Scleroderma

INCREASED BIOSYNTHESIS OF TRIPLE-HELICAL TYPE I AND TYPE III PROCOLLAGENS ASSOCIATED WITH UNALTERED EXPRESSION OF COLLAGENASE BY SKIN FIBROBLASTS IN CULTURE

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ABSTRACT To assess potential abnormalities in collagen metabolism in systemic scleroderma, skin fibroblast lines from patients with this disease were established and compared to control cell lines derived from healthy subjects. For studies on the biosynthesis of procollagen, the cells were incubated with [^{14}C]proline in a medium supplemented with ascorbic acid and β-aminopropionitrile, and the synthesis of nondialyzable [^{14}C]hydroxyproline, in relation to DNA or cell protein, was taken as an index of procollagen formation. Five of eight scleroderma fibroblast cell lines demonstrated procollagen biosynthesis rates significantly higher than the controls, and the mean rate of procollagen synthesis by scleroderma fibroblasts was about twice that of the control cells. Control experiments demonstrated that the specific activity of the intracellular free proline was not different in scleroderma and control fibroblasts, and the mean population doubling times of the scleroderma and the control fibroblast cell lines were the same. The relative synthesis of the genetically distinct procollagens was examined by isolating type I and type III procollagens from the cell culture medium using DEAE-cellulose chromatography. The ratios of type I/III procollagens in scleroderma cell lines did not differ from the controls. The helical stability of the collagenous portion of type I and type III procollagens, estimated by the resistance of ^{14}C-collagen to limited proteolytic digestion with pepsin under nondenaturing conditions, was the same in both scleroderma and control cultures. The capacity of the cells to synthesize enzymatically active and immunologically reacting collagenase was also studied; no marked differences in these parameters could be observed. The results suggest that cultured skin fibroblasts from patients with scleroderma demonstrate a metabolic abnormality expressed as increased synthesis of type I and type III procollagens in a normal ratio. This abnormality may play a role in the excessive accumulation of collagen in the skin and other organs affected in scleroderma.

INTRODUCTION

Systemic scleroderma (progressive systemic sclerosis) is a clinical condition of unknown etiology characterized by the diffuse accumulation of collagen in the skin, subcutaneous tissues, and a variety of internal organs (2–4). The mechanistic details of collagen accumulation in the affected tissues have not been clarified (4). Early evidence demonstrated an increased rate of collagen synthesis in the skin of patients with scleroderma (5). This conclusion was based on the observations that the activity of prolyl hydroxylase, a key enzyme in collagen biosynthesis, was increased in skin biopsy specimens taken from patients with active disease (6, 7). Furthermore, the incorporation of radioactive proline into cutaneous collagen and the synthesis of radioactive hydroxyproline were also increased under tissue culture conditions (8, 9). The conclusion that collagen biosynthesis might be increased in patients with active scleroderma was further supported by the demonstration of new, labile cross-links in vivo, an observation consistent with excessive synthesis of collagen (10). However, it has not been resolved whether each individual fibroblast is activated to produce more collagen or whether the collagen produc-

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tion per cell is normal but more fibroblasts are present in the diseased skin.

This question has recently been examined employing skin fibroblasts in culture but with conflicting results. Two studies have suggested that the synthesis of collagen per cell may be increased in patients with scleroderma (11–13). In contrast, another study indicates that the synthesis of collagen by scleroderma fibroblasts is not different from that of the control cells (14). In addition, it has also been suggested that the activity of collagenase, the enzyme initiating collagen breakdown in vivo, is decreased in scleroderma (15). Therefore, the accumulation of collagen in the skin and other tissues could be explained by decreased degradation rather than increased deposition of collagen fibers.

In the present investigation we have employed skin fibroblasts in culture to examine certain parameters of collagen metabolism in patients with systemic scleroderma. We have assessed (*a*) the rate of procollagen synthesis; (*b*) the genetic types of the newly synthesized procollagen molecules; (*c*) the stability of the triple-helical conformation of the newly synthesized collagen, and (*d*) as an indicator of one aspect of collagen degradation, the synthesis of enzymatically active collagenase and immunoreactive enzyme protein. The results demonstrate that scleroderma fibroblasts in culture synthesize increased amounts of procollagen, but the relative proportions of type I and type III procollagens remain unchanged. No marked changes in collagenase synthesis or activity could be demonstrated.

METHODS

Patients. Fibroblast cultures were established from the skin of nine patients with systemic scleroderma after obtaining informed consent. The clinical information regarding these patients is given in Table I. Control cultures were initiated from healthy volunteers or were purchased from the American Type Culture Collection, Rockville, Md.

Human skin fibroblast cultures. The primary cultures of human skin fibroblasts were initiated from a 3-mm skin punch biopsy. Cells were subcultivated in Dulbecco's modified Eagle's medium and glutamine (KC Biologicals) with 30 mM Hepes buffer, pH 7.6, and containing 20% fetal calf serum, 200 U/ml of penicillin and 50 μg/ml of streptomycin. The cells were used in the third to fifth serial passage.

To assess collagen and collagenase production, identical secondary cultures of human skin fibroblasts were initiated from a single pool of cells in 75-cm² flasks (Falcon Plastics, Oxnard, Calif.) or in 100-mm plates by trypsinization and plating at 5×10^5 cells/flask. Medium containing serum was changed at 2-d intervals until the desired growth stage was reached.

Collagen biosynthesis. For these studies cells were incubated with [^{14}C]proline under conditions which we have previously shown to be optimal for collagen biosynthesis (16). The cells were labeled on day 1 or 2 after the cultures had reached visual confluency. 50 μg/ml ascorbic acid was added to the culture medium 4 h before labeling and the labeling was initiated by placing the cultures in 7 ml of Dulbecco's modified Eagle's medium containing 20% dialyzed fetal calf serum, 50 μg/ml ascorbic acid, 20 μg/ml β-aminopropionitrile fumarate, 200 U/ml penicillin, 50 μg/ml streptomycin, and 30 mM Hepes buffer, pH 7.6. 3 μCi/ml of the radioactive proline was added, and the cultures were incubated for 20 h at 37°C. Under these conditions, the incorporation of [^{14}C]proline into newly synthesized protein as well as the synthesis of [^{14}C]hydroxyproline have been shown to be linear up to 24 h; incubation of parallel control cultures have indicated a variability of ±18% (SD).[1] At the end of the labeling period the medium was removed, and the cell layer was rinsed with 3 ml Dulbecco's modified Eagle's medium; the rinse was combined with the medium. The medium fraction was then placed in an ice bath and 1 ml of stock solution containing protease inhibitors was added to give final concentrations of 20 mM Na_2EDTA, 10 mM *N*-ethylmaleimide, and 1 μM phenylmethylsulfonylfluoride. Aliquots of the media were dialyzed against running tap water for assay of total ^{14}C-radioactivity and [^{14}C]hydroxyproline (see below). Part of the medium was precipitated with 114 mg/ml ammonium sulfate (20% of saturation), for DEAE-cellulose chromatography or limited proteolytic digestion with pepsin (see below). The cell layer was rinsed three times with 10 ml of Hanks' balanced salt solution, and the cells were scraped with a rubber policeman into 5 ml of 0.4 M NaCl, 0.1 M Tris-HCl, pH 7.5, containing 20 mM Na_2EDTA, 10 mM *N*-ethylmaleimide, and 1 μM phenylmethylsulfonylfluoride. The cells were sonicated at 60 Hz for 30 s. Aliquots of the cell homogenates were dialyzed against running tap water for assay of total ^{14}C-protein and [^{14}C]-hydroxyproline. In addition, aliquots of the cell fraction were dialyzed against 0.15 M NaCl, 0.001 M Tris-HCl, pH 7.4, for assay of DNA and total cell protein (see below).

Chromatographic procedures. For DEAE-cellulose chromatography, the ^{14}C-proteins recovered from the medium by ammonium sulfate precipitation were dissolved in starting buffer consisting of 2 M urea and 1 mM Na_2EDTA in 0.025 M Tris-HCl, pH 7.5, and dialyzed against the same buffer. The samples were then chromatographed on DEAE-cellulose using a linear gradient from 0 to 0.22 M NaCl in 0.025 M Tris-HCl buffer, pH 7.5, and containing 1 mM Na_2EDTA, as described elsewhere (17, 18).

For gel filtration on agarose, the ^{14}C-proteins digested with pepsin (see below) were denatured by heating at 100°C for 5 min in the presence of 10 M urea, 20 mM EDTA, 10 mM *N*-ethylmaleimide, and 1 μM phenylmethylsulfonylfluoride. The samples were chromatographed on 6% agarose column in 1 M $CaCl_2$, 0.05 M Tris-HCl, pH 7.5, at 22°C, as described elsewhere (19).

Chemical and enzymatic treatments. For pepsin digestion, the ^{14}C-proteins isolated from the culture media by ammonium sulfate precipitation were dissolved in 0.5 M acetic acid and then dialyzed against the same buffer. 100 μg/ml of twice crystallized pepsin (Worthington Biochemical Corp., Freehold, N. J.) was added and the samples were incubated for 3 h at 4°C. At the end of the incubation, the pH of the digests was brought to 8.5 to inactivate the pepsin, and the samples were dialyzed against 0.4 M NaCl, 0.1 M Tris-HCl, pH 7.5. The pepsin-resistant ^{14}C-collagen molecules were then isolated by gel filtration, as described above.

Collagenase assay. To determine collagenase expression in normal and scleroderma human skin fibroblasts, the cells were washed three times in a total volume of 100 ml of Hanks' balanced salt solution. The cultures were then maintained for

[1] Uitto, J., K. L. Polak, and B. A. Booth. Manuscript in preparation.

TABLE I
Clinical Characteristics of Patients with Scleroderma

Culture code	Sex	Age	Clinical findings
		yr	
Control			
WUN 76135	M	26	None
CRL 1119	M	15	
WUN 76131	F	27	
CRL 1141	M	3	
CRL 1121	M	3	
CRL 1224	F	40	
WUN 76130	F	20	
WUN 78321	F	25	
WUN 77248	F	28	
WUN 77250	F	25	
WUN 77251	F	36	
Scleroderma			
WUS 76118	M	56	4 yr duration; progressive; Raynaud's with acrosclerosis and distal bone resorption; pulmonary fibrosis.
WUS 77269	F	43	1 yr duration; rapidly progressive Raynaud's; widespread thickening of skin with flexural contractures; restrictive pulmonary disease with pulmonary hypertension; pericardial thickening.
WUS 77213	M	18	4 yr duration; progressive; acrosclerosis with digital ulcerations and distal bone resorption; esophageal hypomotility; mild myositis.
WUS 77221	F	49	½ yr duration; progressive; acrosclerosis; arthralgias; abnormal liver function tests.
WUS 77284	F	47	11 yr duration; Raynaud's; acrosclerosis with distal bone resorption; restrictive pulmonary disease; esophageal and small bowel hypomotility.
WUS 77222	M	56	2 yr duration; rapidly progressive; restrictive pulmonary disease; pericardial effusion; decreased small bowel motility.
WUS 77266	F	43	1 yr duration; acrosclerosis; mild restrictive pulmonary disease; no gastrointestinal disease.
WUS 78313	F	14	4 yr duration; progressive; Raynaud's; fingertip ulcerations; acrosclerosis; distal demineralization on bones; no gastrointestinal or pulmonary disease.
WUS-MP	F	61	17 yr duration; acrosclerosis; cutaneous calcinosis and ulcerations; mild esophageal involvement.
CRL 1108	F	46	Clinical status unknown; purchased from American Type Culture Collection.

24 h in 7–10 ml of serum-free Dulbecco's modified Eagle's medium and glutamine containing 30 mM Hepes buffer, pH 7.6. The medium was harvested, buffered to a final concentration of 50 mM with 1 M Tris-HCl, pH 7.5, and stored at −70°C until used. The cell layer was harvested for determination of cell pellet protein and DNA content. Total collagenase activity in the cell culture medium was assessed by activating procollagenase proteolytically with trypsin, as described pre-

viously (20), and after which enzyme activity was measured using native reconstituted [^{14}C]glycine-labeled collagen fibrils containing ≅3,000 cpm/substrate gel (21).

Immunoreactive human skin collagenase was measured by a slight modification of the double-antibody radioimmunoassay previously reported (22). The procollagenase used as the unlabeled standard and for iodination in the radioimmunoassay was purified to homogeneity from cell culture medium as described previously (23). This same enzyme preparation was used to produce monospecific antiserum to the enzyme (24).

In addition to quantitating collagenase synthesis in fibroblast cultures, short-term explant cultures were established from the affected skin of two scleroderma patients for comparison with those from the skin of six control subjects. The tissue (15–30 mg wet wt) was cut into pieces ≅2 mm in size and cultured in Leighton tubes on a ^{14}C-labeled native reconstituted collagen substrate (5,000 cpm/substrate gel) for 48 h at 37°C (25). At the end of the incubation period, tissue fragments were removed and the contents of the tube were centrifuged. The amount of enzymatically degraded collagen fragments released into the supernate were determined by counting in a liquid scintillation spectrometer (25).

Other assays. To measure the synthesis of [^{14}C]hydroxyproline and to determine the incorporation of ^{14}C-radioactivity into proteins, the dialyzed medium and cell samples were hydrolyzed in 6 M HCl at 110°C for 24 h. The [^{14}C]-hydroxyproline in the hydrolysate was then separated from [^{14}C]proline on a 10 × 0.75-cm column of Beckman type W-2 polystyrene resin (Beckman Instruments, Inc., Fullerton, Calif.), eluted with 0.2 M sodium citrate buffer, pH 3.24. 1.0-ml fractions were collected, and 0.4-ml aliquots were counted in 3 ml of ACS counting solution using a Beckman LS 3155 P liquid scintillation counter (Beckman Instruments, Inc.). Alternatively, in some experiments, the [^{14}C]hydroxyproline was assayed by a specific radiochemical method (26).

To measure the specific activity of the intracellular free proline during the incubation with [^{14}C]proline, aliquots of the cell homogenate were precipitated with an equal volume of trichloroacetic acid, and the [^{14}C]proline and the total proline contents of the supernate were determined by a modification of the method described by Troll and Lindsley (27).

For assay of DNA and total cell protein, aliquots of the cell homogenates were dialyzed against 0.15 M NaCl, 0.001 M Tris-HCl, pH 7.5. DNA was measured by the method of Burton (28) and the protein was assayed according to Lowry et al. (29).

The growth kinetics of control and scleroderma fibroblasts were determined by seeding cultures from a single large pool at low density. Replicate cultures were fed serum-containing medium every 2 d for the length of the experiment. Duplicate cultures were harvested daily for cell counts.

RESULTS

Rate of procollagen biosynthesis. In this study, the synthesis of radioactive hydroxyproline, in relation to DNA or total cell protein, was taken as an index of collagen formation. Incubation of fibroblast cultures demonstrated that the rate of collagen synthesis in cell lines derived from patients with scleroderma was clearly increased when compared to control cell lines. The mean hydroxyproline synthesis in scleroderma fibroblast lines was about twice that in the corresponding controls (Table II). No significant correlation between the age of the control subjects and the rate of [^{14}C]hydroxyproline synthesis was noted. Furthermore, control experiments have also demonstrated that the rate of ^{14}C-procollagen synthesis by cultured fibroblasts is essentially the same in the age range from 3 to 81 yr. When examined individually, five of the eight scleroderma cell lines demonstrated values higher than mean plus 2 SD of the controls (Table II). It should be noted, that several of these scleroderma fibroblast cell lines

TABLE II

The Rate of Procollagen Biosynthesis by Scleroderma Fibroblasts in Culture

Cell line	[^{14}C]Hydroxyproline			
	dpm/μg DNA/h	*% of the controls*	*dpm/mg protein/h*	*% of the controls*
Controls (n = 11)				
Mean±SE	127.0±7.2	100	2,572.3±202.4	100
Scleroderma*				
WUS 77221	434.2‡	342	7,177.3‡	279
CRL 1108	414.1‡	326	10,823.4‡	420
WUS 77213	365.6‡	288	5,878.2‡	229
WUS 77284	178.0‡	140	4,338.0‡	169
WUS 76118	174.6‡	137	5,475.5‡	213
WUS 77266	172.3	135	3,295.0	128
WUS 77269	126.3	99	3,439.7	134
WUS 77222	117.6	93	1,087.0	42
Mean±SE	247.8±47.0§	195	5,189.3±1,039.0§	202

* The individual values represent means of two parallel determinations.

‡ These values are greater than the mean + 2 SD of the controls.

§ Statistically different from the controls ($P < 0.05$).

were incubated in three separate experiments with similar results. Examination of the secreted [^{14}C]-hydroxyproline-containing polypeptides separately from [^{14}C]hydroxyproline in the cell fraction demonstrated that after a 20-h labeling period >80% of the newly synthesized collagen was found in the extracellular space both in the control and scleroderma cell cultures. This result is consistent with the observation that essentially all collagenous protein in the medium is recovered as procollagen (see below).

Because the rate of incorporation of radioactive proline into protein is affected by several factors, control experiments were performed to exclude possible sources of misinterpretation. In particular, the specific radioactivity of the intracellular free proline in all cell cultures used for the assay of procollagen biosynthesis was tested. There was no significant difference in the specific radioactivity of the intracellular free proline between scleroderma lines and the control cells (Table III). In further experiments, the mean population doubling times were determined in control and scleroderma fibroblast cultures. As shown in Table IV, the mean population doubling time for the scleroderma fibroblasts was not different from the control cell lines. Thus, the differences in collagen synthesis between these two groups of cells can not be accounted for by differences either in the intracellular proline pool or in the kinetics of growth.

Synthesis of type I and type III procollagens. Cultured human skin fibroblasts synthesize and secrete two major genetic types of procollagen, type I and type III (17, 30). To estimate the relative synthesis of these procollagens, ^{14}C-procollagen in the medium was partially purified by ammonium sulfate precipitation and type I and type III procollagen molecules were then isolated by DEAE-cellulose chromatography. The elution patterns of ^{14}C-protein were qualitatively similar in samples obtained both from scleroderma and control fibroblasts (Fig. 1). In all chromatograms two major peaks of radioactivity, peaks A and B, were observed. On the basis of their [^{14}C]hydroxyproline content, susceptibility to digestion by bacterial collagenase, α-chain composition as estimated from CM-cellulose chromatography after limited pepsin proteolysis, and cyanogen bromide peptide mapping, peaks A and B have been identified as containing type I and type III procollagen, respectively (30).[1] Because peak B occasionally has some noncollagenous [^{14}C]proline-containing proteins, the isolated type I and type III procollagens were estimated by determining the [^{14}C]-hydroxyproline in peaks A and B (Fig. 1A). The distribution of radioactivity between type I and type III procollagen was relatively constant, and the mean ratio of [^{14}C]hydroxyproline in type I and type III procollagen in scleroderma cell cultures did not differ from the corresponding value observed in control cell lines (Table V).

The helical stability of the genetically distinct types of collagen was also measured by subjecting the secreted ^{14}C-procollagen, partially purified by ammonium sulfate precipitation, to limited pepsin proteolysis, and then separating the pepsin-resistant collagen polypeptides by gel filtration under denaturing conditions (Fig. 2). The separation of type I collagen α-chains from the corresponding polypeptides of type III collagen is based on the fact that human type III collagen molecules contain interchain disulfide bonds in their triple-helical portion and they

TABLE III
*The Specific Activity of Intracellular Free Proline in Scleroderma Fibroblasts**

Cell line	Free [^{14}C]proline	Free [^{12}C]proline	Free [^{14}C]proline/ free [^{12}C]proline
	cpm × 10^{-3}/ flask	*μg/flask*	*cpm × 10^{-3}/μg*
Controls (n = 8)	86.6±2.9	1.27±0.09	68.2±3.4
Scleroderma (n = 8)	79.0±3.4‡	1.07±0.11‡	73.8±3.1‡

* The values are mean±SE.
‡ Not statistically different from the controls.

TABLE IV
Growth Kinetics of Scleroderma Fibroblasts

Cell line	Population doubling time
	*d**
Controls	
WUN 76135	3.44±0.63
CRL 1119	3.21±0.05
WUN 76131	2.85±0.05
CRL 1141	3.05±0.31
CRL 1121	3.34±0.45
CRL 1224	2.99±0.64
WUN 76130	2.55±0.53
Mean±SE	3.04±0.11
Scleroderma	
WUS 76118	2.96±0.55
WUS 77269	2.63±0.58
WUS 77213	3.83±0.79
WUS 77221	2.98±0.30
WUS 77284	2.17±0.13
WUS 77222	2.73±0.17
WUS 77266	3.72±0.13
Mean±SE	3.00±0.22‡

* Mean±SE.
‡ The values are not statistically different from the controls.

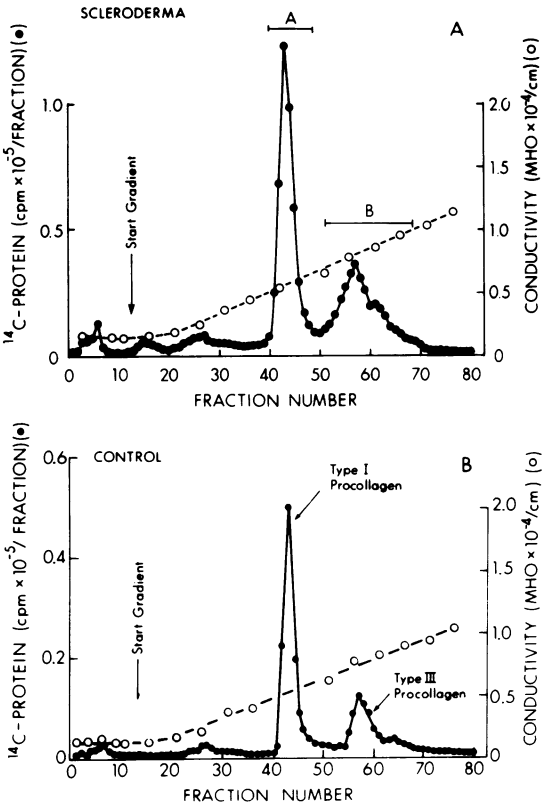

FIGURE 1 DEAE-cellulose chromatography of medium ^{14}C-protein synthesized by human skin fibroblasts in culture. Scleroderma and control fibroblasts in confluent monolayer cultures were incubated with [^{14}C]proline, as indicated in Methods. After a 20-h incubation, the medium ^{14}C-protein was isolated and chromatographed on DEAE-cellulose. The protein was eluted with a linear gradient from 0 to 0.22 M NaCl in 2 M urea and 0.025 M Tris-HCl, pH 7.5. The two major peaks of radioactivity, A and B, have been shown to contain type I and type III procollagen, respectively, as indicated in Fig. B; the genetic identity of these procollagens has been confirmed by cyanogen bromide peptide mapping, amino acid analysis, and α-chain composition (see text). To quantitate the relative ratios of type I and type III procollagens, the fractions indicated by the horizontal bars were pooled and assayed for [^{14}C]hydroxyproline. (A) ^{14}C-Protein synthesized and secreted by a scleroderma fibroblast cell line. (B) ^{14}C-Protein synthesized and secreted by a control cell line.

chromatograph in the γ-chain position with an apparent mol wt of 285,000 (31, 32). In contrast, the polypeptide chains of type I collagen lack such disulfide bonds and they elute from the agarose column in the α-chain position with an apparent mol wt of 94,000 daltons. Chromatography of the pepsin-resistant ^{14}C-proteins in the cell culture media demonstrated that the ratio of type I and type III collagen molecules, which were in a stable triple-helical conformation, was the same both in scleroderma and control cell lines (Table VI). On the basis of these experiments the synthesis of both type I and type III procollagens was increased in scleroderma

TABLE V
Relative Synthesis of Type I and Type III Procollagens by Scleroderma Fibroblasts

Cell line	Relative amounts of procollagen*	
	Type I	Type III
Controls (n = 9)	83.8±4.0	16.2±0.8
Scleroderma (n = 8)	80.5±6.5‡	19.5±1.6‡

* The values are percent of [^{14}C]hydroxyproline in type I and type III procollagen peaks separated by DEAE-cellulose chromatography (Fig. 1 and Methods); mean±SE.
‡ The values are not statistically different from the controls.

fibroblast cultures, but the newly synthesized procollagens did not differ in their helical stability from the controls.

The type I and type III collagen α-chains isolated by gel filtration on $CaCl_2$-agarose were also examined by assaying the relative contents of [^{14}C]hydroxyproline and [^{14}C]proline in the molecule. In both type I and type III collagens, from 42 to 51% of the [^{14}C]proline residues incorporated into the polypeptide chains were converted to [^{14}C]hydroxyproline. This suggests that the degree of prolyl hydroxylation in the newly synthesized procollagen polypeptides is not abnormal in scleroderma.

Assay of enzymatically active and immunologically reacting collagenase. Collagenase production was assessed in the scleroderma patients in two different ways. In initial experiments in vitro collagenase activity was measured by incubating skin explants on radioactive collagen. Collagenase activity in two scleroderma explant cultures was 136 and 157 cpm/mg wet wt, respectively, compared to 155±24 cpm/mg wet wt for six different control cultures (P = NS).

In further experiments collagenase production by scleroderma fibroblasts, using the same cell lines which were examined for the rate of procollagen biosynthesis was compared to that of control human skin fibroblast lines. First, immunoreactive collagenase protein was determined using a specific radioimmunoassay. As a group, fibroblast cultures from patients with systemic scleroderma displayed a mean value of immunoreactive enzyme protein of 121±27% of control (mean±SE) when based on the medium protein and 113±29% of control when based on the content of cellular protein (Table VII). Individually, however, one cell line (Expt. 4, WUS 78313) displayed only about one-third as much immunoreactive enzyme protein as the control cultures from the same experiment. Because collagenase is found in the cell culture medium in an inactive proenzyme form (20, 23), collagenase activity was determined, in both control and scleroderma fibroblast cultures, after maximal enzyme activation by

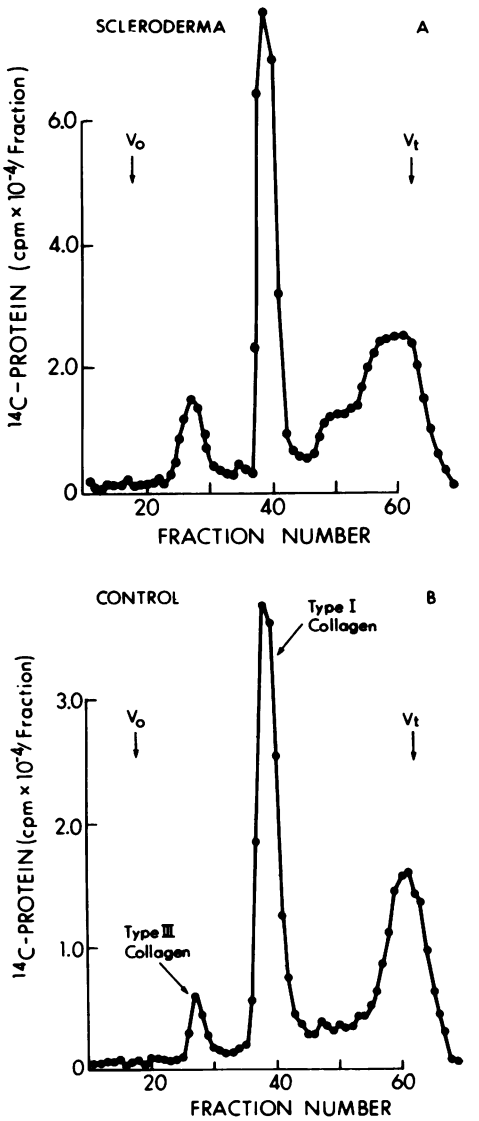

FIGURE 2 Assay by gel filtration of triple-helical type I and type III collagens synthesized and secreted by human skin fibroblasts in culture. Scleroderma and control fibroblasts were incubated with [^{14}C]proline as indicated in Fig. 1 and in Methods. After a 20-h incubation, the medium ^{14}C-protein was subjected to limited proteolytic digestion by pepsin and chromatographed on 6% agarose under denaturing conditions, as described in the text. As indicated in Fig. B, the type III collagen molecules, which contain interchain disulfides, chromatograph earlier than the α-chains of type I collagen. (A) ^{14}C-Protein in the medium of a scleroderma fibroblast cell line. (B) ^{14}C-Protein in the medium of a control cell line.

trypsin proteolysis. Again as a group, collagenase activity per unit immunoreactive protein was 98±8% of control values (Table VII) indicating that the catalytic efficiency of the enzyme was also apparently normal in the cell cultures derived from scleroderma patients.

TABLE VI
The Ratio of Triple-Helical Type I and Type III Collagens Synthesized and Secreted by Scleroderma Fibroblasts

Cell line	Relative amounts of collagen*	
	Type I	Type III
Controls (n = 5)	79.6±6.2	20.4±1.4
Scleroderma (n = 6)	85.7±4.0‡	14.3±0.7‡

* The values are percent of ^{14}C-radioactivity in type I and type III collagens separated by gel filtration after limited pepsin proteolysis (Fig. 2 and Methods); mean±SE.
‡ The values are not statistically different from the controls.

DISCUSSION

In the present study, the capacity of cultured scleroderma fibroblasts to synthesize both collagen and collagenase, the enzyme required to initiate collagen degradation, has been assessed in the same cell lines. Our results indicate that the rate of procollagen synthesis by skin fibroblasts in culture is increased in patients with scleroderma, a finding which would explain the major pathologic feature of the disease, i.e., excessive accumulation of collagen in a variety of organs, including the skin and subcutaneous tissue.

Previous studies exploring the metabolic events leading to collagen accumulation have yielded conflicting results. Although most studies have shown increased biosynthesis of collagen (8–13), in one series, an increased rate of collagen synthesis by fibroblasts in culture was not found (14). It has also been suggested that the rate of collagen degradation is diminished in scleroderma as a result of decreased collagenase in the clinically affected skin (15). Our observations do not support the latter finding irrespective of whether collagenase synthesis was assessed in skin explants or in fibroblast cultures.

The apparent discrepancy among these studies may have several explanations. First, it is clear that the rate of collagen synthesis in scleroderma skin depends on the clinical stage and the activity of the disease (5). Increased synthesis is frequently observed in the active stages of the disease, whereas collagen synthesis is normal or even decreased in the more advanced, fibrotic stages. A second explanation for the differing results may be found in the conditions employed for collagen biosynthesis in cell cultures. The concentrations of such components of the culture medium as ascorbic acid (16, 33) or serum (16, 34) may in part explain the differences. In the present study, culture conditions optimal for collagen synthesis (16) in which 50 μg/ml ascorbic acid and 20% fetal calf serum were included in the culture medium were employed. A recent report suggested that scleroderma fibroblasts in culture can be stimulated by 10% fetal calf serum to

TABLE VII
Production of Collagenase by Scleroderma Fibroblasts

Cell line	Immunoreactive collagenase*				sp act*	
	ng/μg medium protein	*% of the controls*	*ng/μg cell protein*	*% of the controls*	*cpm/ng‡*	*% of the controls*
Expt. 1						
Controls	15.3±4.2		8.1±3.1		18.0±4.6	
WUS 76118	18.8±5.2	(123)	8.0±3.0	(99)	14.0±3.1	(78)
WUS 77269	17.3±10.8	(113)	5.0±2.7	(62)	18.8±5.0	(104)
Expt. 2						
Controls	16.5±5.3		8.6±2.9		18.0±16.0	
WUS 77213	12.0±7.7	(73)	8.4±3.2	(98)	ND	—
WUS 77221	47.5±40.8	(288)	8.4±6.6	(98)	22.0±4.0	(122)
Expt. 3						
Controls	44.4±11.0		15.6±3.4		16.4±3.4	
WUS 77284	38.0±8.0	(86)	15.3±5.3	(98)	11.6±3.4	(71)
WUS 77222	49.8±29.4	(112)	18.5±10.1	(119)	19.2±12.4	(117)
Expt. 4						
Controls	5.5±1.7		2.8±0.5		15.8±2.8	
WUS 77266	8.1±3.7	(147)	8.4±3.7	(300)	13.4±6.2	(85)
WUS 78313	1.6±0.1	(29)	0.8±0.3	(29)	17.4±5.4	(110)
Mean±SE		121±27		113±29		98±8

* Mean±SE.

‡ Procollagenase was activated by employing a range of trypsin concentrations (0.1–2.0 μg trypsin/100 μl enzyme sample) to ensure complete proteolytic activation. After 10 min at 25°C, a fivefold excess of soybean trypsin inhibitor was added and the assay was carried out on ^{14}C-labeled collagen substrate gels (21). Collagenase activity is expressed as the total counts per minute ^{14}C-collagen solubilized per nanogram immunoreactive enzyme protein in a 5-h assay at 37°C.

produce more collagen than control cells (34). However, our preliminary studies[2] employing several fibroblast lines used in this report have failed to demonstrate significant differences between control and scleroderma fibroblasts in the sensitivity to stimulation by varying concentrations of fetal calf and human serum.

In the present study, the increased biosynthesis of procollagen was characterized further by examining the distribution of newly synthesized type I and type III procollagens in the culture medium. The ratio of these two genetically distinct procollagens was unaltered in scleroderma. In normal human skin ≅80–85% of the collagen is type I, whereas some 10–15% of the collagen consists of type III (19, 32). The remaining 5% or less consists of minor forms of collagen, such as type IV, A-B, and type I-trimer collagens (19, 35, 36). Thus in vitro, both normal human fibroblasts and cells derived from scleroderma skin synthesize type I and type III procollagens in a ratio of ≅5:1, a value reflecting the relative rates of in vivo procollagen synthesis. In this regard our observations do not corroborate a previous immunofluorescence study (37) suggesting that the proportions of genetically distinct collagens in the skin may vary according to the histopathologic stage of the disease, whereas the results are in agreement with a recent biochemical study (38) demonstrating no difference in the relative proportions of dermal type I and type III collagens in scleroderma.

The newly synthesized type I and type III procollagens were also examined with respect to the stability of the triple-helical portion of the molecule. As judged by their resistence to limited proteolytic digestion with pepsin under nondenaturing conditions, the collagen portion of both type I and type III procollagens appeared to be in a stable triple-helical conformation. Furthermore, analysis of the [^{14}C]-hydroxyproline and [^{14}C]proline in the triple-helical collagen portion of the type I and type III procollagen molecules demonstrated no significant differences in the degree of prolyl hydroxylation between scleroderma and the control cultures. On the basis of these observations, it appears that the increased biosynthesis of procollagen by the scleroderma fibroblasts is directed towards the synthesis of both type I and type III procollagens in the same relative ratio. These

[2] Uitto et al. Unpublished observations.

procollagens also appear to be similar to the corresponding molecules synthesized by the control fibroblast cultures.

The precise mechanisms involved in increased procollagen synthesis by scleroderma fibroblasts are not fully understood. It has been suggested that immunologic mechanisms, mediated through lymphokines, can stimulate collagen synthesis by these cells and, therefore, may play a role in scleroderma (39–43); however, the significance of such lymphocyte-mediated events requires further clarification. If the alterations in the rate of collagen biosynthesis were caused by immunological events, then theoretically, immunosuppressive agents might be useful in the early prevention of this disease. Alternatively, some of the newer developments in the control of collagen production, such as use of proline analogues which specifically inhibit the synthesis of triple-helical collagen molecules (44), might also be of value in the management of scleroderma.

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