Acta Derm Venereol (Stockh) 1989; 69: 277–283

Biochemical and Immunohistochemical Comparison of Collagen in Granuloma Annulare and Skin Sarcoidosis

A. OIKARINEN,[1] T. KINNUNEN[1] and M. KALLIOINEN[2]

[1]*Department of Dermatology and Collagen Research Unit, and* [2]*Department of Pathology, University of Oulu, Oulu, Finland*

Collagen was studied by biochemical and immunohistochemical means in 5 patients with granuloma annulare (GA) and 3 with cutaneous sarcoidosis (SA). The solubility of collagen from the lesional skin in acetic acid was higher than that of collagen from unaffected skin from both patients and control subjects. Collagen concentration in the skin lesions, measured in terms of hydroxyproline content, was reduced in 3 patients with granuloma annulare and one with sarcoidosis, but the ratio of type III/I collagen was unchanged vis-à-vis non-affected skin. The collagen concentration in non-affected skin of both GA and SA-patients was also lower than in controls. The most typical immunohistochemical finding was the association of type III procollagen and fibronectin with granulomas in the lesional skin of both GA and SA cases. The activity of prolyl hydroxylase, a key enzyme in collagen biosynthesis, was markedly increased in the lesional skin, indicating that collagen synthesis in vivo was also increased. Surprisingly, collagen synthesis was not increased in cell culture studies. This could be due to cell selection as observed previously in scleroderma. Another possibility could be that various mediators released in vivo from inflammatory cells activate fibroblasts. However, when cells are subcultivated, this effect is not maintained. In conclusion, marked changes in collagen could be observed in granuloma annulare and skin sarcoidosis, reflecting increased turnover of collagen in vivo.

(Accepted January 31, 1989)

Acta Derm Venereol (Stockh) 1989; 69: 277–283.

A. Oikarinen, Department of Dermatology, University of Oulu, SF-90220 Oulu 22, Finland.

Collagen alterations are frequently found in certain granulomatous skin diseases. Focal degeneration of collagen and inflammation and fibrosis can be found in granuloma annulare (GA), with inflammatory cells surrounding the areas of collagen degeneration (1). GA has been well characterized ultrastructurally, but the biochemical changes in collagen, the most abundant protein in the connective tissue of the dermis (2), have not been studied particularly well. For these reasons, biochemical and immunohistochemical changes in collagen were studied in skin and fibroblast cultures from lesional and unaffected sites in cases of GA and the results compared with those found in another granulomatous disease, cutaneous sarcoidosis (SA).

PATIENTS AND METHODS

Patients and skin biopsies

Biopsy samples were taken under local anaesthesia from affected and non-affected (site-matched) skin of 8 patients. Clinical data on the patients are shown in Table I.

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

Samples for light microscopy and immunohistochemical studies were excised from the patients' lesional skin. Those for light microscopy were fixed in 4% phosphate-buffered formaldehyde, processed routinely and embedded in paraffin wax. 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 (Chemical Co, St. Louis, Mo) to enhance the availability of the antigens (3). Endogenous peroxidases were inactivated by exposing the sections to 0.1% hydrogen peroxide, and peroxidase–antiperoxidase staining for type III procollagen and fibronectin was performed *ad modum* Sternberger (4). The antibody to pro-III collagen was kindly donated by Drs J. and L. Risteli, Oulu. Purification of the antigen and preparation of the antibody were as described earlier (5). The concentration of the antibody was 0.31 mg/ml and it was used in 1:25 dilution. The anti-fibronectin was obtained from Dako A/S Copenhagen. Normal rabbit serum and phosphate-buffered saline were used in place of the primary antibody for control stainings.

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

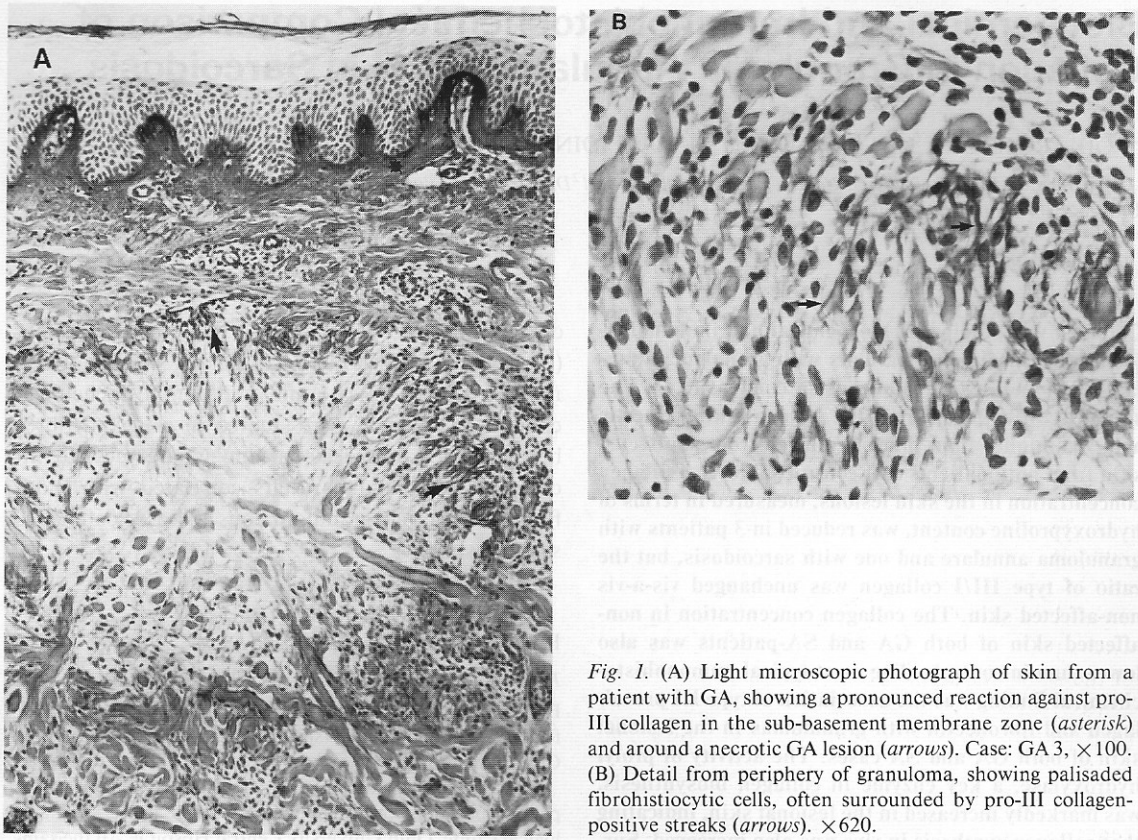

Fig. 1. (A) Light microscopic photograph of skin from a patient with GA, showing a pronounced reaction against pro-III collagen in the sub-basement membrane zone (*asterisk*) and around a necrotic GA lesion (*arrows*). Case: GA 3, ×100. (B) Detail from periphery of granuloma, showing palisaded fibrohistiocytic cells, often surrounded by pro-III collagen-positive streaks (*arrows*). ×620.

Collagen biosynthesis

Fibroblasts at early confluency were labelled with [^{14}C]proline and total non-dialysable radioactivity and [^{14}C]hydroxyproline in the medium were assayed as described (6) after 24 h incubation. The cell layer was used for the assay of total cell protein (7) and DNA (8). Part of culture medium after [^{14}C]proline labelling was used to assay the distinct collagen types. The medium fractions were digested with pepsin, and collagenous proteins were precipitated by NaCl, dialysed and used for sodium dodecyl sulphate (SDS) gel electrophoresis (9). The gel electrophoresis was performed using 8% polyacrylamide gels with and without delayed reduction with 2-mercaptoethanol (10). The collagen polypeptides were visualized by fluorography (11) and bands representing α1(III), α1(I) and α2(I) were quantified densitometrically.

Assays of prolyl hydroxylase and collagenase activities

Cells at early confluency were trypsinized, homogenized, and supernatants used to assay prolyl hydroxylase activity as described (12). Prolyl hydroxylase activity was also assayed directly from the 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 was collected. Medium fractions were treated briefly with trypsin and used for collagenase assays using type I collagen as substrate (13).

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

The amount of hydroxyproline, a measure of collagen was determined by a specific colorimetric assay (14). The intra-assay variation was 7.0±5.6% and interassay variation, 11.0±7:4%.

To measure acid and pepsin soluble collagen, the skin specimens were homogenized in 0.5 M acetic acid, extracted, and centrifuged. The supernatant, containing acid-soluble collagen, was then used to assay hydroxyproline. The pellet was treated twice with pepsin, and the pepsin-soluble material was used for the assay of hydroxyproline (14). Genetically distinct collagen types were measured as described below. The pellet was further used for the assay of hydroxyproline. To assay collagen types I and III, the pepsin-soluble material was precipitated with 4.4 M NaCl and the precipitates used for 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.

Other assays

Fibroblasts at early confluency were labelled with [^{35}S]methionine in serum-free DMEM for 16 h. The medium and cell fractions were collected and proteins precipitated with $(NH_4)SO_4$ (320 mg/ml). The precipitated material was then analysed using 6% SDS-gel electrophoresis after reduction with mercaptoethanol. A [^{14}C]methylated protein mixture was used as standard. For statistical analyses, Student's t-test was used.

RESULTS

Histological and immunohistochemical studies

The lesions of the granuloma annulare (GA) cases featured focal degeneration and necrosis of collagen surrounded by histiocytes, sometimes in a palisading arrangement, and fibroblasts with a few lymphocytes. The elastic bundles were disrupted and lost within the lesions. Cutaneous sarcoidosis took the form of epithelioid cell granulomas with giant cells and lymphocytes in the deep and central parts of the dermis. One of the sarcoidosis cases (SA 3) appeared as a cicatricial sarcoid with a huge number of foreign body giant cells, and polarizing particles, but showed only a few epithelioid cells.

The anti-pro III collagen antibody displayed a weak general staining throughout the dermis but an intense or moderate reaction around the lesions of granuloma annulare and sarcoidosis as well as in the upper dermis just beneath the epidermis (Figs. 1, 2). Fibronectin appeared to be quite evenly distributed throughout the dermis and only occasionally could a trace of a stronger staining be detected around the GA and SA lesions (not shown).

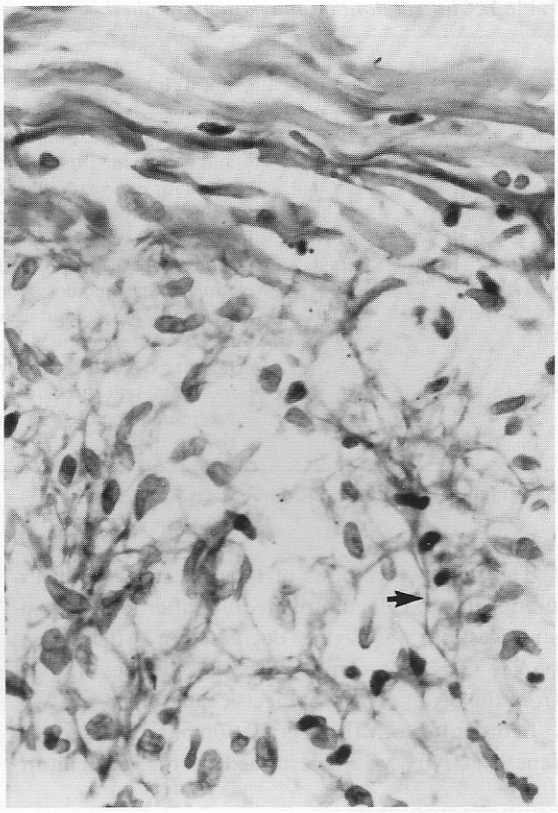

Fig. 2. The photomicrograph shows faintly stained pro-III collagen-positive strands around epithelioid cells in the periphery of a granuloma of a sarcoidosis patient (*arrows*). Case: SA 2, ×500.

Biochemical studies on skin biopsies

The collagen concentration, solubility of collagen in acetic acid and pepsin-digestible fractions, collagen types and prolyl hydroxylase were measured from lesional and unaffected skin samples from the patients (Table II, Fig. 2). Collagen concentration, measured in terms of hydroxyproline, had decreased in the lesional skin of 3 patients with GA and one with SA, in comparison with non-affected skin or controls. The solubility of collagen in acetic acid was increased in all the biopsies derived from lesional skin as compared with unaffected skin (Table II). The solubility of collagen in pepsin-digestible fraction was increased in 4 patients with GA, and in 2 patients with SA (Table II), and the concentration of DNA, measuring cellularity was increased in the lesional skin samples. The proportion of type III collagen, as analysed by gel electrophoresis, was 34% in lesional skin (30.1% in unaffected skin) from the GA patients and 24% in that from the SA cases (36.0% in unaffected skin) and 36.2% in controls. The ratio $\alpha 1(I):\alpha 2(I)$ was 2.3 ± 0.5 in GA and 2.9 ± 1.0 in SA.

The activity of prolyl hydroxylase in the lesional skin had increased 4.1-fold in GA ($p < 0.025$) and 1.6-fold in SA (Fig. 3).

Cell cultures

Collagen synthesis, measured in terms of the synthesis of [^{14}C]hydroxyproline in fibroblasts, was not altered in the GA or SA patients, and the ratio of [^{14}C]hydroxyproline to total radioactivity was unchanged, indicating that the relative synthesis of collagen was unchanged (Table III). The activity of prolyl hydroxylase was similar in fibroblasts derived from either lesional and unaffected skin (Table III).

Table I. *Clinical data on the patients studied*

Code	Sex	Age (years)	Clinical findings	Site of biopsy
GA 1	F	19	Lesions for one year on upper and lower extremities and abdomen. Type I diabetes for 8 years	Abdomen
GA 2	F	25	Lesions for one year on lower extremities and abdomen	Upper arm
GA 3	F	25	Lesions for 3 years on upper extremities	Upper arm
GA 4	F	51	Lesions for 2 years on upper extremities	Upper arm
GA 5	F	50	Lesions for 2 years on right lower extremity	Lower leg
SA 1	F	68	Nodular skin sarcoidosis for 16 years on face and upper extremities. Systemic steroids; prednisolone 10 mg/day	Upper arm
SA 2	M	49	Lung sarcoidosis noticed 16 years ago. For several years, nodular sarcoidosis on lower and upper extremities	Knee
SA 3	F	36	For 2 years, scar sarcoidosis on right knee. Thyroid carcinoma operated 5 years earlier, thyroxin 0.1 mg twice a day	Knee

GA: granuloma annulare; SA: sarcoidosis.

The relative synthesis of type III collagen, measured from the cell culture medium after limited pepsin digestion, was slightly decreased in two cell lines (GA 1, GA 2), as compared with cell lines derived from the SA or control subjects (Table III).

In order to study the degradation of collagen, collagenase activity was determined in the cell culture medium after brief trypsin treatment. This collagenase activity was similar in three cell lines derived from the lesional skin (GA 1, GA 2, SA 1) to that in corresponding cell cultures derived from unaffected skin or from control subjects (not shown).

The cells were also labeled with [^{35}S]methionine and medium and cell proteins analysed by 6% SDS-

Table II. *Collagen and DNA concentrations and solubility of collagen in affected (A) and non-affected (NA) skin from the patients*

	Hypro[a] µg/mg ww		Acid[b] Soluble %		Pepsin[b] Soluble %		DNA[c] µg/mg ww	
Code	A	NA	A	NA	A	NA	A	NA
GA 1	12.2	16.5	3.0	0.8	30.3	17.4	0.96	0.65
GA 2	14.0	21.3	2.0	0.9	28.8	24.0	1.54	0.68
GA 3	9.8	14.3	8.4	2.8	60.7	23.5	nd	nd
GA 4	11.6	10.0	2.3	2.0	21.5	27.4	nd	nd
GA 5	9.7	9.5	2.8	2.3	25.2	20.6	nd	nd
Mean ±S.D.	11.5±1.8	14.3±4.9	3.7±2.7	1.8±0.9	33.3±15.7	22.7±3.8	1.25	0.67
SA 1	6.3	16.8	2.4	1.3	42.0	31.0	2.77	0.73
SA 2	15.6	14.2	nd	1.3	22.1	18.3	nd	nd
SA 3	16.1	11.2	2.5	nd	44.0	65.9	nd	nd
Mean ±S.D.	12.7±5.5	14.1±2.8	2.45	1.3	36.0±12.1	38.4±24.6	2.77	0.73
Controls mean ±S.D.	22.7±7.8		1.8±0.7		30.6±15.3		0.79±0.20	

[a] Hydroxyproline (hypro) was assayed as described, and values expressed as µg hypro/mg wet weight of skin.
[b] Skin biopsy specimens were sequentially extracted with 0.5 M acetic acid (acid soluble) and pepsin as described. Hydroxyproline was assayed in acid-soluble, pepsin-soluble and insoluble fractions. The ratio of the amount of hydroxyproline in each fraction to the total amount of hypro was calculated and expressed as a percentage.
[c] DNA was assayed as described and the values expressed as µg/mg wet weight.

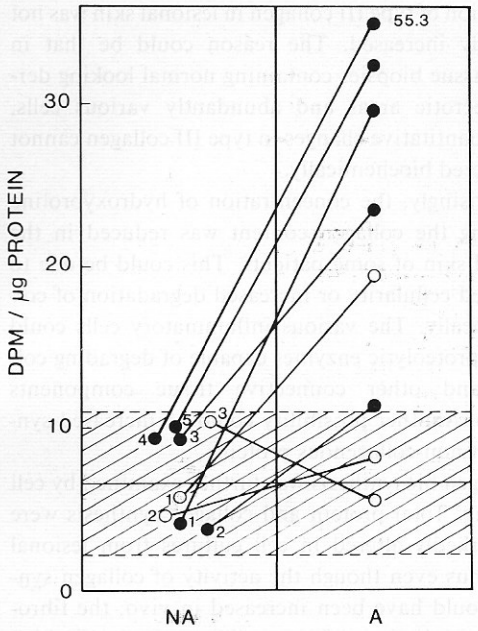

Fig. 3. Prolyl 4-hydroxylase activity in the non-affected (*NA*) and affected (*A*) skin of the patients. The activity was determined as described in Patients and Methods. The values are expressed as dpm per µg cytosol, protein. ——, Mean value for the controls; – – – limits of their means ±2 S.D. Symbols: ●, Granuloma annulare; ○, sarcoidosis. Note: The numbers beside the symbols refer to the patients as listed in Table I.

gel electrophoresis after reduction. Extra protein bands migrating at the 50 kD position could be seen in the medium of three cell lines (GA 1, GA 2, SA 1) (Fig. 4).

DISCUSSION

The present results indicate a modified collagen metabolism in GA with an increase in the solubility of collagen in acetic acid and pepsin. Since newly synthesized collagen is more soluble (2), this increased solubility may indicate that collagen synthesis in vivo is increased in the lesional skin of these patients. Two additional findings support the hypothesis that the synthesis of collagen is increased locally in the lesional skin of GA. Firstly the activity of prolyl hydroxylase, a key enzyme in the intracellular biosynthesis of collagen (12, 15) was increased. Previous research has indicated that the activity of this enzyme is increased under conditions in which the collagen synthesis increases. It should be noted, however, that some of the prolyl hydroxylase activity found in GA patients could be derived from inflammatory cells (16) found in the lesional skin (1, 17).

Secondly, immunohistochemical tests employing antibodies to type III procollagen and fibronectin indicate that type III collagen propeptide and fibronectin can be seen in the granuloma areas. It has been shown previously that type III collagen is actively synthesized during wound healing and early fibrosis

Table III. Total incorporation of [^{14}C]proline, synthesis of [^{14}C]hydroxyproline, activity of prolyl hydroxylase (PH) and relative synthesis of type III collagen in fibroblast cultures

Cell line (code)	Total incorporation[a] (dpm×10^{-6}/mg DNA)		[^{14}C]hydroxyproline[a] (dpm×10^{-6}mg DNA)		PH[b] (dpm/µg protein)		Type III[c] collagen	
	Affected	Non-affected	Affected	Non-affected	Affected	Non-affected	Affected	Non-affected
GA 1	4.30	nd	0.78	nd	138	nd	0.124	nd
GA 2	2.90	2.84	0.53	0.55	325	315	0.169	nd
SA	3.74	2.97	0.52	0.35	215	235	0.227	0.341
Controls mean ±S.D.	4.68±1.7		1.01±0.28		192±45		0.215±0.071	

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

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

[c] The relative synthesis of type III collagen was determined in [^{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 represent the ratios α1(III)/α1(I)+α2(I)+α1(III).

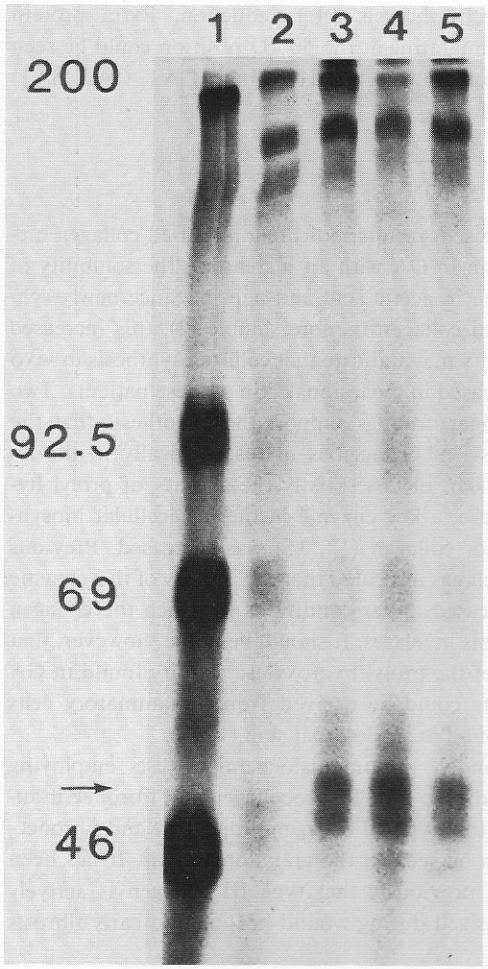

Fig. 4. Analyses of [^{35}S]methionine-labelled medium proteins from cell cultures. The cells were labelled with [^{35}S]methionine in serum-free DMEM for 16 h and medium fractions were precipitated and analysed using 6% SDS-gel electrophoresis after reduction. Lane 1: [^{14}C]methylated protein standard mixture are shown in the right; Lane 2: control cell line; Lane 3: SA 1 (affected); Lane 4: GA 1 (affected); Lane 5: GA 2 (affected). The posititons of the polypeptide chains in the 50 kD region are shown. The protein standards used were: myosin, 200 kD; phosphorylase. B, 92.5 kD; albumin, 69.0 kD; ovalbumin, 46.0 kD; carbonic anhydrase, 30 kD; and lysozyme, 14.3 kD.

(2, 15, 18). Especially in the case of GA, ultrastructural studies have revealed thin fibrils of newly synthesized collagen, further confirming the activity of collagen synthesis in vivo (19).

The association of type III procollagen antibodies to thin fibrils in lesional skin suggests that type III collagen is actively synthesized in vivo. However, the proportion of type III collagen in lesional skin was not markedly increased. The reason could be that in whole tissue biopsies containing normal looking dermis, necrotic areas and abundantly various cells, small quantitative changes in type III collagen cannot be detected biochemically.

Surprisingly, the concentration of hydroxyproline reflecting the collagen content was reduced in the affected skin of some patients. This could be due to increased cellularity or increased degradation of collagen locally. The various inflammatory cells could release proteolytic enzymes capable of degrading collagen and other connective tissue components (20–23). Another possibility could be increased synthesis of non-collagenous proteins.

Collagen biosynthesis was further examined by cell culturing. Total protein and collagen synthesis were not markedly altered in cell cultures from lesional skin. Thus even though the activity of collagen synthesis could have been increased in vivo, the fibroblasts in culture conditions did not maintain high synthesis activity. This could be due to cell selection, as noted in scleroderma in which increased or normal collagen synthesis activity can be seen in vitro in cell cultures (24). Another possibility is that various mediators released from inflammatory cells, e.g. macrophages (25), activate fibroblasts in vivo (26, 27). However, in cell cultures, after 3–6 subpopulations, this effect is lost. Further studies employing in situ hybridization techniques may establish whether in GA and SA fibroblasts are actively synthesizing collagen.

ACKNOWLEDGEMENTS

The authors thank Mrs Eeva Lehtimäki, Ms Helmi Konola and Mrs Marja Tolppanen for expert technical assistance and Mrs Marja-Terttu Tolonen for excellent secreterial 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.

REFERENCES

1. Lever WF, Schaumburg-Lever G. Granuloma annulare. In: Histopathology of the skin, 6th edn. Philadelphia: JB Lippincott, 1983; 234–236.
2. Uitto J, Ryhänen L, Tan EML. Collagen: Its structure, function and pathology. In: Fleischmajer R, ed. Progress in disease of the skin. New York: Grune & Stratton, 1981; 103–141.

3. Burns J, Dixon AJ, Woods JC. Immunoperoxidase localization of the fibronectin in glomeruli of formalin fixed paraffin embedded level tissues. Histochemistry 1980; 67:73–78.
4. Sternberger LA. The unlabeled antibody peroxidase–antiperoxidase (PAP) method. In: Immunocytochemistry, 2nd edn. New York: John Wiley & Sons, 1979; 104–109.
5. Niemelä O, Risteli L, Parkkinen J, Risteli J. Purification and characterization of the N-terminal propeptide of human type III procollagen. Biochem J 1985; 232: 146–150.
6. Juva K, Prockop DJ. Modified procedure for the assay of H^3- or C^{14}-labelled hydroxyproline. Anal Biochem 1966; 15: 77–83.
7. Bradford MM. A rapid and sensitive method of quantitation of microgram quantities of protein utilizing the principle of protein-binding dye. Anal Biochem 1976; 72:248–254.
8. Brunk CF, Jones KC, James TW. Assay for nanogram quantities of DNA in cellular homogenates. Anal Biochem 1979; 92: 497–500.
9. Laemmli UK. Cleavage of structural proteins during assembly of bacteriophage T_4. Nature 1970; 227: 680–685.
10. Sykes B, Puddle B, Francis M, Smith R. The estimation of two collagens from human dermis by interrupted gel electrophoresis. Biochem Biophys Res Commun 1976; 72: 1478–1480.
11. Bonner WM, Laskey RA. A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. Eur J Biochem 1974; 46: 83–92.
12. Kivirikko KI, Myllylä R. Post-translational enzymes in the biosynthesis of collagen: intracellular enzymes. In Cunningham LW, Fredrikson DW, eds. Methods in enzymology, structural and contractile proteins; Part A: Vol. 82. New York: Academic Press, 1982; 245–304.
13. Ryhänen L, Rantala-Ryhänen S, Tan EML, Uitto J. Assay of collagenese activity by a rapid, sensitive and specific method. Collagen Rel Res 1982; 2: 117–130.
14. Kivirikko KI, Laitinen O, Prockop DJ. Modification of a specificy assay for hydroxyproline in urine. Anal Biochem 1962; 19: 249–255.
15. Prockop DJ, Kivirikko KI, Tuderman L, Guzman NA. Biosynthesis of collagen and its disorders. N Engl J Med 1979; 301:13–23, 77–85.
16. Myllylä R, Seppä H. Studies of enzymes of collagen biosynthesis and the synthesis of hydroxyproline in macrophages and mast cells. Biochem J 1979; 182: 311–316.
17. Barrie HJ, Bogoch A. The natural history of the sarcoid granuloma. Am J Pathol 1953; 29: 451–469.
18. Bornstein P, Sage H. Structurally distinct collagen types. Ann Rev Biochem 1980; 49: 957–1003.
19. Wolff HH, Maciejewski W. The ultrastructure of granuloma annulare. Arch Dermatol Res 1977; 259: 225–234.
20. Lazarus GS, Daniels JR, Brown RS, Bladen HA, Fullmer HM. Degradation of collagen by a human granulocyte collagenolytic system. J Clin Invest 1968; 47: 2622–2629.
21. Gadek JE, Fells GA, Wright DG, Chrystal RG. Human neutrophil elastase functions as a type III collagen collagenase. Biochem Biophys Res Commun 1980; 95: 1815–1822.
22. Ohlsson K, Olsson I. The neutral proteinases of human granulocyte: isolation and partial characterization of two granulocyte collagenases. Eur J Biochem 1973; 36: 473–481.
23. Werb Z, Gordon S. Elastase secretion by stimulated macrophages. Characterization and regulation. Exp Med 1975; 142: 361–377.
24. Botstein GR, Sherer GK, LeRoy EC. Fibroblast selection in scleroderma. An alternative model of fibrosis. Arthritis Rheum 1982; 25: 189–195.
25. Umbert P, Winkelman RK. Granuloma annulare. Direct immunofluorescence study. Br J Dermatol 1976; 95: 487–492.
26. Johnson RL, Ziff M. Lymphokine stimulation of collagen accumulation. J Clin Invest 1976; 58: 240–252.
27. Postlethwaite AE, Kang AH. Induction of fibroblast proliferation by human mononuclear leucocyte-derived proteins. Arthritis Rheum 1983; 26: 22–27.