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AiO Print Ltd., Odense, Denmark

Acta Derm Venereol (Stockh) 1991; Suppl. 162: 3–12

Collagen Biosynthesis in Lichen Sclerosus et atrophicus Studied by Biochemical and In situ Hybridization Techniques

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Collagen was studied by biochemical and immunohistochemical techniques and by in situ hybridization in four patients with lichen sclerosus et atrophicus (LSA). The solubility of collagen in acetic acid and pepsin was increased in the lesional skin of the LSA patients when compared with their non-affected skin or samples from control subjects, indicating that a marked proportion of the collagen was newly synthesized. Collagen synthesis was slightly increased in fibroblasts derived from the lesional skin of the LSA patients. In situ hybridization with human sequence-specific cDNAs to type I procollagen demonstrated active fibroblasts in lesional skin of LSA patients, further confirming a high level of collagen synthesis in LSA. Remarkably, active fibroblasts were not associated with inflammatory cell infiltrates noted in LSA skin nor did they express transforming growth factor β($TGF\beta_1$). Our results indicate that despite the degeneration of connective tissue, there is an active regeneration process in LSA with significant collagen synthesis.

Acta Derm Venereol (Stockh) 1991; suppl. 162.

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Collagen alterations are frequently found in certain degenerative and granulomatous skin diseases. Homogenization of collagen can be seen in lichen sclerosus et atrophicus (LSA), in which inflammatory cells occur close to areas where marked collagen changes are seen (1–3). LSA has been well characterized ultrastructurally, but the biochemical changes and synthesis of collagen, the most abundant connective tissue protein in the dermis (4), has not been thoroughly studied.

PATIENTS AND METHODS

Patients and skin biopsies, immunohistochemistry and cell culture

Biopsy samples were taken under local anaesthesia from the affected and non-affected (site-matched) skin of patients. Clinical characterization of the patients are given in Table I.

Control samples were taken during therapeutic operations on age-matched patients at the Dermatology Clinic, Oulu University Central Hospital, Finland. All the samples were taken in accordance with the Declaration of Helsinki.

Samples were excised from the lesional skin of the patients for light microscopy and immunohistochemical studies. The light microscopy samples were fixed in 10% phosphate-buffered formalin, processed routinely and embedded in paraffin. Sections were cut at 5 μm and stained with H & E and Verhoeff van Gieson stains. The sections for immunohistochemistry were deparaffinized and treated with 0.4% pepsin (Sigma Chemical Co, St. Louis, MO) to enhance the availability of the antigens (5). Endogenous peroxidases were inactivated by exposing the sections to

Table I. *Clinical data on LSA patients.*

Code	Sex	Age (years)	Clinical findings	Site of biopsy
LSA 1	F	69	Lesions on the lower extremities and abdomen for two years	Abdomen
LSA 2	F	62	Lesions on the trunk and lower extremities for six years Concurrent rheumatoid arthritis	Abdomen
LSA 3	F	56	Lesions on the trunk for 20 years	Abdomen
LSA 4	F	77	Lesions on the trunk for 10 years	Abdomen

Abbreviations: LSA; Lichen sclerosus et atrophicus.

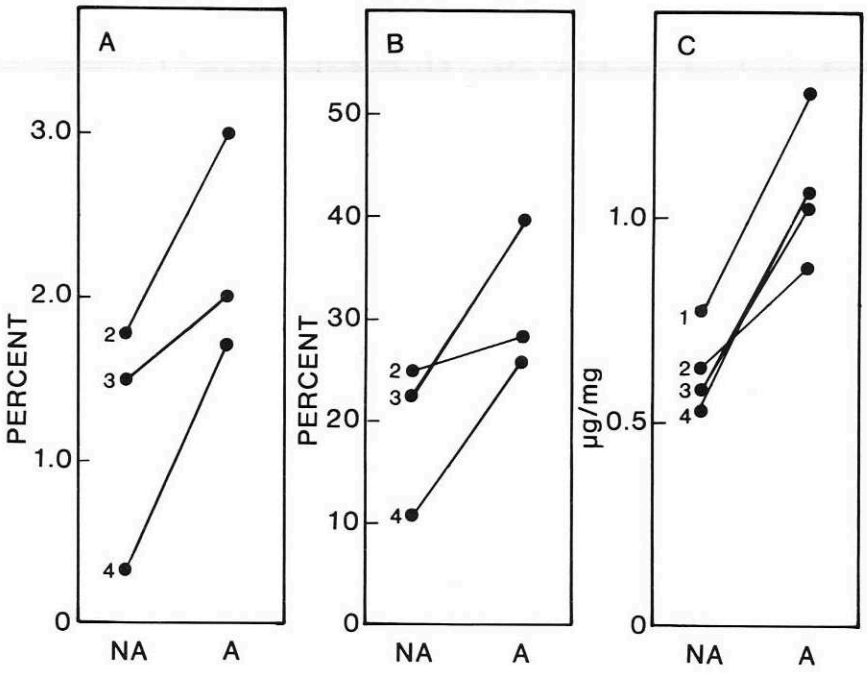


Fig. 1. The solubility of collagen and DNA concentration in affected (A) and non-affected (NA) skin from LSA patients. Skin biopsy specimens were sequentially extracted with 0.5 M acetic acid (acid soluble) and pepsin as described, hydroxyproline then being assayed in the acid-soluble, pepsin-soluble and insoluble fraction. The ratio of the amount of hydroxyproline in each fraction to the total amount of hydroxyproline was calculated and expressed as a percentage. In fig A percent of acid-soluble collagen; in fig B percent of pepsin-soluble collagen and in fig C DNA concentration µg/mg wet weight are shown.

0.1% hydrogen peroxide, and peroxidase-antiperoxidase staining for type III procollagen and fibronectin was performed according to the method of Sternberger (6). The antibody to pro αIII collagen was kindly donated by Drs. J. and L. Risteli, Oulu. The purification of the antigen and preparation of the antibody has been described earlier (7). The anti-fibronectin was obtained from Dako A/S Copenhagen. Normal rabbit serum and phosphatebuffered saline were used in place of the primary antibody for the control stainings.

Primary cell cultures were established by routine methods and subcultivated on plastic culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 50 µg/ml of ascorbate, 290 µg/ml L-glutamate, penicillin (100 U/ml) and streptomycin (100 µg/ml). Analyses of the fibroblast cultures were carried out at 4–8 passages of subcultivation.

Collagen biosynthesis

Fibroblasts at early confluency were labelled with (^{14}C)proline and total non-dialyzable radioactivity and (^{14}C)hydroxyproline were assayed in the medium after 24 h incubation as described (8). The cell layer was used for the assay of total cell protein (9) and DNA (10). Part of the culture medium after (^{14}C)proline labelling was used for the assay of the distinct collagen types. The medium fractions were digested with pepsin, and collagenous proteins were precipitated with NaCl, dialyzed and subjected to SDS-gel electrophoresis (11) performed using 8% polyacrylamide gels with and without delayed reduction with 2-mercaptoethanol (12). The collagen polypeptides were visualized by fluorography (13) and the bands representing α1(III), α1(I) and α2(I) were quantified densitometrically.

Assay of prolyl hydroxylase and collagenase activities

Cells at early confluency were trypsinized, homogenized and the supernatants used for the prolyl hydroxylase (PH) activity assay as described (14). Prolyl hydroxylase activity was also assayed directly from skin samples after homogenization and centrifugation. For the assay of collagenase activity, fibroblasts were cultured in serum-free DMEM for 6 h and the medium collected. The medium fractions were then treated briefly with trypsin and used for collagenase assays with type I collagen as the substrate (15).

Analyses of total collagen, acid-soluble and pepsin-soluble collagen, and collagen types from skin biopsies

The amount of hydroxyproline, a measure of the collagen present, was determined by a specific colorimetric assay (16). The relative amounts of acid and pepsin-soluble collagen in skin specimens were assayed as described (17). Types I and III collagen were assayed by precipitating pepsin-soluble material with 4.4 M NaCl and subjecting the precipitates to 8% SDS-gel electrophoresis with and without delayed reduction with 2-mercaptoethanol. The collagen polypeptides were visualized by staining with Coomassie Brilliant Blue and quantified densitometrically.

RNA extraction and Northern blotting

Total RNA was extracted from skin specimens as described (18). Aliquots of total RNA were fractionated on 1% agarose/formaldehyde gels, blotted onto GeneScreen Plus transfer membranes, and hybridized under stringent conditions as suggested by the manufacturer of the membrane (New England Nuclear). After hybridization, the filters were washed and the bound probe was detected by autoradiography at −70°C using X-ray films and intensifying screens.

In situ hybridization

Details of the technique used have been published earlier (19). Sections from the same paraffin blocks as were used for staining and immunohistochemistry were used for in situ hybridization. For in situ hybridization the sections were pretreated with proteinase K and HCl, and acetylated. The hybridizations were performed at 42°C for 24 h using ^{35}S-deoxy (thio)ATP-labelled probes, followed by washing, autoradiography for 5–11 days, and staining of the sections with hematoxylin.

Hybridization probes. The following cDNA probes were used: pHCAL1 for human pro α1(I) collagen mRNA (20, 21), pHFS3 for human pro α1(III) collagen mRNA (19) and pBC1 for human TGF-β1 mRNA (22). For in situ hybridization, specific restriction fragments of clones pHCAL1, pHFS3 and pBC1 were isolated as described previously (19, 23) and random-primed using (^{35}S)deoxy (thio)ATP. As a negative control, Bg1I-derived fragments (sizes 100–790 bp) of bacteriophage DNA were labelled similarily. For Northern hybridiza-

Table II. *Total incorporation of [^{14}C]proline, synthesis of [^{14}C]hydroxyproline, activities of prolyl hydroxylase (PH), and collagenase in fibroblast cultures.*

Cell line (code)	Total incorporation[a] (dpm×10^{-6}/μg DNA)		[^{14}C]hydroxyproline[a] (dpm×10^{-6}/μg DNA)		Type III[b] collagen		PH[c] (dpm/μg protein		Collagenase[d] activity	
	Affected	Non-affected	Affected	Non-affected	Affected	Non-affected	Affected	Non-affected	Affected	Non-affected
LSA1	6.32	7.48	1.58	1.13	0.244	0.175	243	239	1.5	2.1
LSA2	6.71	5.73	1.00	1.26	0.111	0.198	nd	245	nd	20.7
LSA3	6.74	3.53	1.25	0.47	0.099	0.239	117	204	3.9	15.3
LSA4	10.82	3.41	2.10	0.75	0.190	0.259	147	160	nd	nd
Mean±S.D.	7.65±2.12	5.04±1.95	1.48±0.48	0.90±0.36	0.161±0.068	0.217±0.037	196±66	212±39	2.3±1.4	14.5±8.6
Controls	mean±SD	4.68±1.07	1.01±0.28		0.215±0.071		192±45		14.8±6.6	

[a] Fibroblast cultures from patients and normal human subjects were labelled with [^{14}C]proline and total radioactivity and [^{14}C]hydroxyproline were then assayed from the culture medium as described.

[b] The relative synthesis of type III collagen was determined from ^{14}C proline labelled medium collagen after pepsin treatment and 8% SDS-gel electrophoresis using delayed reduction. α1(I), α2(I) and α1(III) were quantified densitometrically. The values are the ratio α1(III)/α1(I) + α2(I) + α1(III).

[c] Fibroblasts at early confluency were harvested and used for the assay of PH as described.

[d] Medium fractions were used for the assay of collagenase activity after brief trypsin activation using type I collagen as the substrate. The results are expressed as degradation of [^{3}H]proline labelled type I collagen, dpm×10^{-2}/h/μg DNA.

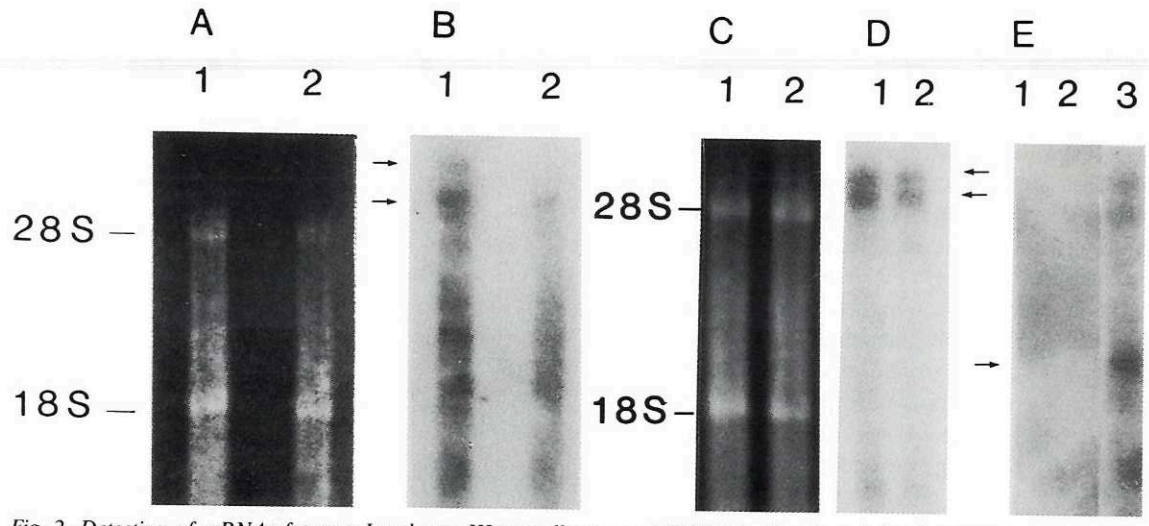


Fig. 2. Detection of mRNAs for type I and type III procollagens and TGF-B by Northern hybridization. Total RNA was extracted from skin samples. Samples of 14 μg of total RNA were electrophoresed under denaturing conditions and transferred to Gene Screen Plus filters. The filters were prehybridized and hybridized with the following nick-translated ^{32}P–CTP-labelled probes! pHCAL1 for human pro α1(I) collagen mRNA (specific activity appr. 2×10^9 cpm/μg; exposure time 4 days) (panel B), pHFS3 for human pro α1(III) collagen mRNA (specific activity 2×10^9 cpm/μg; exposure time 15 days) (panel D), and pBC1 for human TGF-β mRNA (specific activity 2×10^9 cpm/μg; exposure time 28 days) (panel F). Panels A and C show ethidium bromide stained ribosomal RNAs, the28S rRNA and the 18S rRNA. Messenger RNA for pro α1(I) collagen is detected as two bands (6.5 and 5.3 kb), for pro α1(III) as two bands (6.0 and 5.4 kb) and for TGFβ as one band (2.5 kb band). Note the strong hybridization with the TGF-β cDNA probe in the fetal skin sample (panel E) lane 3. In panels A-D, the sample in lane 1 is from unaffected skin, and sample in lane 2 is from lesional skin. In panel E, the sample in lane 1 is from unaffected skin, in lane 2 from affected skin of LSA patients and in lane 3 from fetal skin.

tions, the isolated insert fragments were labelled by the randompriming method (Amersham) using (^{32}P) dCTP.

Assay of DNA synthesis, proliferation and attachment of fibroblasts

DNA synthesis activity was estimated by determining the incorporation of (^{3}H)thymidine into the trichloroacetic acid-precipitable material (24). To study the attachment and proliferation of fibroblasts, equal numbers of fibroblasts were inoculated into cell culture wells and cell number and the amount of DNA measured after 24 h and 6 days.

Other assays

Fibroblasts at early confluency were labelled with (^{35}S)methionine in serum-free DMEM for 16 h and medium and cell fractions collected. Proteins were precipitated with $(NH_4)_2SO_4$ (320 mg/ml). The precipitated material was then analyzed by 6% SDS-gel electrophoresis after reduction with mercaptoethanol, using a (^{14}C)methylated protein mixture as the standard.

Student's t-test was used for the statistical analyses.

RESULTS

Histological and immunohistochemical studies

The biopsy samples showed the histological findings of LSA. The anti-pro III collagen antibody reacted with a narrow zone beneath the epidermis and around cutaneous appendages but only a weak overall staining or no reaction at all was present throughout the dermis. Fibronectin appeared in substantial amounts in basement membrane areas and was moderately distributed in the other parts of the LSA dermis. The degenerated areas did not demonstrate any change in the staining intensity when compared with non-affected skin (not shown).

Biochemical observations on the skin biopsies

Collagen concentrations, the solubility of collagen in acetic acid and pepsin-digestable fractions, collagen types and prolyl hydroxylase were measured in lesional and non-affected skin samples from the patients. Collagen concentration, measured in terms of

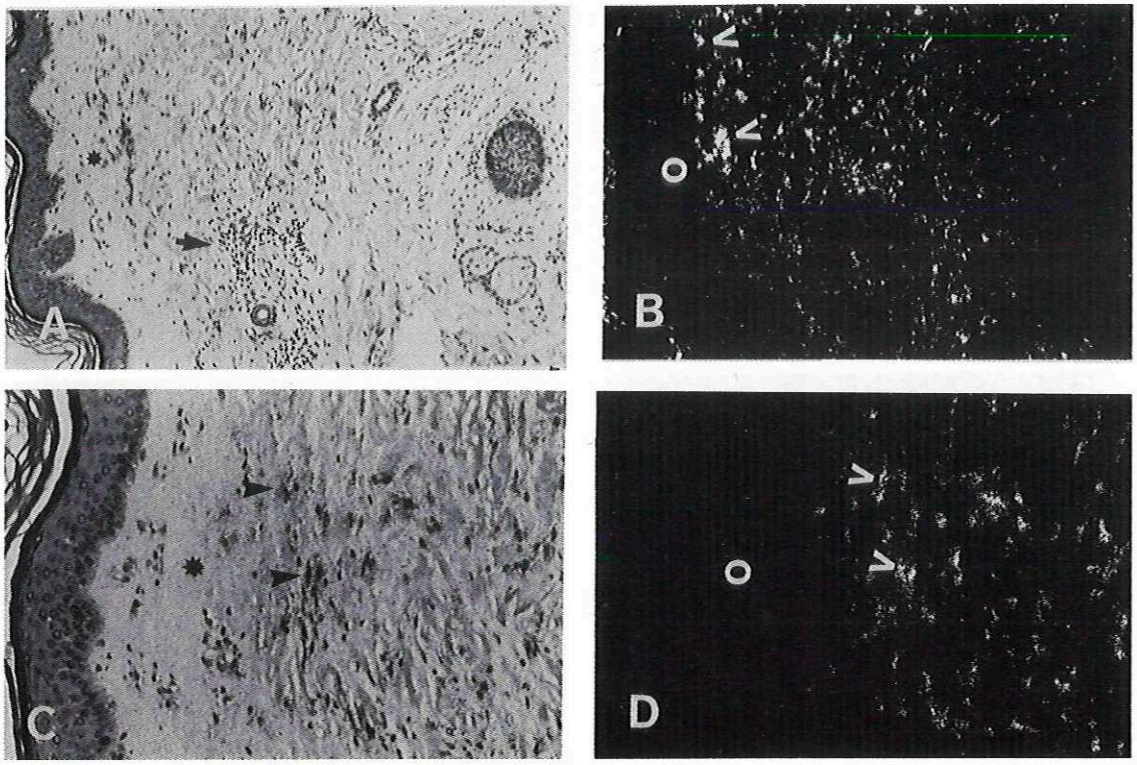


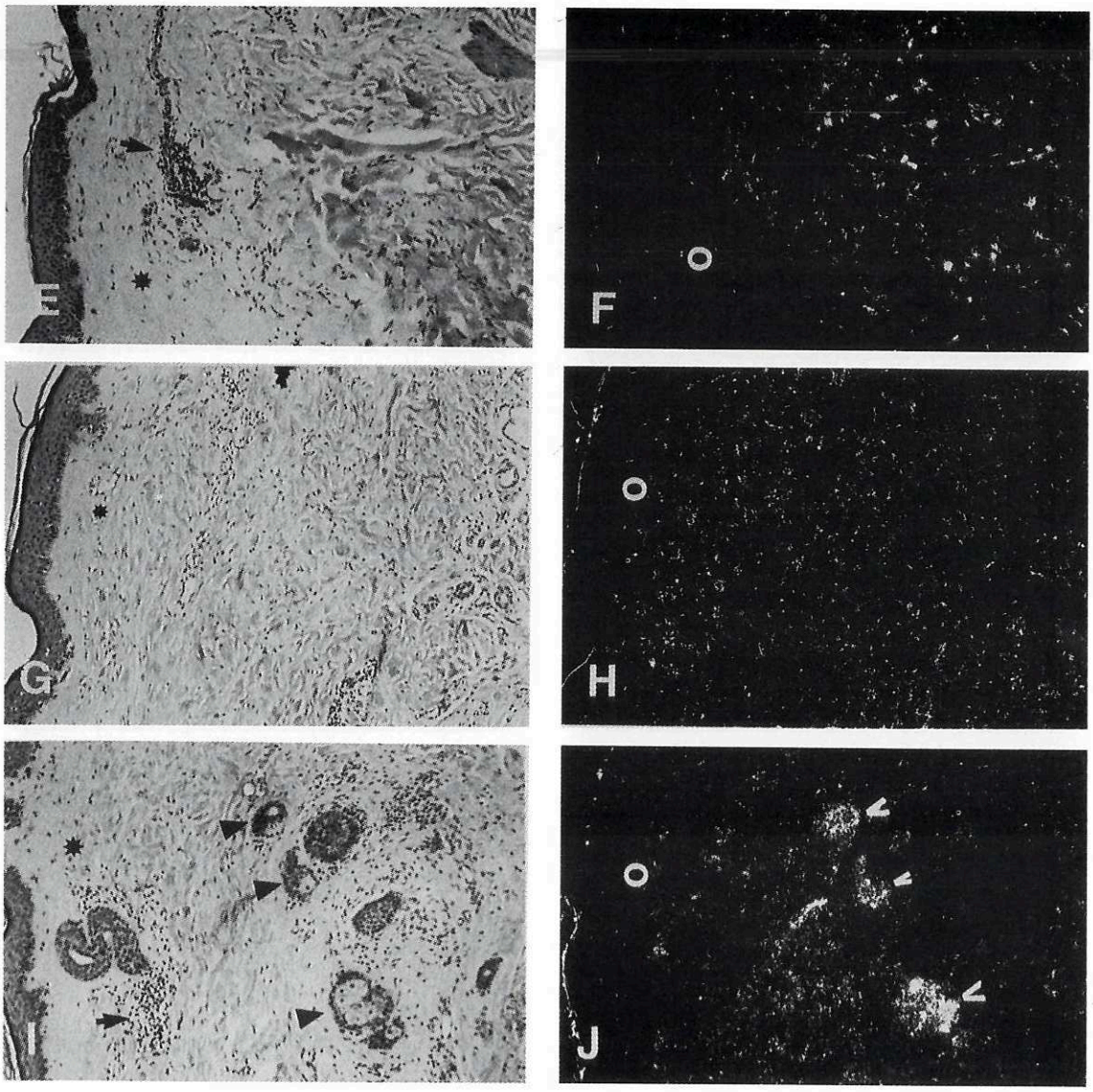
Fig. 3. In situ hybridization of LSA skin samples with type I procollagen cDNA (A–F), type III procollagen cDNA (G–H) and with TGFB cDNA (I–J).
The specific activity of the pro α(I) collagen cDNA probe was 1×10^9 cpm/μg, the specific activity of TGFβ$_1$ cDNA probe was 9×10^8 cpm/μg.
Figs A, C, E, G and I show the bright field of samples and figsB, D, F, H and J the dark field of the corresponding samples. In figs A, B, C, D, G, H, I and J, the sample was obtained from lesional skin of case LSA 1, and in figs E and F from lesional skin of case LSA2.
The length of exposure was 11 days in figs A–F and 5 days in figs G–J.
The arrow heads indicate the specific grains in fibroblasts. Note the different distribution of grains for pro α1(I) collagen mRNA in cases LSA1 and LSA2 (Figs A, B, E and F respectively). Pro α1(I) mRNA was not seen in the degenerated zone (in bright field figures, 0 in dark field figures). Pro α1(I) mRNA was not found near inflammatory cells (marked by arrows in figs A and E).*
Messénger RNA of TGFβ was found primarily in adnexae (arrow-head in figs I and J) and not in the degenerated zone or near inflammatory cells (arrow).
Magnification×100 (figs A, B, E–J), ×250 (figs. C, D).

hydroxyproline, was 23.6±4.8 μg/mg wet weight in affected skin and 22.1±6.2 in non-affected skin of patients with LSA. The solubility of collagen in acetic acid ($p < 0.063$) and pepsin-digestable fractions ($p < 0.119$) were slightly increased in the biopsies derived from the lesional skin of the LSA patients when compared with the non-affected skin, and the concentration of DNA, measuring cellularity, was also increased in the lesional skin ($p < 0.008$) (Fig. 1).

Type III collagen accounted for 30% of the total collagen and the ratio α1(I): α2(I) of type I collagen was 2.2 in the lesional skin of LSA patients. Prolyl hydroxylase activity was similar in the affected (7.1±1.1 DPM/μg protein) and non-affected skin (7.6±2.2) of the LSA patients.

Cell cultures

DNA synthesis rate measured by thymidine incorporation was slightly increased in fibroblasts derived from lesional skin. However, when the proliferation rate was followed for 6 days, the number of cells was less in cultures obtained from lesional skin (statistically not significant) (not shown). The attachment of cells from affected skin seemed to be also slightly decreased when compared to non-affected or control cells (not shown).



The total incorporation of (^{14}C)proline into the non-dialyzable fraction was slightly increased in the fibroblasts derived from the lesional skin of the LSA patients ($p < 0.249$) and the synthesis of (^{14}C)hydroxyproline, reflecting collagen synthesis, was similarly slightly increased ($p < 0.183$) (Table II). The ratio of (^{14}C)hydroxyproline to total radioactivity was unchanged, however, indicating that the relative synthesis of collagen was not altered (Table II). The activity of prolyl hydroxylase was similar in the fibroblasts from the lesional and the unaffected skin, and in the controls.

The relative synthesis of type III collagen, measured from the cell culture medium after limited pepsin digestion, had decreased slightly in two cell lines, LSA 2 and 3, when compared with the cell lines derived from the unaffected skin (Table II). The ratio of $\alpha 1(I)$: $\alpha 2(I)$ synthesized was 1.67 ± 0.13 in affected fibroblasts and the corresponding value in nonaffected cells was 1.68 ± 0.08, indicating that, qualitatively, the gene expression of type I collagen was similar in the affected and non-affected skin of LSA patients.

Collagenase activity, reflecting the degradation of

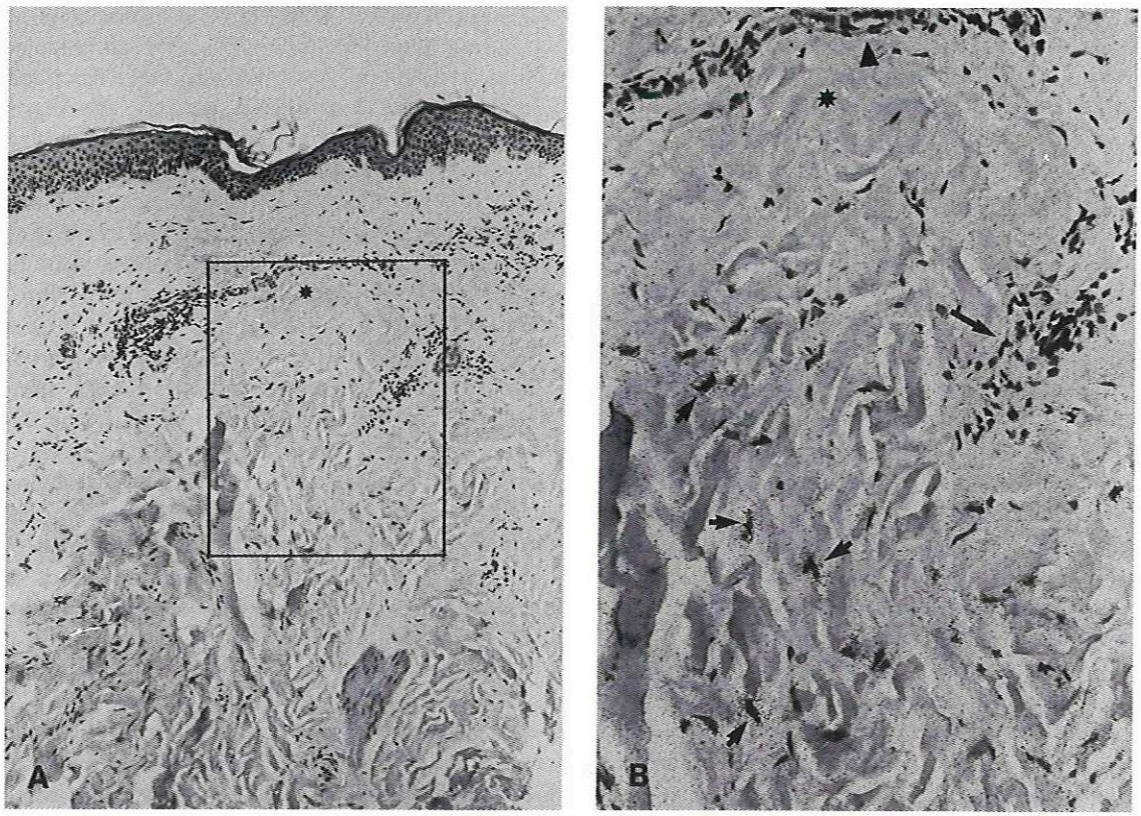


Fig. 4. In situ hybridization of a LSA skin sample with type I procollagen cDNA probe (case LSA2) demonstrating active fibroblasts in the mid and lower dermis. Note that active fibroblasts are not within the degenerated area (asterisk) or near inflammatory cells (long arrow and arrowhead in fig. B). There is a marked heterogeneity of activity of fibroblasts. Some fibroblasts contain numerous positive grains (short arrows), while in others, no signals are observed. Magnification×200 (A) and ×500 (B).

collagen was studied in the cell culture medium after brief trypsin treatment, and was found to be lower in two cell lines derived from lesional skin, LSA 3 and LSA 4, than in the corresponding cell cultures from the unaffected skin of the LSA patients ($p < 0.025$) (Table II).

No marked differences could be observed between affected and non-affected fibroblasts after labelling with (^{35}S)methionine and analysis of medium and cell proteins by 6% SDS-gel electrophoresis after reduction.

Demonstration of α1(I), α1(III) collagen and TGFB mRNAs in LSA skin samples by Northern blotting and in situ hybridization

Northern blotting. LSA skin samples were used for isolation of total RNA as described, and aliquots of 14 μg of total RNA were analyzed by agarose gel, transferred to filters and hybridized with cDNA probes for pro α1(I) and pro α1(III) collagens and $TGFB_1$ (Fig. 2).

The hybridization signal with pro α1(I) and pro α1(III)cDNA probes could be observed in LSA samples. The mRNA of pro α1(I) collagen migrated as two bands (6.5 and 5.3 kb) and for pro α1(III) collagen also as two bands (6.0 and 5.4 kb) (Figs. 2B, 2D respectively). In contrast, the mRNA of TGFβ yielded only a very faint signal in adult skin, in affected or non-affected samples. However, in fetal skin TGFB could be clearly demonstrated by Northern blotting (Fig. 2E, lane 3).

In situ hybridization: LSA samples were used for in situ hybridization as described using cDNAs for pro α1(I), and pro α1(III) collagens and TGFB (Figs. 3–4). Hybridization studies using pro α1(I) collagen cDNA revealed active fibroblasts in LSA skin samples. Active fibroblasts were observed in the upper

and mid dermis in case LSAl (Fig. 3A–D), or in the lower dermis in case LSA 2 (Fig. 3E, F, Fig. 4A, B). In situ hybridization of normaI skin samples studied under identical conditions did not demonstrate as active fibroblasts as in LSA lesions.

The degenerated zone was devoid of active fibroblasts. In case LSA 1, however, active fibroblasts were seen in close proximity to the lower border of the degenrated zone (Fig. 3C, D). Interestingly, active fibroblasts were not associated with inflammatory cells present in LSA skin samples (see Fig. 3A, B, E, F). In case LSA 2, active fibroblasts were observed in regions of thick collagen bundles, where the histological picture resembled sclerodermatous skin (Fig. 4). It should be noted that fibroblasts demonstrated marked heterogeneity: some contained high amounts of specific grains demonstrating active collagen synthesis, while others were devoid of grains (Fig. 4).

The hybridization with pro α1(III) cDNA did not reveal any marked changes in affected skin (Fig 3G, H). $TGF\beta_1$, which is a connective tissue synthesis activating factor, was not associated with active fibroblasts in LSA skin. In contrast, positive signals were observed in adnexae, i.e. in sebaceous glands and sweat ducts (Fig. 3I, J).

DISCUSSION

The results indicate that collagen metabolism is altered in LSA. The solubility of collagen in acetic acid and pepsin was increased in the lesional skin of LSA patients, and since newly synthesized collagen is more soluble (4), this may indicate that a marked proportion of the collagen observed in vivo is newly synthesized in the lesional skin of the patients. Histological reports mention that thin, newly synthesized collagen fibrils are visible in lichen sclerosus et atrophicus (25).

Cell cultures of fibroblasts derived from the lesional skin of the LSA patients showed marked heterogeneity, collagen synthesis being increased in vitro in three out of the four cell lines. Thus it is possible that there may be areas affected by LSA in which collagen synthesis is increased in vivo. This was confirmed using in situ hybridization technique, where it is possible to visualize active fibroblasts synthesizing collagen. The usefulness of in situ hybridization for the localization of metabolically active connective tissue cells is supported by a number of studies, in vivo and in vitro, which show that collagen production is regulated predominantly at the transcriptional level (26–28), although modulation at translational level has also been described (29). In lesional skin of LSA patients, fibroblasts containing high amounts of messenger RNA of type I procollagen could be found. Especially interesting was that the fibroblasts showing the highest amounts of type I procollagen mRNA were not found near inflammatory cells present in the lesional skin. In order to study the mechanism of activation of fibroblasts in LSA, the messenger RNA of TGFβ, was studied utilizing in situ hybridization techniques. TGFβ, which is chemotactic to various cells, stimulates collagen and fibronectin production, and decreases proteolysis (30–34). It has been suggested that TGFβ is one of the major factors stimulating connective tissue formation in wound healing and fibrotic processes (35, 36). In the lesional skin of LSA patients, TGFβ was not found in regions of fibroblasts actively synthesizing collagen. In contrast, mRNA of TGFβ was very abundant in adnexae of the skin such as sweat ducts, and sebaceous glands. This is in agreement with a recent study, in which TGFβ transcripts could be seen in skin adnexae (37). However, TGFβ in that study was also found in infiltrating cells in scleroderma and in other inflammatory skin disorders (37). Our results indicate that TGFβ is not associated to inflammatory cells of LSA, and suggest that other mediators may be more important in activating fibroblasts in LSA skin. Another possibility could be that in LSA skin, TGFβ may have initiated activation of fibroblasts in early stages of the disease process, and permanently altered the collagen synthesis in LSA fibroblasts. When skin biopsies are taken, the fibrotic process is ongoing, but expression of TGFB is no longer detectable as suggested also in some cases of progressive systemic scleroderma patients (38).

It appears that the disease process of LSA is not only the degeneration of connective tissue, but also an increased synthesis of collagen similar to scleroderma (see 39). This suggests that LSA and scleroderma are possibly related (2, 40). The cause of tissue homogenization noted in LSA skin, however, remains to be elucidated.

ACKNOWLEDGEMENTS

The authors thank Mrs Eeva Lehtimäki, Ms Helmi Konola and Mrs Marja Tolppanen for their expert technical assistance and Ms Anna Autio for her excellent secretarial work. The helpful comments made by Professor Matti Hannuksela are also gratefully appreciated.

This work was partially supported by a grant from the Medical Research Council of the Academy of Finland.

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