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Alterations in Scleroderma Fibroblast Surface Glycoproteins Associated with Increased Collagen Synthesis

VELI-MATTI KÄHÄRI,[1] JYRKI HEINO,[1] HANNU LARJAVA[2] and EERO VUORIO[1]

[1]*Department of Medical Chemistry and* [2]*Department of Periodontology, University of Turku, Turku, Finland*

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Fibroblasts were cultured from affected and unaffected skin sites of 6 patients with localized scleroderma. As a parameter of fibroblast activation, collagen synthesis and cellular proα1(I)collagen mRNA levels were measured. Cell surface glycoproteins were labelled with the periodate/borohydride method and fractionated electrophoretically. A distinct reduction in the relative amount of surface glycoproteins in the 120 kDa region was observed in two affected cell lines producing increased amounts of collagen and in one affected cell line with normal collagen production when compared to unaffected fibroblast lines. Other, non-systematic alterations in the surface glycoproteins of the affected cell lines were also detected. Compared to unaffected and healthy control fibroblasts no alterations in the surface protein profiles were seen in the other three affected cell lines. These cells did not show an increase in collagen production either. The results suggest that the activation of collagen synthesis found in scleroderma fibroblasts might be connected with alterations in the normal cell surface glycoprotein pattern. *Key words: Collagen; mRNA; Membrane glycoproteins.* (Received June 27, 1986.)

V.-M. Kähäri, Department of Medical Chemistry, University of Turku, Kiinamyllynkatu 10, SF-20520, Turku, Finland.

Scleroderma is a connective tissue disorder characterized by excessive deposition of collagen in skin (localized scleroderma) and also in various internal organs (systemic scleroderma). Fibroblasts cultured from progressive scleroderma lesions exhibit characteristically an increase in collagen synthesis (1–3) associated with elevated cellular levels of the corresponding mRNAs (4, 5).

It has been suggested that selection of a high-collagen-producing fibroblasts subpopulation could account for the excessive accumulation of collagen in scleroderma (6). Recent studies show that activated scleroderma fibroblasts gradually reduce their collagen production to normal values upon subculturing (7, 8). This could in part be explained by the selection theory, but equally well by a gradual reduction in any endogenous activation mechanism.

Cell surface glycoproteins possess important roles in many cellular functions e.g. cell adhesion, cell- recognition, cell matrix interactions and growth regulation (9). In a recent study, certain differences were observed in surface protein patterns between cultured systemic scleroderma fibroblasts and control fibroblasts (10). The purpose of this work was to extend these studies to localized scleroderma (morphea) and to investigate whether the surface glycoprotein pattern of cultured scleroderma fibroblasts is in connection with the activation of collagen synthesis by comparing the fibroblasts cultured from affected and unaffected skin of the same patient with each others and with skin fibroblasts from healthy controls.

Abbreviations: cDNA, complementary DNA; mRNA, messenger RNA; kDa, kiloDalton.

MATERIALS AND METHODS

Cell lines

Punch biopsies were taken from the edge of the scleroderma lesion (affected cells) and from symmetrically located uninvolved skin site (unaffected cells) of six patients with histologically confirmed localized scleroderma (morphea) as well as from four healthy volunteers (aged 25–73 years). Details about the patients are shown in Table I. Fibroblast cultures were started by the explantation method and maintained in Dulbecco's medium supplemented with 10% fetal bovine serum (both from Gibco-Biocult, Paisley, Scotland), streptomycin (50 μg/ml) and penicillin (100 IU/ml) as described earlier (11).

Measurement of collagen synthesis

Confluent fibroblast cultures in early passages (3rd or 4th) were used. Cells were labelled in 25 cm^2 cell culture flasks (Nunc, Roskilde, Denmark) for a period of 24 hours with 5 μCi/ml of L-[G-^{3}H]proline (NET-285, 5.0 Ci/mmol, New England Nuclear, Boston, MA) in glutamine-free cell culture medium supplemented with ascorbate and β-amino-propionitrile (50 μg/ml each). At the end of the labelling period the media were removed, the cell layers were detached with trypsin (25 μg/ml; Trypsin 1:250, Difco, Detroit, MI) and aliquots were counted in Bürker chamber. The amount of radioactive hydroxyproline in the labelling media collagen was measured using the method of Juva and Prockop (12). The rate of collagen synthesis for each cell line was calculated as the amount of radioactive hydroxyproline in labeling media per cell. Alteration in collagen synthesis was expressed as ratio of collagen synthesis by affected / unaffected fibroblasts of each patient.

Measurement of proα1(I)collagen mRNA levels

Cytoplasmic RNAs were isolated from the same fibroblasts used for ^{3}H-proline labelling by the method described previously (13). Serial dilutions of cytoplasmic RNAs were dotted onto nitrocellulose filters, baked and prehybridized as described earlier (14). Filters were hybridized with a nick-translated radioactive cDNA probe specific for human proα1(I)collagen mRNA (15), washed and exposed to Kodak X-Omat film with intensifying screens. Densitometric scans of the films were performed to quantitate the intensity of hybridization.

Analysis of cell surface proteins

Cell surface glycoproteins were labelled with the periodate/borohydride method (16). Briefly, confluent fibroblast cultures in 57 cm^2 cell culture dishes were scraped off and suspended in 1 ml of ice cold phosphate buffered saline (pH 7.4). In order to determine the trypsin-resistance of cell surface glycoproteins, duplicate cultures of some cell lines were also detached with trypsin treatment (Bovine Trypsin, Type XI, 25 μg/ml, Sigma Chemical Copany, St. Louis, MO) for 15 min at 37°C. The cells were then pretreated with 10 μl of 0.1 M $NaIO_4$ on ice for 10 min in the dark. After washing with phosphate buffered saline (pH 7.4), 5 mCi of $NaB[^3H]_4$ (TRK.45, 10 Ci/mmol, Radiochemical Centre, Amersham, U.K.) was added to a suspension containing the cells in 0.5 ml of phosphate buffered saline (pH 8.0). The cells were incubated at room temperature for 30 min and washed three times with phosphate buffered saline (pH 7.4). Labelled glycoproteins were solubilized in 0.2 ml of phosphate buffered saline (pH 7.4) containing 1% Triton X-100 on ice.

The radioactivity of each sample was measured by liquid scintillation counting. Equal amounts of the samples were fractionated by polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulphate, in homogenous 6% gels both without reduction and after reduction with 0.1 M mercaptoethanol (17). The gels were processed for fluorography (18) and exposed to Kodak X-Omat film.

RESULTS

Collagen synthesis and proα1(I)collagen mRNA levels of fibroblasts cultured from scleroderma lesions (affected cells) and from symmetrically located uninvolved skin (unaffected cells) were compared (Table II, Fig. 1). Two out of six affected scleroderma cell lines (KLU and SLU) showed distinctly increased collagen synthesis and mRNA levels, while the other four affected cell lines exhibited normal or reduced collagen synthesis and mRNA levels compared to unaffected cells (Table II). Alterations in collagen synthesis

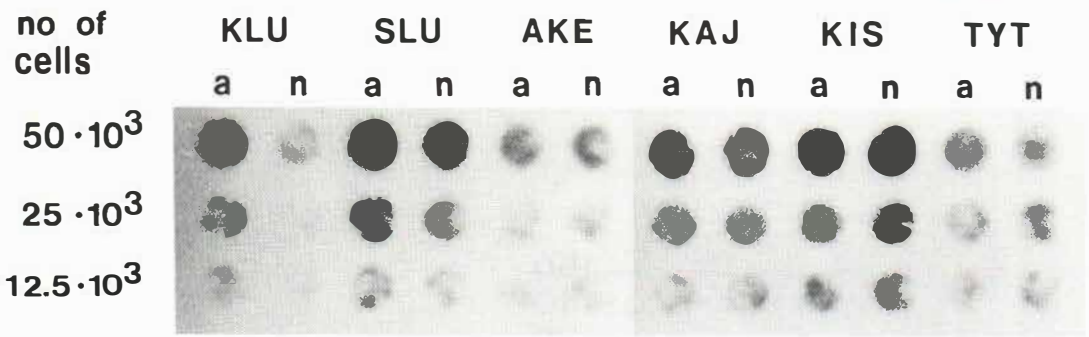

Fig. 1. Serial dilutions of cytoplasmic RNA samples from affected (a) and unaffected (n) fibroblasts of scleroderma patients were dotted onto nitrocellulose filter, baked, prehybridized and hybridized with a nick-translated cDNA probe fo human proα1(I)collagen mRNA. Filter was exposed to x-ray film and the intensity of hybridization was measured densitometrically.

correlate rather well with the data on the activity of the disease and the age of the lesion biopsied (Table I).

Cell surface glycoproteins of the fibroblasts were labelled with the periodate/borohydride procedure and fractionated electrophoretically. The main glycoprotein bands had the approximate M_rs of 140 000, 120 000, 85 000 and 55 000 (Figs. 2 and 3). Some degree of variation was detected in the relative amounts of several minor proteins between different cell lines.

Table I. *Clinical data of the scleroderma patients*

Patient	Sex	Age (years)	Duration and activity of the disease (years)	Age of the lesion (years)
KLU	F	12	10, active progression	1
SLU	F	5	0.5, active progression	0.5
AKE	F	29	20, slow progression	1
KAJ	F	6	0.75, slow progression	0.75
KIS	F	49	4, inactive	1
TYT	F	70	4.5, inactive	4

Table II. *Collagen synthesis and mRNA levels of scleroderma fibroblasts*

Rate of collagen synthesis was measured as radioactive hydroxyproline in labelling media protein per cell. Cytoplasmic dot hybridizations were performed to measure the proα1(I)collagen content per cell. The results are given as ratios of affected / unaffected of the values obtained

Cell line	Collagen synthesis	proα1(I)collagen mRNA
KLU	3.65	3.04
SLU	3.31	1.49
AKE	0.66	0.93
KAJ	0.55	1.09
KIS	1.19	1.01
TYT	0.62	1.04

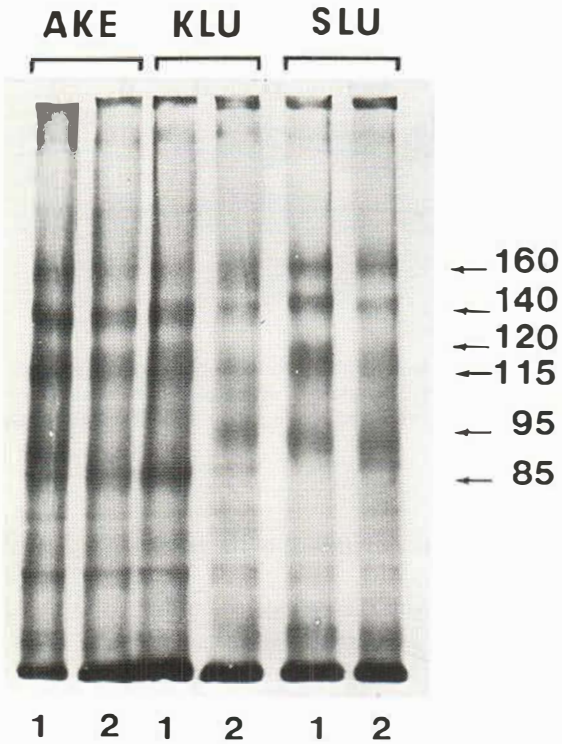

Fig. 2. SDS-polyacrylamide gel electrophoresis of periodate/borohydride labeled surface glycoproteins of six scleroderma cell lines. AKE, KLU and SLU are scleroderma patients. Cell lines marked with 1 were cultured from unaffected skin and cell lines marked with 2 from affected skin.

Major changes in the surface glycoprotein patterns were found in the two affected cell lines showing increased collagen synthesis and in one affected cell line with normal collagen synthesis when compared to unaffected cell lines (Figs. 2 and 3). All the other cell lines, including cells from the skin of healthy controls, exhibited a diffuse band in the 120 kDa region representing glycoproteins with approximate M_rs from 115000 to 120000. The affected cell lines with increased collagen synthesis showed a sharper band representing mainly the 115 kDa glycoproteins, and a decrease in the relative amount of glycoprotein with an approximate M_r of 120000. This was the only alteration common and specific to the activated cell lines. The overall incorporation of label into surface glycoproteins did not differ between affected and unaffected cell lines. Therefore the observed differences represent both relative and absolute changes in surface proteins.

The affected cells of patient KLU showed increased amount of label in the 95 kDa region and decreased amount of label in the 85 kDa region compared to unaffected cells, while the affected cells of patient SLU exhibited increased amount of radioactivity in the 90 kDa region (Fig. 2). These alterations were reproducible in several labelling experiments performed.

In order to test the trypsin resistance of cell surface glycoproteins some cells were treated with trypsin before the labelling procedure. This treatment degraded mainly glycoproteins with M_rs over 140000 and a prominent 55 kDa glycoprotein band, while other glycoproteins, including those in the 120 kDa region remained intact (Fig. 3).

Some changes in the cell surface glycoprotein profiles were seen after reduction with mercaptoethanol. The broad glycoprotein band in the 85–95 kDa region migrated to the 95–100 kDa region, but the mobilities of glycoproteins in the 120 kDa region remained unchanged (Fig. 3B).

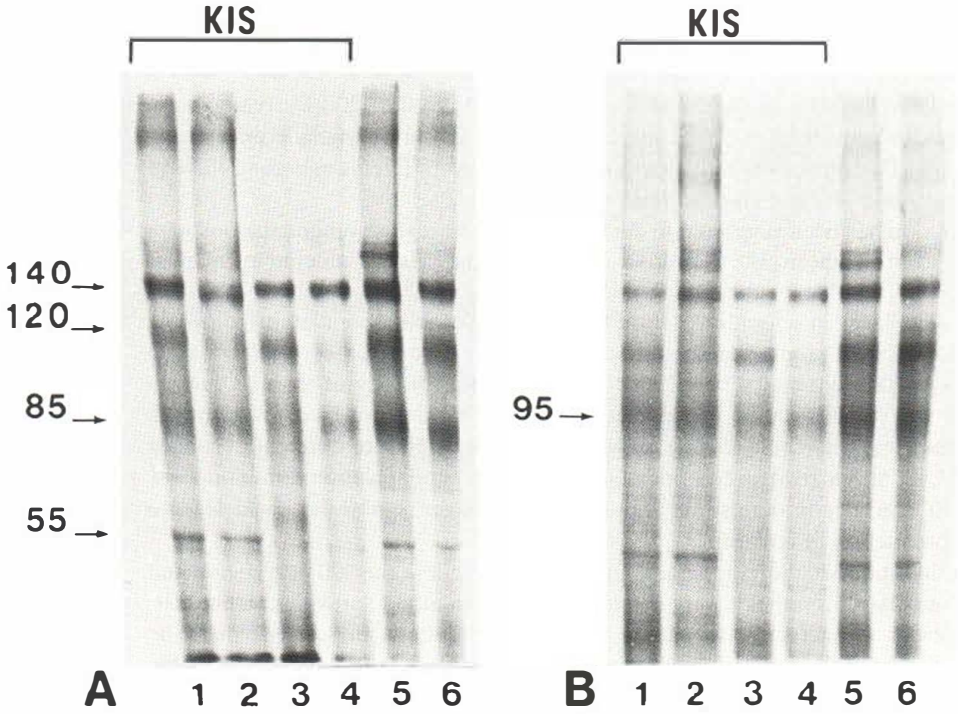

Fig. 3. SDS-polyacrylamide gel electrophoresis of periodate/borohydride labelled surface glycoproteins of fibroblasts fractionated without reduction (panel A) and after reduction with mercaptoethanol (panel B). The samples were from unaffected (lanes 1 and 3) and from affected skin (lanes 2 and 4) of the patient KIS. Samples of the lanes 5 and 6 are from two healthy controls. Cell lines 3 and 4 were treated with trypsin (25 μg/ml) for 15 min at 37°C before the labelling procedure.

DISCUSSION

It has been well established that skin fibroblasts cultured from actively progressing scleroderma lesions produce elevated amounts of collagen (1–3). Increased collagen synthesis has been shown to be due to elevated cellular levels of the corresponding mRNAs, suggesting increased transcription rates of type I collagen genes or increased half-lives of these mRNAs or a combination of the two (4, 5). The reasons for the activation of collagen synthesis are not known. Scleroderma cell lines with increased collagen synthesis gradually reduce their production of collagen to normal levels upon subculturing (7, 8). In this study two out of six scleroderma fibroblast lines showed distinct activation of collagen synthesis, while the other four were inactive. This supports the earlier findings, that great variability exists in collagen production of scleroderma fibroblasts depending on the activity of the disease (2, 7, 11).

For further characterization of scleroderma fibroblasts, we used the periodate/borohydride method, which labels the sialic acid residues of external membrane glycoproteins while leaving intracellular glycoproteins unlabelled (19). Membrane glycoprotein patterns of various cell types have been characterized by this method (16, 20, 21). In this study we compared the surface glycoprotein patterns of fibroblasts from active and inactive scleroderma lesions to those of fibroblasts from unaffected skin of the same patients and healthy controls. The main scleroderma-related change in our cell lines was reduction in the relative amount of glycoprotein in the 120 kDa region. A similar change has also been

reported in fibroblasts from patients with progressive systemic sclerosis by SundarRaj et al. (10). Their cell lines were not studied for collagen production. In our study the alteration in the 120 kDa region was most significant in the affected cell lines showing increased collagen synthesis (Figs. 2 and 3). Glycoproteins in the 120 kDa region were trypsin-resistant and non-reducible with mercaptoethanol. In polyacrylamide gel electrophoresis, the 120 kDa region obviously consists of several glycoproteins migrating close to each other. Functions of these glycoproteins are uncertain.

Even normal skin has been suggested to contain different fibroblast subpopulations with respect to collagen synthesis (2). Selective increase in the relative amount of a high-collagen-producing fibroblast subpopulation has been proposed to account for increased accumulation of collagen in scleroderma (6). Furthermore, fibroblast populations, differing with respect to collagen production and growth characteristics, have been isolated from human gingiva by flowcytometry based on the C1q-receptor density of the cell surface (23). Our observations of variation in the relative amounts of minor surface glycoproteins between different cell lines could also be explained by the existence of different fibroblast subpopulations. In the activated cell lines, selection of a subpopulation exhibiting low amounts of 120 kDa glycoproteins and high levels of collagen synthesis, could lead to increased net production of collagen.

The finding that the reduction in the amount of glycoprotein in the 120 kDa region and the activation of collagen synthesis were connected with each others, provides an interesting new hypothesis for defective regulation of collagen synthesis in scleroderma. The mechanisms involved in the regulation of collagen synthesis are poorly understood. Some investigators have suggested that N-terminal propeptides of type I and type III collagen selectively inhibit collagen synthesis at translational level (23, 24) and that scleroderma fibroblasts would be less sensitive to this regulation (25). Since the N-terminal propeptides are liberated extracellularly, they are likely to require a surface receptor to reenter the cell. Such a receptor could have been down-regulated in scleroderma fibroblasts due to increased collagen production, causing insensitivity to the possible feedback regulatory mechanisms. Alternatively, a primary decrese in the receptor density could explain the increase in collagen synthesis due to lack of feedback regulation. However, further experiments are needed to test the relevance of this hypothesis.

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