same age and sex as the scleroderma patients. We suggest that the increase in serum XT activity observed is a direct consequence of the elevated proteoglycan biosynthesis in the fibrotic skin and organ systems. An elevation of dermatan sulfate and chondroitin sulfate concentrations in fibrotic skin has been shown by a diversity of authors (Fleischmajer and Perlish, 1972; Bashey *et al*, 1984; Juhlin *et al*, 1986; Higushi *et al*, 1994). As XT is the initial enzyme involved in the synthesis of dermatan sulfate, heparan sulfate, and chondroitin sulfate proteoglycans, an increased level of XT is an inevitable prerequisite for rapid synthesis of proteoglycans. Our cell culture experiments have demonstrated that XT is secreted into the extracellular space together with chondroitin sulfate proteoglycans, where it is attached to the core protein of the proteoglycan (**Fig 2**). Therefore, we conclude that the elevation of XT activities observed in serum samples of scleroderma patients is a direct consequence of the increased proteoglycan biosynthesis in sclerotic organ systems and not due to a release from the destroyed tissue during the inflammatory process.

Regarding the clinical classification of the investigated specimens we found increased serum XT activities in female patients with the diffuse form of SSc in comparison with women with limited SSc (**Fig 4**). Diffuse SSc was clinically characterized by a more

rapid and severe progression of fibrotic processes in skin and inner organs than limited SSc. Higushi *et al* (1994) proposed a nexus between sclerotic alterations and the glycosaminoglycan content in skin. Our results indicate that elevated serum XT activities occur due to the larger number of affected organs and the accelerated fibrotic processes in patients with diffuse cutaneous SSc.

Our longitudinal studies have shown that XT activities in serum samples of SSc patients remain at an increased level with alterations of less than 11% over a period of 1 y (**Table II**). During this period, however, no significant changes of extent and severity of sclerotic lesions have been observed. The high XT activities observed in the serum of patients suffering from SSc can either be a consequence of an elevated biosynthesis of XT, an increased release of XT from fibroblasts during sclerotic processes or a reduced rate of degradation of the enzyme. As data on the half-life of XT in blood are not yet available a decreased rate of degradation cannot be excluded. An elevated release of XT from destroyed cartilage tissue was observed in the synovial fluid during chronic inflammatory joint diseases (Kleesiek *et al*, 1987). An increased biosynthesis of XT during sclerotic processes could be demonstrated using northern blot analysis of mRNA encoding XT. As information on the nucleotide sequence of the enzyme is not yet available detailed studies on mRNA levels for XT will have to be performed in the future. The increased amounts of dermatan sulfate and chondroitin sulfate in skin biopsies from scleroderma patients and the simultaneous secretion of XT and chondroitin-6-sulfate proteoglycans into the extracellular space, however, let us conclude that an increased rate of biosynthesis of XT is the reason for the high XT activities in the serum of patients with SSc.

SSc is characterized by fibrotic alterations of parenchymal and connective tissue. These inflammatory processes are accompanied by excessive accumulation of collagen fibrils and proteoglycans in the extracellular matrix of skin and other organ systems. The resulting increased rate of proteoglycan biosynthesis reflects the activity of the sclerotic process. As XT is the initial enzyme involved in the biosynthesis of glycosaminoglycan chains, elevated XT activity is more a measure for an actually increased rate of proteoglycan synthesis than for the total amount of proteoglycans in the involved organ systems. Therefore, XT is a marker of disease activity in SSc, whereas the determination of the skin score reflects the disease severity.

In our study, no significant correlation of XT activity in blood and skin score values in female SSc patients was observed. These findings are in accordance with the fact that a pronounced disease severity does not necessarily imply an elevated sclerotic activity. Patients with extended sclerosis might show a reduced sclerotic activity as soon as the fibrotic alterations of the organs involved are finished. This final condition of the sclerotic process then results in a diminishing biosynthesis of proteoglycans and collagen fibrils. As XT is a measure for the actual rate of proteoglycan synthesis the extended disease severity might not necessarily result in elevated XT activities in these SSc patients.

Our results show that the increase of XT in blood is closely related to sclerotic processes in patients with scleroderma and demonstrate that this enzyme is an adequate diagnostic marker suitable for the determination of sclerotic activity in SSc.

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