The association between changes in skin echogenicity and the fibroblast production of biglycan and versican in systemic sclerosis

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Abstract

Objective

To investigate a possible association between the longitudinal changes in skin involvement and the fibroblast production of proteoglycans in vitro, among patients with early and untreated systemic sclerosis (SSc).

Methods

In 11 patients, 6 with diffuse cutaneous systemic sclerosis (dSSc) and 5 with limited cutaneous systemic sclerosis (lSSc), and in 6 controls skin thickness and skin echogenicity of the forearm was measured by high frequency (20 MHz) ultrasound. A skin biopsy was taken from the area of the ultrasound measurements, and from cultivated fibroblasts the production of the proteoglycans versican, perlecan, biglycan and decorin were measured. To investigate longitudinal changes in skin involvement, the ultrasound examination was repeated after 1-3 years.

Results

Compared to controls, SSc patients had increased skin thickness at the first evaluation. Patients with dSSc had lower skin echogenicity than both patients with lSSc and the controls. Patients with greater changes in skin thickness and skin echogenicity produced more versican, whereas the production of biglycan and decorin was higher only in patients with greater changes in skin echogenicity. There was a negative correlation between fibroblast production of biglycan and disease duration.

Conclusion

High fibroblast synthesis of the proteoglycans versican and biglycan is associated with changes in skin echogenicity and may predict more progressive skin sclerosis in SSc.

Key words

Systemic sclerosis, ultrasound, versican, biglycan.

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This study was supported by grants from the Österlund Foundation, the Kock Foundation, Anna Greta Crafoord Foundation, The Swedish Rheumatism Association, Gustaf V's 80 years Fund, the Heart-Lung Foundation, the Medical Faculty of the University of Lund and the Swedish Medical Research Council (S0898) and (11550).

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Received on June 25, 2001; accepted in revised form on November 19, 2001.

Introduction

Systemic sclerosis (SSc, scleroderma) is a systemic connective tissue disease of unknown aetiology. It is characterised by immune abnormalities, microvascular injury, and fibrosis in the skin and internal organs (1). Cutaneous involvement, which is the hallmark of SSc, is partly due to an altered fibroblast function with increased synthesis of the extracellular matrix (2) in both clinically involved and uninvolved skin (3). Three phases of skin involvement can be identified: an early oedematous phase caused by increased amounts of interstitial fluid, an indurative phase during which newly synthesised matrix components are deposited in the skin, and an atrophic phase in which thinning of the abnormal skin occurs (4).

Analysis of the content of the extracellular matrix in SSc has given varying results, probably due to differences in the disease duration, progression rate and biochemical techniques. However, increased production by scleroderma fibroblasts of collagen types I, III, VI, and VII and of the extracellular matrix components, fibronectin and proteoglycans (PG), is well documented (2, 3, 5, 6, 7).

Production of versican, one of the large PG, and of decorin, one of the small PG, are increased in fibroblast cultures from SSc patients in the early stages of the disease (3), whereas decreased production of the small PG, biglycan, was found in the same study (3). Kuroda & Shinkai (8, 9) reported an increased expression of decorin in patients in the early stages of the disease, i.e. a duration of less than one year, but decreased decorin expression in the mid-stages of the disease. Both Kuroda & Shinkai (9) and Izumi *et al*. (10) noticed unaltered decorin production in late disease.

The development of high frequency ultrasound technology has made cross-sectional images possible, which allow not only measurement of skin thickness but also a qualitative assessment of the skin. Dermal echoes are many and variable in most body regions. They originate from the well-organised fibre network, which is also responsible for the tensile properties of the skin. Affections, which erode or disturb this network, cause low reflectancy (11). Although skin echogenicity mainly reflects extracellular fluid content, it is also affected by the orientation of collagen fibres (12). High-frequency ultrasound may be a suitable method to identify the early oedematous phase, during which patients might be susceptible to therapy. Since both decorin and biglycan are hydrophilic and affect the fibrillar architecture (13), we decided to investigate the possible influence of these and other PG on skin involvement among patients with very early and untreated SSc. We selected patients with a shorter disease duration than those in our earlier study (3).

Material and methods

Patients

The cohort consisted of 11 patients, who all fulfilled the American College of Rheumatology criteria for SSc (14). Six patients had diffuse cutaneous systemic sclerosis (dSSc) i.e. truncal scleroderma, and 5 patients had limited cutaneous systemic sclerosis (lSSc) i.e. the absence of truncal scleroderma (1). They all had a short disease duration, mean 12 months (range: 6-72); none had received immunosuppressive treatment and none had any signs of pulmonary fibrosis on chest radiography. Three patients were ANA negative whereas the remaining 8 were ANA positive. One patient had a nucleolar, one had a centromeric, one had a homogeneous, 4 had a speckled, and one patient had both a nucleolar and a homogeneous pattern on indirect immunofluorescence. Two patients with a homogeneous pattern also had anti-topoisomerase I antibodies. Six healthy subjects, matched for age and gender, served as controls (Table I). Fibroblast experiments were performed in triplets with material from one patient with dSSc, from one patient with lSSc and from one healthy person as a control. The concentration of PG in the control subject was arbitrarily set at 1.0 and the PG concentrations of the patients were calculated relative to this control. Twelve females were divided into four triplets, each made up of one patient with lSSc, one with dSSc and one control for later analysis of PG. The fifth,

male only group consisted of one patient with lSSc, two with dSSc and two controls.

Skin assessment

Palpation. Skin involvement was determined by palpation on 24 body areas using the original Rodnan skin score (15). However the rating was modified from a 5-point to a 4-point scale (0-3), where 0 = normal skin, 1 = thickened skin, 2 = thickened skin unable to pinch and 3 = thickened immobile skin. The sum of the scores from all sites gave a total skin score, with a theoretical span from 0 to 72.

Ultrasound. Skin thickness was measured at the forearm, where the biopsies were taken, with a high frequency ultrasound scanner (Dermascan) in which a 20 MHz transducer was mounted in a water chamber. The chamber window was covered with a disposable plastic membrane. Conductive gel was used to ensure satisfactory contact between the crystal face and the skin. The transducer was placed perpendicular to the skin. Two scans of the tissue under investigation were obtained: a one-dimensional A-mode image with different echoes defining the interfaces between the epidermis, dermis and subcutis and a two-dimensional B-mode image with different colours reflecting the different echogenicities of the skin. The measurement was made at a site in the B-mode image where the demarcation lines between the epidermis, dermis and subcutis were parallel and the echoes of the corresponding A-mode image were distinct. With this instrument the mean echogenicity can be estimated for a selected region of interest (16). Skin thickness and skin echogenicity were measured at the same time as when the skin biopsies were obtained. The ultrasound examination was repeated after 1 to 3 years. The differences in skin involvement between the first and repeated ultrasound examination are presented as percentages of the initial value per month, and are referred to as skin thickness and skin echogenicity, respectively. The examiner was unaware of the results from the skin scoring and the previous ultrasound examination.

In healthy subjects there are small differences in skin thickness and skin echogenicity. The authors have data on healthy men and women with a mean ± SD forearm skin thickness of 1.4 ± 0.1 mm and 1.5 ± 0.2 mm, respectively and a mean ± SD skin echogenicity of 39 ± 4 and 35 ± 4, respectively (unpublished observation).

Materials

Cell culture medium was obtained from Gibco, Paisley, UK. ^{35}S-sulphate was purchased from Amersham, UK. Ultra-pure DNA grade agarose came from BioRad laboratories, CA, USA. Dulbecco's modified Eagel's medium (DMEM) was from Sigma Chemicals, St Louis, MO, USA. Ultra-pure guanidinium chloride (8 M) was from ICN Pharmaceuticals, CA, USA. Fetal clone-3 was from HyClone Laboratories, Logan, UT, USA. Chondroitin ABC lyase (E 4.2.2.4) was a product from Siekagaku, Kogyo, Tokyo, Japan. Alcian Blue was bought from Croma, Königen, Germany and PDVF-P Mobilian membranes for blotting were purchased from Millipore, Bedford, UK. All other chemicals were of analytical grade. Antibodies to biglycan and decorin were kindly provided by Dr. Larry Fischer, NIDR, Bethesda, USA.

Fibroblast culture

Among the patients fibroblasts were established from skin biopsies 3 mm in diameter taken from affected skin on the forearm and from an identical site in the controls. The fibroblasts were grown in 25 cm^2 flasks in DMEM supplemented with 10% serum (Fetal clone-3), 1% 2 mM L-glutamine, 50 g Gentamicin and 2.5 g/ml amphotericin B at 37°C in a humidified 5% CO_2/95% air atmosphere. Confluent cultures were trypsinised and split 1:2 before replating. Experiments were performed on cells between passage 4 and 6.

Proteoglycans

At confluence, fibroblast cultures were labelled with 200 Ci/ml ^{35}S-sulphate for 48 h in sulphate-poor (0.1 mMSO_4) DMEM, supplemented with 10% serum. The extraction of PG from the cell medium was performed in 4 M guanidinium chloride. After centrifugation, the PG were precipitated with Alcian Blue. The resulting pellet was dissolved in guanidinium chloride-propanol and the concentration of PG was estimated by the absorption of Alcian Blue at 600 nm (17). The different PG were then separated by agarose (2%) gel electrophoresis (18) and the radioactivity in each band was visualised and quantified by using a Fuji Bas 2000

Table I. Clinical features of 11 SSc patients and 6 controls.

	Controls	lSSc		dSSc	
Gender (male/female)	2/4	2/4		1/4	
		Median (range)			
Age (years)	46 (37-63)	46	(38-75)	48	(37-70)
Disease duration (months)		10	(6-72)	12	(6-24)
Total skin score (points)		12	(8-30)	24	(22-32)
CRP (< 9 mg/l)		< 9	(< 9-88)	< 9	(< 9-66)
Orosomucoid (0.55-1.05 g/l)		0.86	(0.50-1.89)	0.84	(0.70-1.28)
ESR (2-15mm)		24	(6-75)	12	(2-75)
Versican#	1.00	1.94	(0.73-3.41)	0.79	(0.33-1.04)
Perlecan#	1.00	1.51	(0.77-2.30)	0.86	(0.41-2.35)
Biglycan#	1.00	1.24	(0.43-2.29)	0.72	(0.36-1.35)
Decorin#	1.00	0.69	(0.34-4.82)	0.60	(0.32-2.42)
Glycosaminoglycan chains#	1.00*	0.69	(0.40-3.39)	0.67	(0.40-0.92)*

Concentrations of proteoglycans in the control group were arbitrarily set to 1.0 and proteoglycan concentrations of the patients were calculated relative to the control.

* dSSc vs control, p=0.022

Abbreviations: lSSc:limited cutaneous systemic sclerosis; dSSc:diffuse cutaneous systemic sclerosis; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.

Table II. Individual values of thickness and echogenicity of forearm skin at 2 different measurements.

Patient no.	Skin thickness (mm) 1*	2**	skin thickness (%/month)	Skin echogenicity 1*	2**	skin echogenicity (%/month)	Duration/ follow-up (months)
lSSc (n=5)	1.75#§	1.40	0.65	34	44	1.76	10/25
1	1.75			37			72/
2	1.69	1.68	0.02	44.5	28	1.43	10/26
3	1.20	0.90	1.04	29	51	3.16	6/24
4	1.45	1.30	0.26	34	42	0.60	24/39
5	2.20	1.50	1.33	30	45	2.08	6/24
dSSc (n=6)	2.12#§	1.70	0.61	22§§	32	3.89	12/25
6	2.60	2.40	0.30	18	22	0.86	8/26
7	2.45	1.30	1.56	16	36	4.17	24/30
8	2.20	1.60	1.09	17	40	5.41	6/25
9	1.70	1.70	0	25	32	1.33	12/21
10	2.05	1.90	0.61	30	44	3.89	12/12
11	1.70			31			24/
Control (n=6)	1.42##§ 1.23-1.60			36§§ 33-56			

* First ultrasound examination. ** Second ultrasound examination; # Median. ## Median and range; § lSSc (No. 1-5) vs control (p = 0.028); dSSc (No. 6-11) vs control (p = 0.004); §§ dSSc (No. 6-11) vs control (p = 0.004); skin thickness/echogenicity: change in percentage of the initial value per month.

image analyser system. Intensities were normalised for recovery and cell numbers after counting in a Burker chamber. The intraassay variation of PG measurements between different controls in the same run was 10 ± 3%. The amount of versican was calculated by subtracting perlecan from the total amount of large PG after chondroitin ABC lyase digestion (Fig. 2A). Incorporation of ^{35}S-sulphate is considered to be a reliable method for the measurement of PG levels, as it incorporates into the side chain of the PG, which is often heavily sulphated. The only major influx of sulphate into the intercellular pool is from the culture medium. This exchange is rapid, and a constant specific activity is reached within minutes, both in the sulphate and in the 3'-phosphoadenosine-5'phospho-sulphate pools (19).

Degradative methods. Purified PG were precipitated with a 3-fold excess of 90% propanol and 0.4% acetate and recovered by centrifugation at 12,000 g for 20 min in a Biofuge A centrifuge. The pellets were dried and solubilised in an appropriate digestion buffer. Digestions with chondroitin ABC lyase were performed at room temperature for 4 h (20).

Western blot. Versican, perlecan, biglycan and decorin were further identified by Western blot (Fig. 2B). The migration positions of the mentioned PG on Western blot were compared with the respective migration positions of PG on the agarose gel to ensure identification (Fig. 2A). Western blots were made after precipitation with Alcian blue and separation on a 2% agarose gel. After electrophoresis the PG were transferred to PVDF-P membranes. The filters were digested with chondroitin ABC lyase and HNO_2, and treated with antibodies against versican, perlecan, decorin and biglycan. Finally the blots of versican, decorin and biglycan were visualised using a second peroxidase conjugated swine-anti-rabbit antibody. Perlecan was visualised using a second peroxidase conjugated rabbit-anti-mouse antibody (21). Western blot was only used for identification of the different PG. It is not usable for quantification of the different PG as we have visualised the blots by peroxidase.

Chemical analyses

The laboratory parameters measured were serum C-reactive protein (CRP) and orosomucoid and the erythrocyte sedimentation rate (ESR).

Statistical analysis

Median values and range were calculated. Differences between groups were calculated using the Wilcoxon rank sum test and correlations were calculated using Spearman's rho.

Results

Compared to the controls, skin thickness was increased both in lSSc patients (p = 0.028) and dSSc patients (p = 0.004), while skin echogenicity was decreased in dSSc patients (p = 0.004), but not in lSSc patients (Table II). Skin thickness among patients and controls was negatively correlated to skin echogenicity (p < 0.01, Rho = -0.84, Figure 1).

Concentrations of PG in the media from fibroblast cultures did not differ between the controls, and patients with lSSc and dSSc. Control fibroblasts secreted 1.26 (0.58-2.29) dpm/cell of PG, whereas fibroblasts from patients with lSSc and dSSc secreted 1.16 (1.02-3.77) and 0.60 (0.53-2.14) dpm/cell amount of PG, respectively. Further characterisation of the types of PG by electrophoresis (Fig. 2A) and identification by Western blot (Fig. 2B) showed decorin to be the main PG in all three types of cultures (Fig. 2A). Other types of PG secreted were versican, perlecan and biglycan (Fig. 2A and 2B). Compared both to the controls and patients with dSSc, fibroblasts from patients with lSSc produced twice as much versican (Table I), although the difference was not significant (p = 0.14, p = 0.086). Control fibroblast cultures produced more free glycosaminoglycan (GAG) chains than did dSSc cultures (p = 0.022, Table I).

Production of versican, perlecan, biglycan and decorin did not correlate either to skin thickness or to skin echogenicity. To analyse changes in skin thickness and skin echogenicity, the ultrasound examination was repeated after 1 to 3 years in 9 patients. The changes are described as skin thickness and skin echogenicity (Table II). In the whole patient group, the changes in skin thickness by repeated ultrasound mea-

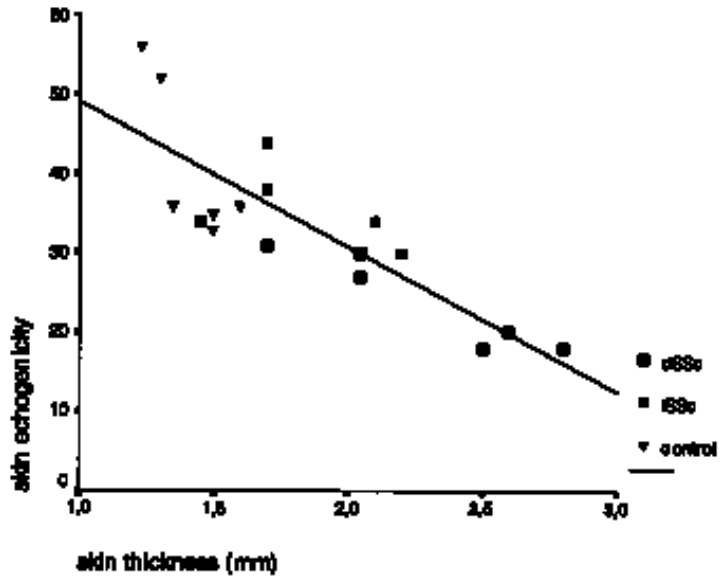

Fig. 1. Correlation between forearm skin echogenicity and skin thickness in controls, and lSSc and dSSc patients examined by high frequency (20 MHz) ultrasound ($p < 0.01$, Rho = -0.84).

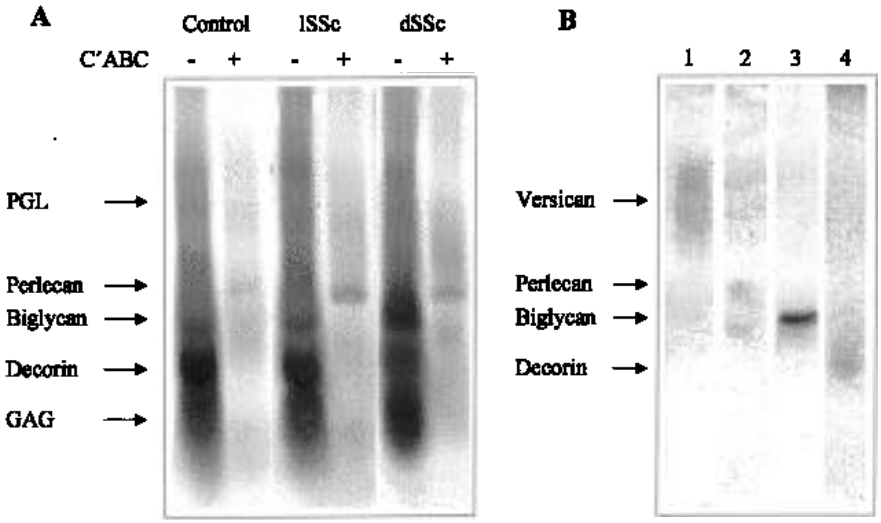

Fig. 2. (**A**) Fibroblast cultures from controls,and lSSc and dSSc patients were incubated with ^{35}S-sulphate and the different types of proteoglycans (versican,perlecan, biglycan and decorin) from the cell medium were separated by agarose gel electrophoresis. Radioactivity in each band was visualised and quantified. The material was subjected to electrophoresis before (-) and after (+) treatment with chondroitin ABC lyase (C'ABC). (**B**) The different types of secreted proteoglycans were identified by Western Blot. PGL includes both versican and perlecan before treatment with C'ABC, and only versican after treatment. Proteoglycans identified are:lane 1 = versican; lane 2 = perlecan,lane 3 = biglycan and lane 4 = decorin.

surement were positively correlated to the production of versican ($p = 0.014$, Rho = 0.86, Fig. 3A). Separate analysis of the results from dSSc and lSSc patients revealed that in dSSc changes in skin thickness were related to the production of versican ($p < 0.01$, Rho = 1.00, Fig. 3A), biglycan ($p < 0.01$, Rho = 1.00, Fig. 3B) and decorin ($p < 0.01$, Rho = 1.00), and changes in skin echogenicity were related to the production of biglycan ($p < 0.01$, Rho = 1.00, Fig. 3D) and decorin ($p < 0.01$, Rho = 1.00), but not to the production of versican (Fig. 3C). In lSSc patients there were positive correlations between changes in skin echogenicity and the production of both versican ($p < 0.01$, Rho = 1.00, Fig. 3C) and biglycan ($p < 0.01$, Rho = 1.00, Fig. 3D), but no correlation with the production of decorin; nor was there any relationship between changes in skin thickness and the production of versican (Fig. 3A), biglycan (Fig. 3B) or decorin.

To analyse further the influence of time on PG production, disease duration was correlated to the different PG. There was a negative correlation between fibroblast production of biglycan and disease duration ($p = 0.033$, and Rho = -0.79, Fig. 4).

To estimate the influence of acute-phase proteins on the development of fibrosis CRP, orosomucoid and ESR were analysed and correlated to PG synthesis and pulmonary function tests. Fibroblast synthesis of versican correlated with ESR ($p = 0.047$, Rho = 0.67), but not to CRP and orosomucoid. Carbon monoxide diffusing capacity was related to orosomucoid ($p < 0.01$, Rho = -0.81) and ESR ($p < 0.01$, Rho = -0.83), but not to CRP (data not shown).

The production of perlecan and free GAG chains did not correlate with the results of examination by ultrasound or acute-phase proteins (data not shown).

Discussion

This study confirms earlier reports of decreased skin echogenicity among dSSc patients with a short disease duration (22). The lower echogenicity could be caused by increased capillary leakage or increased amounts of hydrophilic extracellular matrix. The findings of the present study do not, however, indicate any significant increase in the production of versican, perlecan, biglycan or decorin as the cause of the decreased echogenicity. The combination of increased skin thickness and decreased skin echogenicity in early SSc is probably the result of increased levels of interstitial fluid caused by such early immunological events as endothelial cell perturbation (23), accompanied by an increase in growth factors such as platelet-derived growth factor (23), transforming growth factor- (TGF-) (23) and T-cell activation (24). Such a finding is concordant with the decreased production of free GAG chains in dSSc patients, since free GAG have antiproliferative activity (25) and their decreased production may lead to fibroblast proliferation. Higher echo-

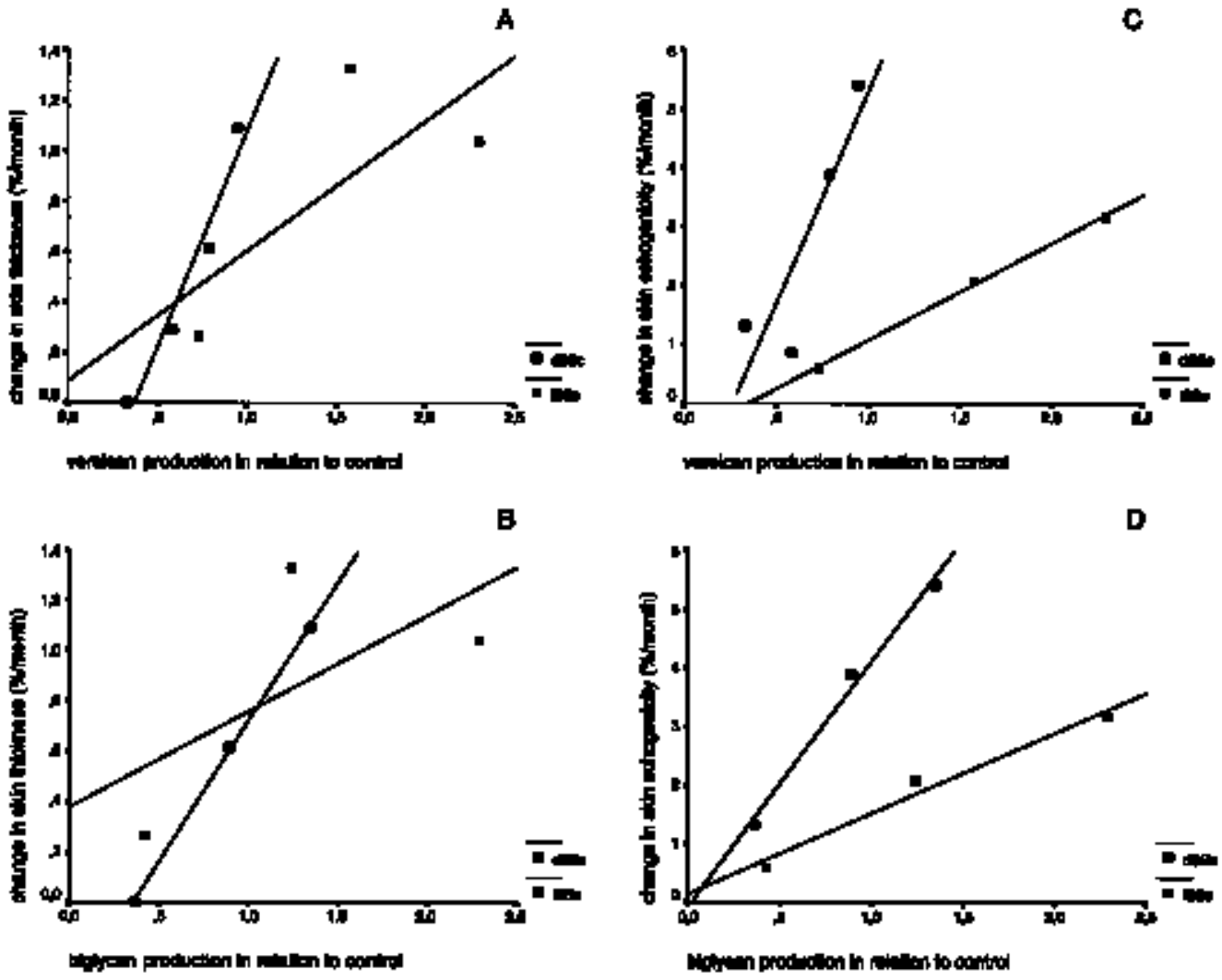

Fig. 3. Changes in forearm skin thickness and skin echogenicity were correlated with the fibroblast production of versican (**A, C**) and biglycan (**B, D**) among patients with lSSc and dSSc examined by high frequency (20MHz) ultrasound.

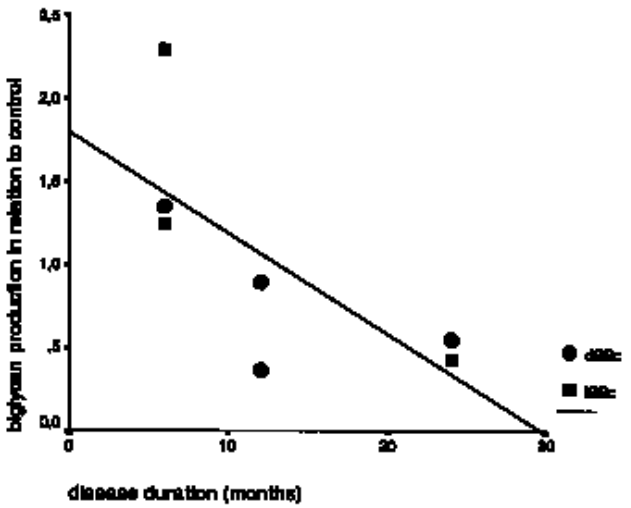

Fig. 4. Correlation between the fibroblast production of biglycan and disease duration among patients with lSSc and dSSc ($p = 0.033$, and $Rho = -0.79$).

genicity at the re-evaluation suggests that the increase in interstitial fluid is replaced by cells and matrix.

Unlike the results of earlier studies by Westergren-Thorsson *et al*. (3) and by Kuroda and Shinkai (8,9), an altered fibroblast production of versican, biglycan or decorin was not found. Differences in disease duration could account for some of the discrepancy, since the median disease duration among patients in the report by Westergren-Thorsson *et al.* was 24 months, i.e. twice as long as in this study, while Kuroda and Shinkai reported different expressions of decorin in patients with early, mid-stage or late disease.

A change in skin echogenicity was related to the fibroblast production of versican in lSSc patients. It has been

suggested that versican, which is highly expressed in fast growing tissues, plays a role in cell proliferation (26). This cell-matrix interaction may be mediated by the interaction of the chondroitin sulphate side chains of versican with the carbohydrate-binding domain of the adhesion molecules L- and P-selectin and CD44 (27). Later, synthesis of collagen type I takes place in a versican-rich provisional matrix, suggesting that versican may influence the progression of the repair process in inflammatory diseases (28). The explanation for the more rapidly increasing skin echogenicity among patients with higher versican production may therefore be found in increased collagen synthesis, to some extent caused by versican itself. The relationship between ESR and the production of versican from cultured fibroblasts also indicates that its synthesis may be influenced by inflammation.

Changes in skin echogenicity also correlated with biglycan production both in lSSc and dSSc patients. Biglycan is situated near cells. It binds growth factors such as TGF- , which is increased in the early stages of SSc (23), thus supporting matrix production by facilitating the delivery and activation of TGF- to its receptors (29). The early occurrence of biglycan is further supported by the negative correlation found between the production of biglycan and disease duration.

A possible role of biglycan in the pathogenesis of SSc has not earlier been reported. However, in the development of peri-bronchial fibrosis among patients with asthma, hyper-responsiveness to methacolin was significantly correlated to the production of biglycan (30,31). An early and transient increase of biglycan has also been reported in animal models, where lung fibrosis was induced by either bleomycin (32) or adenovirus containing cDNA for TGF- (33), and liver fibrosis by thioacetamide (34).

Biglycan production may be stimulated by TGF- (35) and biglycan itself may also affect the inflammatory event, as shown by the development of lung fibrosis after infection with adenovirus containing cDNA for biglycan (36). *In vitro*, biglycan can stimulate the migration of cells (37) and recruit neutrophils through the stimulation of nuclear factor-kappab (NF-kB) and the increased expression of intercellular adhesion molecule-1 (ICAM-1) by its side chains (38). In this study, the correlation of biglycan with changes in skin echogenicity indicates that patients with rapidly changing skin echogenicity are in an early phase of the disease, which is also supported also by the relationship between biglycan production and disease duration.

In the present study orosomucoid and ESR correlated with carbon monoxide diffusing capacity, and ESR also correlated with the fibroblast production of versican, suggesting its synthesis to be inflammation driven (20). These correlations are of clinical interest as both ESR (39) and the carbon monoxide diffusing capacity (39) are predictors of survival in SSc. Both versican and biglycan generate a more loose type of tissue into which inflammatory cells can infiltrate more easily, suggesting that increased synthesis of these PG may be of pathogenic importance. The pathogenic significance of versican may be that it precedes collagen synthesis (28), but it would assume even greater importance if it were also to stimulate collagen synthesis.

Finally, it is intriguing that the altered production of PGs such as versican and biglycan may have profound effects on the properties of the extracellular matrix, which in turn can affect several biological functions such as cell growth, migration, and the organisation of matrix fibres, as well as cytokine activity.

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