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**Research Article**

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# Increased Collagen Synthesis by Scleroderma Skin Fibroblasts In Vitro

## A POSSIBLE DEFECT IN THE REGULATION OR ACTIVATION OF THE SCLERODERMA FIBROBLAST

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**ABSTRACT** Cultures of dividing skin fibroblasts from normal and sclerodermatous human skin have permitted estimations of soluble collagen concentration, net collagen accumulation, cell-doubling times, and the comparison of morphologic and ultrastructural characteristics. In vitro, the scleroderma fibroblast produces more soluble collagen, synthesizes collagen more rapidly, and fourfold more of its protein synthetic activity is directed to collagen production than in the normal skin fibroblast. Cell-doubling times and morphologic and ultrastructural observations of cells in culture have not provided clues to the nature of the biologic defect in the regulation or activation of collagen synthesis by the scleroderma fibroblast.

### INTRODUCTION

Scleroderma (systemic sclerosis) is a multisystem disorder of connective tissue characterized by extensive induration and fibrosis of skin and by a distinctive proliferative reaction of blood vessels in many internal organs (1, 2). Despite numerous histochemical, ultrastructural, and biochemical studies, the pathogenesis of these abnormalities remains unclear. To pursue the mechanism of fibrosis in scleroderma, diploid fibroblasts from the affected skin of subjects with scleroderma have been grown in tissue culture and analyzed for the capacity to synthesize selected components of connective tissue. In an initial report, confluent cultures of scleroderma fibroblasts contained more soluble collagen (hydroxyproline in the media) and glycoprotein (hexosa-

mine and sialic acid) than did paired cultures of normal skin fibroblasts (3). The present report provides further data demonstrating: (a) increased soluble collagen in dividing scleroderma skin fibroblast cultures; (b) increased incorporation of [<sup>14</sup>C]proline into collagen hydroxyproline by scleroderma fibroblasts; (c) similar patterns of cell growth, turnover, and morphology of scleroderma and normal skin fibroblasts; and (d) the persistence of an increased capacity to synthesize collagen by scleroderma fibroblasts after up to 15 subcultures. These data suggest that the disorder scleroderma may represent a basic defect in the biologic regulation or activation of collagen metabolism at the cell level.

### METHODS

Techniques of patient selection, informed consent, skin biopsy, and cell growth in explant cultures were similar to those previously described (3). Scleroderma subjects were chosen for study if (a) forearm skin was hidebound and devoid of epidermal appendages and (b) patients were receiving no medications known to affect connective tissue metabolism (e.g., steroids). Subjects with healthy skin were selected to match the age, race, and sex of scleroderma patients and the biopsy site was duplicated. After informing all subjects of the investigative nature of the procedure, elliptical biopsies were taken under local anesthesia (1% Xylocaine) with sterile precautions and transported to the laboratory in sterile culture dishes. A portion of the biopsy was fixed for histologic evaluation. Under sterile conditions, fat and hair were removed and 1-mm cubes of skin were cut and placed in sterile T-flasks (Bellco Glass, Inc., Vineland, N. J.) under perforated cellophane. These explants were grown in serum-enriched media containing glutamine, penicillin, and streptomycin in a 5% CO<sub>2</sub>, 37°C, 50% humidity atmosphere. Cells were harvested from explant cultures by trypsinization and propagated in monolayer subcultures on glass. The clinical impression of nor-

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mal and scleroderma skin was confirmed by histologic examination in all cases.

The data of the present report was obtained from cell lines developed from eight skin biopsies of five scleroderma patients and eight control biopsies of eight subjects with no apparent skin disease. All cell lines, skin biopsies, and subjects were different from those of the previous report because of a freezer failure which destroyed the earlier cell lines. All patients with scleroderma had progressive cutaneous involvement of 6-12 mo duration.

**Experimental protocol.** In the present study, experiments were begun at the time of subculture from monolayer cultures. Medium was removed and 0.25% trypsin in balanced salt solution was added until >95% of cells were no longer adherent (3-10 min). Cells were counted in an AO-Spencer Bright-Line Hemocytometer (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.), separated from trypsin by centrifugation, and replanted on glass T-60 flasks (Bellco Glass, Inc., 60 cm<sup>2</sup>) at equal densities (6-12 × 10<sup>3</sup>/cm<sup>2</sup> surface area) on day 0 of the experiment. 16 h and 6 days later, medium (CMRL 1066, 80%; fetal calf serum, 10%; human serum, 10% plus glutamine, penicillin, and streptomycin) was removed (day 1 and day 6 medium) and replaced. After removal of medium at day 12, cells were washed with Hank's solution, harvested by layering a solution (1 mg/ml) of purified bacterial collagenase (Worthington Biochemical Corp., Freehold, N. J., type CLS) in saline until no cells remained adherent to the glass surface (viewed by inverted light microscopy). Cells were counted, centrifuged, and the collagenase supernate was assayed for hydroxyproline to determine the cell-associated or insoluble collagen. Cell pe-

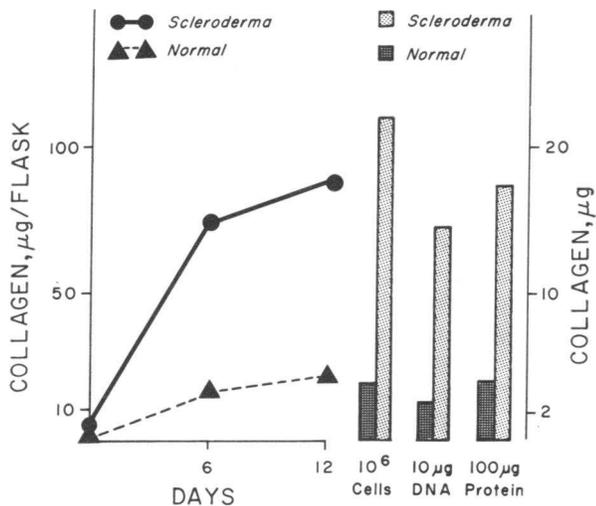


FIGURE 1 Collagen synthesis by normal and scleroderma fibroblasts in a single 12-day experiment. Data at days 1, 6, and 12 are expressed as collagen per flask. After harvest at day 12, data are expressed using the three cell parameters monitored (cell number, cell DNA, and cell protein). Consistent increases in collagen (nondialyzable hydroxyproline × 7.42) in scleroderma cultures were observed at days 6 and 12. Each point is the mean of triplicate flasks. All flasks were maintained for 12 days, and collagen per cell (bar graph on right) refers to the 12-day determinations only.

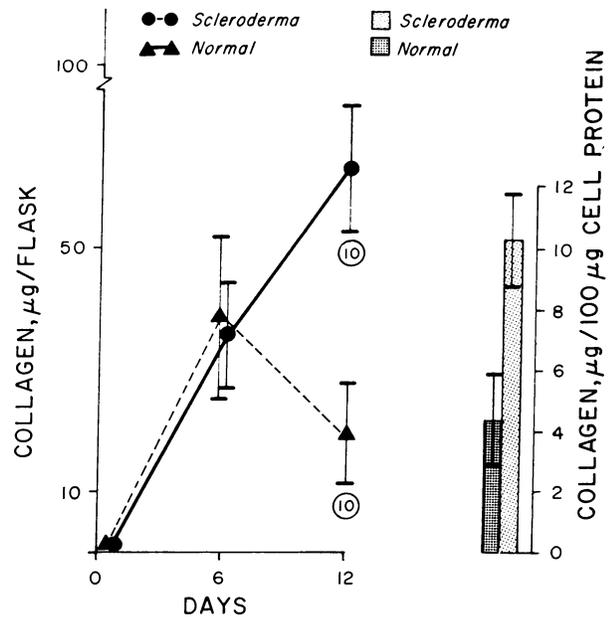


FIGURE 2 A summary of collagen synthesis in 12 day experiments. Each point is the mean of 10 separate experiments (circled numbers). Each experiment is the mean of duplicate or triplicate flasks. The brackets represent the standard error of the mean (SEM). The differences in collagen concentration between scleroderma and normal cultures is most apparent after 12 days.

lets were washed and resuspended in saline; aliquots were analyzed for cell protein (4) and DNA (5).

To determine net collagen synthesis, [<sup>14</sup>C]proline (L-[U-<sup>14</sup>C]proline, New England Nuclear, Boston, Mass., 260 mCi/mM), previously chromatographed (6) to remove labeled hydroxyproline, was added to dividing cultures at day 9 (6-10 μCi/culture). After harvest of both cells and medium at day 12, proline concentration (7), proline radioactivity (8), hydroxyproline concentration (9), and hydroxyproline radioactivity (10) were determined.

**Dialysis of medium.** Medium was dialyzed at 4°C against 300 vol of 0.9% NaCl which contained unlabeled proline and hydroxyproline (0.4 mg/ml) for 48 h and was proline- and hydroxyproline-free for a second 48 h. Dialyses were changed at 8-h intervals. Analytical determinations were carried out after acid hydrolysis.

## RESULTS

**Collagen synthesis.** A representative experiment to assess collagen metabolism in dividing skin fibroblasts is shown in Fig. 1. Cultures of scleroderma skin fibroblasts contain more soluble collagen (medium nondialyzable hydroxyproline) at day 12 than do cultures of normal skin fibroblasts. Differences are significant when expressed both as collagen per flask or as collagen per cell using any of three cell measurements (cell numbers, cell DNA, or cell protein). As shown in Fig. 1, a close correlation was found between the three cell determinations. Consequently, the data of this report are expressed as

TABLE I  
*Determination of Collagen Synthesis by [<sup>14</sup>C]Proline Incorporation into Collagen Hydroxyproline\**

Experiment	dpm/flask		dpm/10 <sup>6</sup> cells	
	Normal	Scleroderma	Normal	Scleroderma
1	9,800	17,400	3,391	9,158
2	8,200	12,200	2,837	6,421
3	8,250	14,200	3,125	7,282
4	8,070	13,200	3,057	6,769
5	14,900	23,000	3,697	3,566
6	15,000	28,100	3,722	4,357
7	12,600	22,300	2,857	3,022
8	1,900	20,800	4,308	2,818
9	44,600	62,900	5,547	7,230
10	32,100	50,900	3,993	5,851
11	34,700	61,700	4,971	7,880
12	40,000	41,800	5,731	5,338
Mean±SEM	20,602±3,886	30,708±5,409	3,936±291.2	5,808±584
<i>P</i>		<0.001		<0.01

\* Trypsinized early subcultures were fed at day 6 and labeled proline was added at day 9 as described in Methods. Radioactivity incorporated into soluble collagen (nondialyzable hydroxyproline) was determined in medium at day 12.

collagen per flask, collagen per 10<sup>6</sup> cells, or collagen per 100 μg cell protein.

Fig. 2 is a summary of 10 separate experiments comparing soluble collagen in scleroderma and normal skin fibroblasts. As in previous experiments, the increased amount of soluble collagen in scleroderma skin fibroblasts is most apparent after 12 days when expressed either as collagen per flask or collagen per 100 μg cell protein. These data are in keeping with previous studies suggesting increased collagen synthesis by the scleroderma fibroblast in confluent cultures (3). The study of dividing cell cultures thus provides a flexible format to study mechanisms of increased collagen accumulation in scleroderma cultures, because mechanical manipulations disturb confluent cultures.

The analysis of medium obtained at day 6 provides additional information. At day 6 (Fig. 2), normal and scleroderma cultures contain soluble collagen in similar amounts; thus the distinct differences at day 12 are due both to a further increase in soluble collagen in scleroderma cultures as well as a decrease in normal cultures. The decrease in soluble collagen in cultures of fibroblasts from normal skin is as yet unexplained; experiments are in progress to study the effect of mixing cells and medium from normal and scleroderma cultures to determine the basis for these changes.

In this context, the increase in soluble collagen in scleroderma skin fibroblast cultures could be viewed as either an increase in net synthesis or a decrease in collagen degradation. To measure net collagen synthesis,

culture pairs were incubated with [<sup>14</sup>C]proline and the incorporation of label into collagen hydroxyproline was measured. These data are shown in Table I. They demonstrate that the net synthesis of soluble collagen is greater in each of 12 scleroderma cultures when compared with its normal culture pair. The combined data are significantly different by Student's *t* test.

Thus the increased soluble collagen in cultures of scleroderma skin fibroblasts is due, at least in part, to an increased capacity of the scleroderma fibroblast to synthesize collagen. The form in which this increased collagen is secreted by the scleroderma fibroblast is unknown and currently under study. Direct measurements of free intracellular proline, carried out in association with ΔC/ΔP determinations (*vide infra*), showed the proline pool of normal and scleroderma fibroblasts to be indistinguishable. Also, collagenase determinations at days 6 and 12 in serum-free normal and scleroderma media have been negative (performed by E. D. Harris). In the absence of alterations of intracellular precursor pool size or of degradative activity, the increased label incorporated into scleroderma soluble collagen would appear to indicate increased net synthesis.

*Effect of subculture.* Paired cultures were studied at the same subpassage in all experiments. The effect of continued subpassage was studied by comparing the soluble collagen produced by subcultures between the 2nd and 11th subpassage, as shown in Fig. 3. The mean duration of each subpassage was 7 days. Soluble collagen in scleroderma cultures remained increased throughout

the 11 subcultures observed; there was the suggestion that the difference between scleroderma and normal fibroblasts was greater in the early subcultures. The enhanced collagen accumulation has been observed in scleroderma cultures for up to 15 subpassages.

The persistent, although declining, differences between scleroderma and normal fibroblasts may be extrapolated to imply increased collagen synthesis by scleroderma fibroblasts *in vivo*. The nature of the defect in regulation or activation of the scleroderma fibroblast and its decreased expression after 11–15 subpassages is consistent with the decline of an activating stimulus (11), the selection of a cell population producing less collagen, or premature cultured cell senescence (12).

**Cell turnover.** The growth behavior of scleroderma fibroblasts was in all respects similar to the behavior of normal fibroblasts. In the experimental format used for determinations of collagen accumulation, with feedings at days 1 and 6, doubling times in 20 culture pairs were  $5.3 \pm 0.4$  (mean  $\pm$  SEM) days in normal and  $4.6 \pm 0.6$  days in scleroderma cultures, an insignificant difference. Doubling times earlier in culture (days 2–5 and 6–8) with medium change at 48-h intervals were  $2.4 \pm 0.2$  and  $2.5 \pm 0.1$  days, again showing no differences between normal and scleroderma cultures.

Morphologic characteristics of normal and scleroderma skin fibroblasts in culture have been compared by light, phase, differential interference, and electron microscopy. Figs. 4 and 5 contain representative regions of dividing fibroblast cultures observed after Wright's stain (Fig. 4a), by differential interference optics (Fig. 4b) and by electron microscopy (Fig. 5). The ultrastructure of the scleroderma skin fibroblast (Fig. 5a) in cell culture could not be differentiated from that of the normal skin fibroblast. A finely fibrillar extracellular matrix (Fig. 5b) was observed in increased amounts surrounding scleroderma fibroblasts when photomicrographs were observed without knowledge of the cell source. The nature of this fibrillar material, found in both normal and scleroderma cultures, awaits further definition. No intracellular ultrastructural differences could be observed between normal and scleroderma skin fibroblasts grown in monolayer cultures, and intracellular collagen fibrils were not seen.

**Collagen and total protein synthesis.** Collagen synthesis in the fibroblast cell lines was evaluated in relation to total protein synthesis, using the approach of Green and Goldberg ( $\Delta C/\Delta P$ , [13]). This approach involves determining the ratio of labeled amino acid incorporated into collagen to that incorporated into all proteins. The results of four experiments are shown in Table II. In normal skin fibroblasts the mean  $\Delta C/\Delta P$  of four experiments was 4%; in scleroderma skin fibroblasts the mean  $\Delta C/\Delta P$  was 18%. Thus in normal fibroblasts

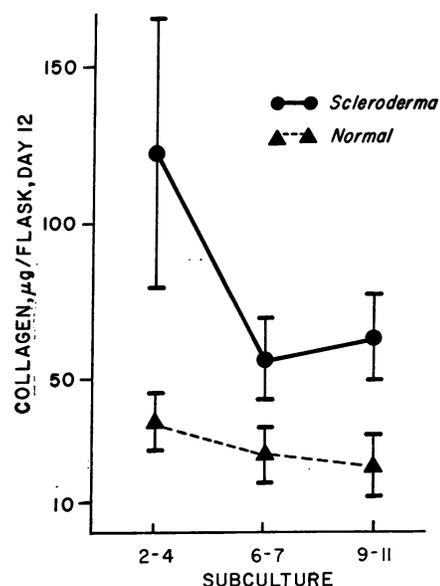
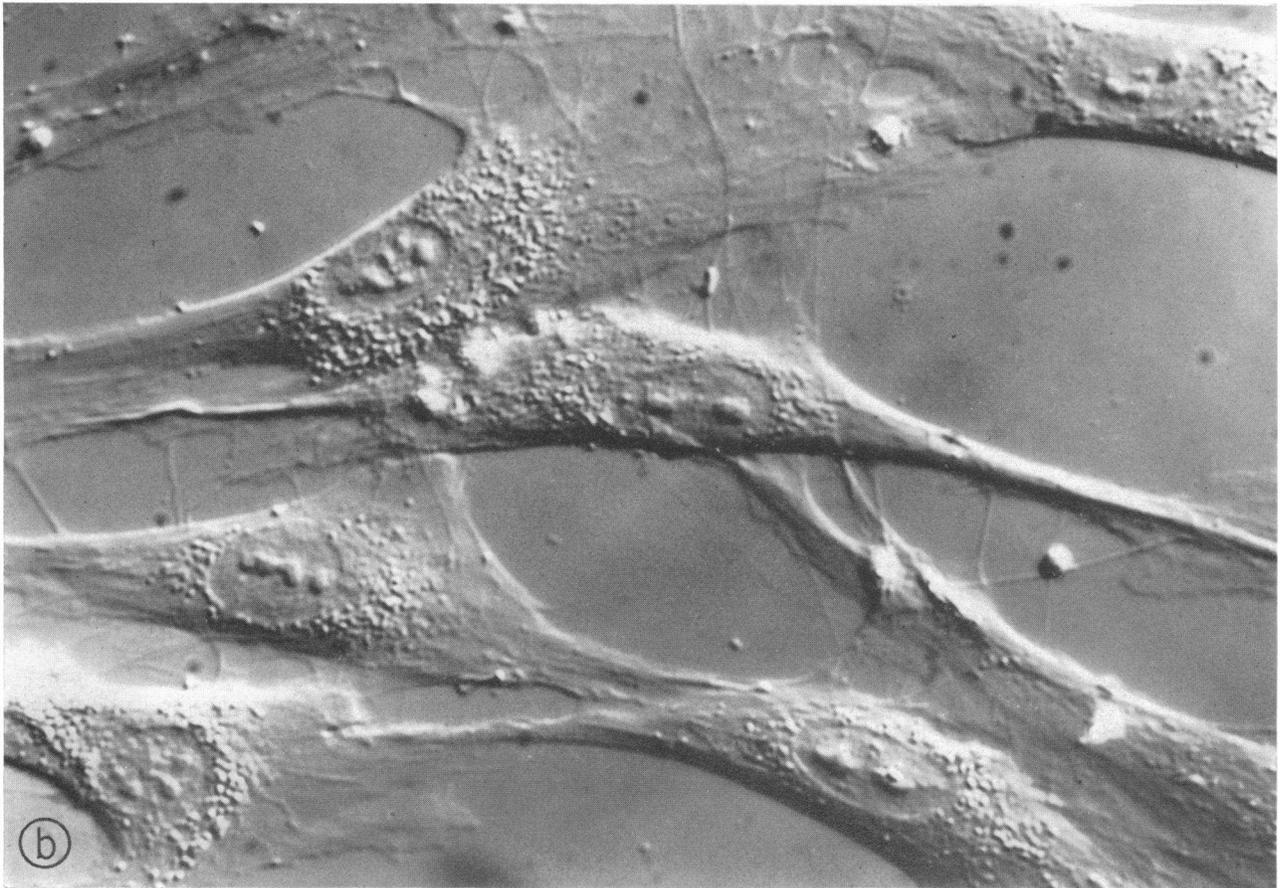
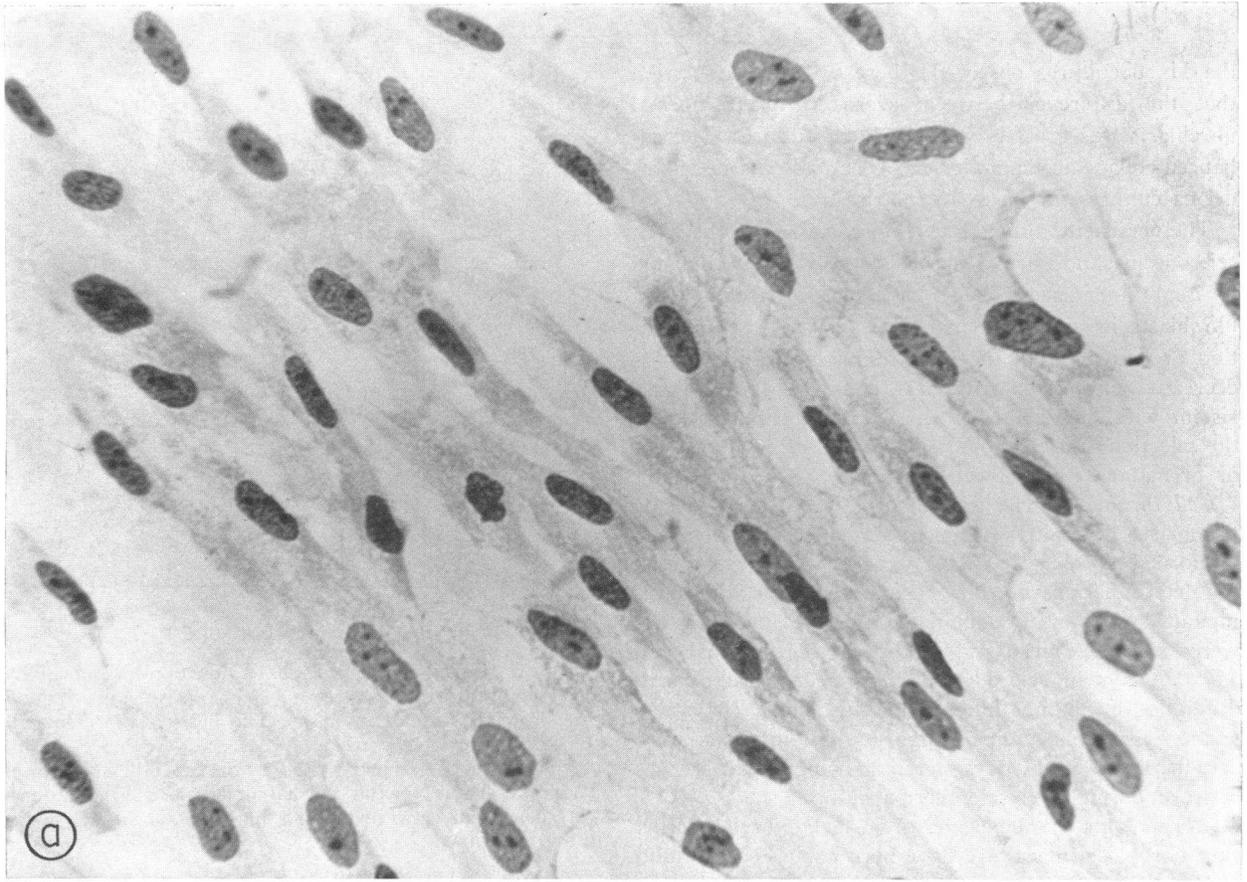


FIGURE 3 Collagen synthesis as a function of subculture. Each point represents three to five experiments of duplicate or triplicate culture flasks. The bracket represents the standard error of the mean. Differences are more marked at subcultures 2–4 than at 6th–7th or 9th–11th subcultures. The differences between normal and scleroderma cultures were consistent between the 2nd and 11th subculture studied in this experiment.

less than 1/20th of the protein synthesized is collagen whereas in scleroderma fibroblasts approximately 1/5th of the protein is collagen. These determinations, carried out on harvested cell pellets of fibroblast cultures, suggest that scleroderma fibroblasts in culture accumulate four times more collagen than do normal skin fibroblast with similar levels of total protein synthesis. Because the already demonstrated levels of collagen secreted into the media are increased in scleroderma fibroblasts, the determination of  $\Delta C/\Delta P$  would underestimate the commitment to collagen synthesis by the amount secreted. This consideration would further enhance the demonstrated differences between scleroderma and normal fibroblasts in cultures.

**Insoluble collagen.** The use of collagenase in the harvest of cells at 12 days permitted the determination of insoluble or cell-associated collagen (hydrolysis of collagenase supernate = insoluble collagen). Probably because of the short time of subculture (12 days), levels of insoluble collagen were often below the level of analytical reproducibility of the hydroxyproline assay. No differences were noted between normal and scleroderma cultures; this is consistent with the data reported previously from confluent cultures in which the levels of insoluble collagen were higher, more reproducible, and the same in normal and scleroderma cultures (3).



## DISCUSSION

The present study provides direct evidence for increased collagen synthesis by skin fibroblasts from patients with scleroderma. These data extend several indirect lines of evidence of an abnormality in connective tissue metabolism in this disorder. This abnormality of collagen synthesis has both pathologic and biologic implications. It can explain the pathological features (diffuse proliferation and fibrosis) of this microvascular and connective tissue disorder and it may represent a defect in the biologic regulation and/or activation of collagen synthesis at the cell level useful in the understanding of the control of connective tissue metabolism.

Early studies of connective tissue in scleroderma focused on the gross structural features of collagen. Scleroderma collagen was identical to normal collagen by amino acid composition (14), X-ray diffraction pattern (15), and banded periodicity by electron microscopy (16). Studies of collagen solubility found neutral salt-soluble collagen, which is newly synthesized collagen, to be increased in some scleroderma skin samples (17). Systematic studies of the nature of the cross-links in collagen from the skin of scleroderma subjects have not been done; preliminary studies have not revealed unusual types of linkages.

Morphologic studies of scleroderma skin have provided variable results. Statements such as "densely packed fibrils" and "eosinophilic changes" have not always been reproducible (18). The observation of a distinctive fibril with definite ultrastructural characteristics has been made by several laboratories, revealing a population of small, beaded fibrils similar to fibrils in embryonic skin and suggestive of newly synthesized collagen fibrils (19, 20). These fibrils have been localized to the subdermal connective tissue (16). Conclusions from morphologic studies are that new collagen synthesis in the subcutaneous tissues leads to the hide-bound induration characteristic of scleroderma skin. The direct demonstration of increased collagen synthesis in the present report confirms these morphologic suggestions.

Selected steps in collagen synthesis have been analyzed in scleroderma. Proline hydroxylase levels have been variably elevated in skin biopsy material from scleroderma patients, as well as skin tissue from several other connective tissue syndromes (21, 22). The incorporation of labeled proline, estimated either as overall incorporation or as hydroxyproline counts, has been

increased in about 50% of the observations made (23). Variations in skin samples may affect these studies; a histologic diagnosis of scleroderma was not always required and the tissue samples could vary significantly in the number of fibroblasts present. Many determinations were based on tissue weight rather than cells present. Thus proline hydroxylase determinations have not given a consistent picture of collagen synthesis, perhaps because the hydroxylation of proline may not be rate-limiting in collagen synthesis. The morphologic, collagen solubility, and proline hydroxylase studies taken together point to increased collagen synthesis in scleroderma; the direct demonstration of increased synthesis in the present report supports the hypothesis that, in scleroderma, fibroblasts produce collagen in excess by unknown mechanisms.

Alterations in noncollagenous connective tissue has long been suspected in scleroderma. Morphologic studies of skin and subcutaneous tissue suggest that the small subcutaneous fibrils are surrounded by an excess of ground substance (14); moreover, areas of fibril-free ground substance have also been observed (24). Initial chemical analyses of scleroderma skin indicated an increase in hexosamine content bound to collagen (25). An increased concentration of hexosamine and sialic acid suggesting increased glycoprotein content has been demonstrated in tissue culture studies (3). These two suggestions of a possible abnormality of glycoprotein metabolism in scleroderma warrant further study.

Two recent studies of glycosaminoglycan concentration in scleroderma skin, using cetylpyridinium chloride precipitation and resolution in buffers of increasing ionic strength, have observed an increase in total glycosaminoglycan concentration. One laboratory found chondroitin-4,6-sulfate (26) whereas the other found dermatan sulfate (27) to be the fraction responsible for the increase. Whether enhanced synthesis or reduced degradation leads to increased glycosaminoglycan concentration remains to be determined. In vitro studies of soluble glycosaminoglycan concentration showed similar levels in normal and scleroderma fibroblast cultures (3).

What role do these abnormalities of connective tissue play in scleroderma? Is the defect in collagen synthesis primary or secondary to the microvascular pathology? Careful analysis of the microvascular lesion suggests that abnormal collagen production could be important

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FIGURE 4 Morphologic observations of normal and scleroderma fibroblasts in vitro. (a) Coverslip preparation of scleroderma skin fibroblasts 6 days after the sixth subpassage. (Giemsa stain, light microscopy  $\times 500$ .) Normal fibroblasts were similar in appearance. (b) Differential interference microscopy of a coverslip preparation of normal skin fibroblasts 6 days after the sixth subpassage. Scleroderma fibroblasts were similar in appearance. (Magnification  $\times 1,000$ .)

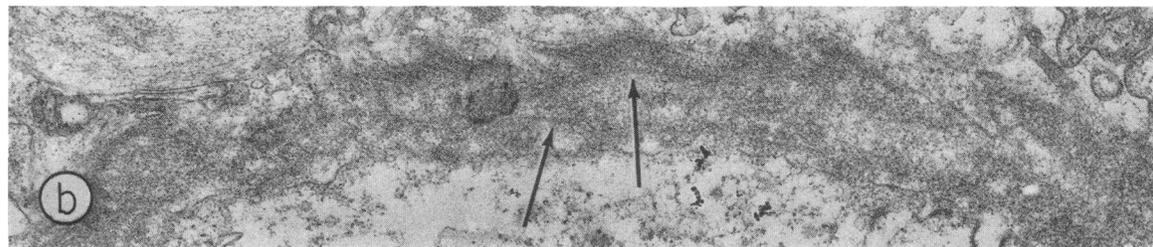
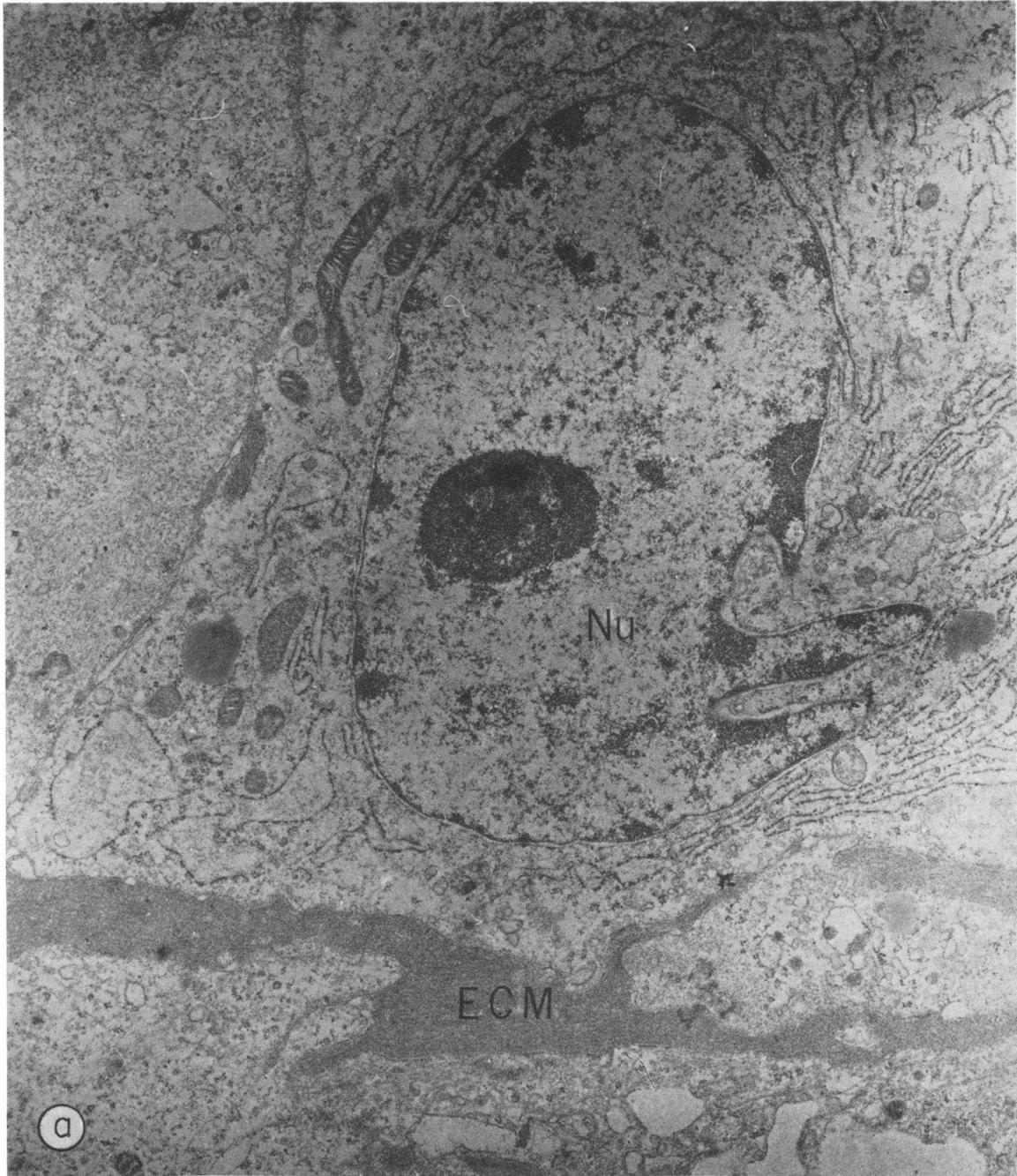


TABLE II  
*Collagen and Protein Accumulation in Normal and Scleroderma  
 Fibroblast Cell Pellets*

Experiment	$\Delta C$ (dpm/flask)		$\Delta P$ (dpm/flask)		$\Delta C/\Delta P$ %	
	NL	SD	NL	SD	NL	SD
1*	23,917	124,706	525,635	759,459	5.0	16.4
2*	42,025	183,811	831,850	459,964	5.1	40.0
3‡	615	3,485	23,831	48,865	2.6	7.1
4‡	685	4,100	40,426	45,150	1.7	9.9
Mean	16,811	78,900	355,435	328,360	3.8	18.1

\* Technique of Peterkofsky and Prockop (8).

‡ Technique of LeRoy, Harris, and Sjoerdsma (10).

Fibroblasts were grown in dividing cultures for 12 days with media change at days 1 and 6. At day 9, 10  $\mu\text{Ci}$  [ $^{14}\text{C}$ ]proline was added to each culture. Cells were washed and harvested mechanically. No attempt was made to separate cells and extracellular insoluble proteins.

in its pathogenesis. Three prominent histological abnormalities are noted in arteries between 150 and 500  $\mu\text{m}$  in diameter. These are: (a) intimal proliferation with deposition of glycoprotein (periodic acid-Schiff and Alcian blue positive), (b) medial thinning, and (c) adventitial thickening with a cuff of collagen fibrils (2). The increased synthesis of collagen by scleroderma fibroblasts would promote the diffusion of soluble collagens through the vessel wall to the intimal surface where collagens are known to stimulate platelet and perhaps leukocyte adhesion and aggregation (28); the release of platelet and leukocyte lysozymes may damage the intima and prompt endothelial reactions which further increase collagen synthesis (29). Thus the intimal proliferation of scleroderma could be initiated by an increased accumulation of collagen. Medial thinning may occur after intimal proliferation and secondary to it; smooth muscle cells have been shown to migrate to the intima and produce connective tissue in response to intimal injury (30). An adventitial cuff of collagen, synthesized and secreted by surrounding fibroblasts, may also impede normal vascular distensibility with consequent vasomotor instability.

Taken together, the adventitial fibrous cuff and the intimal proliferative response can explain the vasomotor and microvascular abnormalities resulting in progressive devascularization in scleroderma. The etiologic and primary pathogenic events are still unknown, but

a defect at the fibroblast level such as the enhanced collagen synthesis demonstrated in the present report is consistent with the clinical events observed in vivo and sufficient to explain the observed pathology.

The level of regulation of these abnormalities of connective tissue metabolism in scleroderma is not known. A promising approach to understand the control of collagen synthesis lies in the identification of procollagen, a precursor of collagen with nonhelical *N*-terminal peptides linked by disulfide bonds (31). These peptides serve as registration peptides in the assembly of collagen fibrils and might also serve to limit new collagen synthesis after their cleavage from collagen by an enzyme or enzymes termed procollagen peptidase. Evidence for a control function lies in the high levels of new collagen synthesis by fibroblasts from children with an inherited disorder of connective tissue associated with a deficiency of procollagen peptidase. The reduced levels of procollagen peptides, together with the increased levels of collagen synthesis, prompt the hypothesis that these peptides might be regulatory in collagen synthesis (32).

It has been a striking feature of human fibroblasts in tissue culture that most of the collagen secreted into the media is in the form of polymeric procollagen (33-34). The formation of procollagen by scleroderma fibroblasts has not yet been studied, but procollagen provides a potential control mechanism of the collagen

FIGURE 5 (a) Electron photomicrograph of scleroderma fibroblast in cell culture. The scleroderma fibroblast was similar to normal fibroblasts in subcellular structure and organization. The amount of extracellular matrix around scleroderma fibroblasts (arrows) was greater than around normal fibroblasts. (Uranyl acetate-lead citrate stain,  $\times 15,000$ .) (b) A higher magnification of the extracellular matrix around the scleroderma fibroblast. There is a finely fibrillar appearance to the extracellular material. (Uranyl-acetate-lead citrate stain,  $\times 50,000$ .)

synthesis of scleroderma fibroblasts. Procollagen levels in scleroderma fibroblast cultures are currently under study in this laboratory.

The scleroderma fibroblast can now be approached as a cell in which the regulation of connective tissue synthesis is defective. Possibilities for the site of this defect include: (a) the production of abnormal connective tissue components unable to exercise feedback inhibition; (b) the incomplete cleavage of connective tissue components, such as procollagen, the products of which cleavage normally exert feedback control; (c) an inability of the scleroderma fibroblast to respond to the usual stimuli for feedback control; or (d) an abnormality of control at the transcriptional or translational level. These possibilities are amenable to experimental testing; thus the scleroderma fibroblast provides an opportunity to study the mechanism of connective tissue regulation from both a biological and a pathological point of view.

The scleroderma fibroblast can also be approached as a cell in an exaggerated state of activation. Connective tissue activation has been studied in the synovial tissue of subjects with rheumatoid arthritis, using hyaluronate synthesis, lactate formation, and glucose uptake as estimates of fibroblast activation (11). A connective tissue activating peptide, whose activity may be mediated through a prostaglandin and/or cyclic AMP, has been described which induces metabolic hyperactivity in cultured normal synovial cells resembling that seen in rheumatoid synovitis. Such a state of connective tissue activation could explain the present observations in scleroderma. Cell-mixing experiments may help to select the most appropriate path for future investigation.

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