CONNECTIVE TISSUE SYNTHESIS BY SCLERODERMA SKIN FIBROBLASTS IN CELL CULTURE

By E. CARWILE LEROY

(From the Department of Medicine, Columbia University College of Physicians and Surgeons, and the Edward Daniels Faulkner Arthritis Clinic of the Presbyterian Hospital, New York 10032)

Scleroderma (systemic sclerosis) is a disorder of connective tissue affecting skin and a variety of internal organs (1, 2). Despite remarkable variation in the site, rate, and degree of involvement in the individual subject, tight skin is the clinical hallmark of the disease. Repeated studies using a variety of techniques (histochemical, electron microscope, and biochemical) have failed to define the defect in skin connective tissue; its etiology and pathogenesis are unknown (3). There remains considerable disagreement concerning the thickness of scleroderma skin (4), the appearance of its connective tissue fibers (3), and the relative extractability of collagen from it (5, 6). Promising studies of both collagen and carbohydrate biochemistry either have not been confirmed or remain insufficiently characterized (5, 7). These direct studies of skin biopsy material are limited by both technical and clinical considerations.

Abnormalities of connective tissue that persist in cell culture could be approached from the standpoint of the nature of the abnormal tissue and the mechanism of its development. Tissue culture techniques have been applied effectively to the metabolic study of both heritable (Hurler's syndrome, galactosemia, acatalasemia) and acquired (rheumatoid arthritis, amyloidosis) disorders of man (8, 9).

The present study reports the establishment in vitro of cell strains of skin fibroblasts from subjects with scleroderma paired with strains from subjects with normal skin. A consistent increase in soluble collagen was observed in scleroderma skin cell cultures. Glycoprotein content, estimated by hexosamine and sialic acid determinations, was also increased. Low levels of uronic acid were observed. These are the basis for attributing the hexosamine changes to glycoprotein, and they suggest that the scleroderma skin cells were not synthesizing chondromucoprotein in quantity. The significance of these observations and the advantages and limitations of the cell culture technique are considered.

Materials and Methods

The cell culture techniques were developed from those of the laboratory of Dr. C. W. Castor, with his generous help (9, 10). All reagents were of analytical quality or higher.

Culture Materials.—The media used to develop cell lines consisted of: CMRL-1066 (lyophilized, reconstituted in double-distilled water), 80%; fetal calf serum, 20%; and penicillin

(50 μg/ml), streptomycin (50 μg/ml), and L-glutamine (20 mM/ml) (Grand Island Biological Co., Grand Island, N. Y.). Cells were cultured in T-flasks with V bottoms (Bellco Glass, Inc., Vineland, N. J.). Pronase (B grade), trypsin (0.25% sterile), and collagenase (CLS, 200 units/mg) were obtained from Calbiochem, Los Angeles, Calif., Grand Island Biological Co., and Worthington Biochemical Corp., Freehold, N. J., respectively.

Skin Specimens.—Scleroderma skin was obtained by biopsy. Control skin was obtained at operation from subjects with no apparent skin disease. Control skin was matched with scleroderma skin for the age and sex of the subject and the anatomic site from which the specimen was taken, with one exception (see Subjects Studied).

Cell Culture Technique.—For primary explants, fresh sterile skin was cleaned of subcutaneous fat, cut into 1-mm pieces, placed in T-flasks under perforated cellophane sheets with media, and grown for 2–4 wk at 37°C in an atmosphere of 5% CO_2 and air. Explants were subcultured when a "swirl" of fibroblasts appeared around each explant. Monolayer subcultures were obtained from explant cultures by treating the latter with trypsin (1 mg/ml in balanced salt solution) at 37° for 5–30 min to free cells from the glass surface. Cells were separated by centrifugation and resuspended in growth media by repeated aspiration into a pipette. Monolayer cultures were grown in serum dilution bottles until the stationary phase of growth was observed microscopically.[1]

Cell Culture Harvest.—After experimental periods of 7, 9, or 12 days, the stationary cultures were divided into media and cell fractions. The media were aspirated from the culture bottles, centrifuged to remove particulate matter, measured volumetrically, and stored at 4°C. The cell monolayer was washed with saline, harvested with a rubber policeman, and homogenized at 0°C in 2 ml of saline with a Duall tissue homogenizer with a motor-driven pestle (Kontes Glass Co., Vineland, N. J.). Homogenization periods of 10–15 sec were alternated with 1–2-min cooling periods to prevent local heating. Companion experiments were carried out to compare the use of cell counts and DNA measurements to estimate the cells present. After the media had been removed, the cell layer was treated with collagenase (1 mg/ml saline, 30 min, 37°C), which dispersed the cells uniformly and allowed reproducible counts in a Neubauer hemocytometer (Neubauer Manufacturing Co., Minneapolis, Minn.).

Media and Cell Homogenate Analyses.—The media were analyzed for hydroxyproline, hexosamine, uronic acid, and sialic acid; the cell homogenates, for DNA and hydroxyproline, as follows:

Hydroxyproline: The procedure of Prockop and Udenfriend (12) was the primary technique used; all determinations were carried out in duplicate or triplicate and monitored for interfering materials at OD 450. Selected samples were also assayed by the modified procedure of LeRoy et al. (13) to check for materials previously shown to interfere with authentic hydroxyproline determination. The two procedures agreed within 5%; interfering material (OD 450) was not observed. In separate determinations, fetal calf serum was found to contain only small quantities of the previously described collagen-like protein of serum (13).

DNA: DNA was determined in the cell pellet as an estimate of cells by the procedure of Burton (14). The standard was calf thymus DNA (Pierce Chemical Co., Rockford, Ill.), DNA was not detectable in the media.

Hexosamine, uronic acid, and sialic acid: The recognized interference of large quantities of protein was avoided by extensive digestion of the media with pronase (23°C, 72 hr, 125 μg enzyme/ml media) followed by separation of the carbohydrate-containing glycopeptide peak on a Sephadex G-25 column (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) with volatile buffers (15). The carbohydrate-containing excluded peak was lyophilized. Weighed aliquots were used for carbohydrate determinations.

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[1] For this study the stationary phase is defined as the presence of both dividing (mitosis observed) and dying (floating or pyknotic) cells in the same confluent culture (11).

Hexosamine: Initial determinations were carried out using the column procedure of Boas (16) and the direct procedure of Swann and Balazs (17). Using pronase-treated media, these procedures gave indistinguishable results; the Swann and Balazs procedure was used routinely. All determinations were carried out in duplicate and were monitored for interfering material at OD 565. The standard was galactosamine (J. T. Baker Chemical Co., Phillipsburg, N. J.).

Uronic acid: The technique of Dische (18) was used with pronase-treated media. The standard was D-glucuronic acid (J. T. Baker Chemical Co.).

Sialic acid: The procedure of Warren (19) was used, with pronase-treated media. The standard was N-acetyl neuraminic acid (J. T. Baker Chemical Co.).
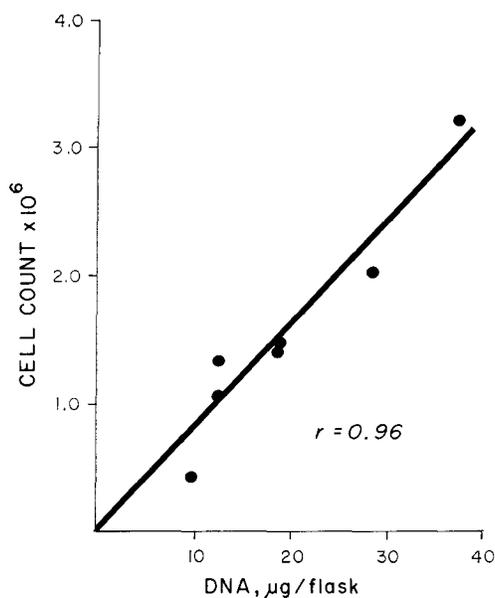

FIG. 1. Plot of cell count in relation to DNA determination in control and scleroderma cell cultures. The correlation coefficient (r) was 0.96. The regression equation was $y = 0.08 \, x +$ ($-$ 0.06). See Materials and Methods for description of techniques.

RESULTS

Validity of Procedures.—The validity of DNA measurements as an estimate of cells was confirmed by relating DNA values to total cell counts after extensive digestion with collagenase to free the cells from the extracellular matrix. In Fig. 1, cell counts are plotted against DNA measurements; there is a linear correlation between the two ($r = 0.96$). DNA was used routinely as a measure of cells present in a monolayer culture; analyses for connective tissue components were expressed as a function of the DNA content of the culture (20). The data in Fig. 1 indicate a mean DNA level of 11–12 pg/cell, a value consistent with previous determinations (21).

Subjects Studied.—Selected characteristics of the patients from whom skin

specimens were obtained are shown in Table I. Each specimen of scleroderma skin obtained at biopsy was paired with a sample of control skin matched for the age and sex of the donor and the anatomic site from which the specimen was taken. Cultures were then developed and studied in identically handled pairs. An exception to the anatomic matching of biopsies was an upper-thigh skin biopsy obtained from a young man with scleroderma. This specimen was

TABLE I

Clinical Data

	Age	Sex	Site	Operation
	yr			
Control (9)	20–57	8 F, 1 M	Wrist (8), forearm (1)	Varied*
Scleroderma‡ (9)	16–62	7 F, 2 M	Wrist (7), forearm (1), thigh (1)	Biopsy

* Procedures from which normal skin was obtained included: wrist fusion for trauma, ganglionectomy (2), median nerve release for carpel tunnel syndrome, open reduction of Colles' fracture (2), tendon lysis for DeQuervain's stenosing tenosynovitis, synostosis for paralytic poliomyelitis, and tenosynovectomy for rheumatoid arthritis.

‡ All subjects had scleroderma (systemic sclerosis), with involvement of the skin at the site of biopsy by both clinical and histologic criteria.

TABLE II

Cell Culture Data

	Mean primary explant culture time	Monolayer no.	Mean duration of subcultures*	Experimental period		
	days (range)		*Days (range)*		*days*	
Control (9)‡	33	3.2	30.0	7 (3),	9 (2),	12 (4)
	(25–57)	(1–6)	(7–89)			
Scleroderma (9)	30	2.3	35.3	7 (3),	9 (2),	12 (4)
	(21–40)	(1–4)	(7–80)			

* The mean duration of monolayer subcultures is the time from the original trypsin dispersion of primary explants to the beginning of the experimental period. The total days divided by the number of subcultures gives the mean duration.

‡ Number of cell cultures in each group.

in all respects similar to the specimens from the wrists and forearms of scleroderma subjects. The indications for surgery in the control subjects are shown in Table I. Skin from control subjects was clinically and histologically normal. The skin of the biopsy site in all the scleroderma subjects was hidebound, and histological findings were compatible with the diagnosis of scleroderma.

Establishment of Cell Cultures.—Parameters of cell growth are shown in Table II. The time required to establish primary explants was similar in the control and scleroderma groups. In the monolayer subcultures, however, skin

fibroblasts from scleroderma subjects required slightly longer to reach the stage of confluence than did control cells (Table II); consequently, the duration of the subcultures was slightly longer and the number of subcultures was slightly less in the scleroderma group. These qualitative observations, suggest that, in these experiments, scleroderma skin fibroblasts grew more slowly than control skin fibroblasts. Determinations of cell-doubling times are being carried out to study this possibility.

Collagen Synthesis.—Hydroxyproline determinations were used as estimates of soluble collagen in the media and of insoluble collagen in the cell pellets,

TABLE III

Collagen Assay

Experiment	Soluble		Insoluble		Total	
	Control	Scleroderma	Control	Scleroderma	Control	Scleroderma
			μg *hypro*/μg *DNA**			
1 (12 days)‡	0.5	4.0	0.2	0.4	0.7	4.4
2 (12 days)	1.3	3.4	0.4	1.0	1.7	4.4
3 (9 days)	0.8	2.9	0.2	0.4	1.0	3.3
4 (12 days)	0.4	1.6	–	–	–	–
5 (7 days)	0.3	1.2	0.03	0.1	0.3	1.3
6 (7 days)	0.6	1.1	0.5	1.4	1.1	2.5
7 (9 days)	0.2	1.1	0.4	0.4	0.6	1.5
8 (12 days)	0.1	0.7	0.4	0.2	0.5	0.9
9 (7 days)	1.0	0.6	0.1	0.2	1.1	0.8
Mean ± SEM	0.6 ± 0.1	1.8 ± 0.4	0.3 ± 0.1	0.5 ± 0.2	0.9 ± 0.2	2.4 ± 0.5
P	<0.005		NS§		<0.01	

* Expressed as the net increase in hydroxyproline per μg DNA (media hydroxyproline subtracted).

‡ Number of days cells were exposed to the particular medium analyzed.

§ NS, not significant at the 5% level, i.e., $P > 0.05$.

as shown in Table III. Media from scleroderma skin fibroblasts contained increased quantities of soluble collagen in eight of nine experiments, the increase ranging from 20 to 800%. These data are significantly different ($P < 0.005$) from control measurements, using Student's t test.

Glycoprotein Synthesis.—The net increase in hexosamine and sialic acid was determined in five culture pairs as an estimation of glycoprotein synthesis; these data are shown in Table IV. In four of five culture pairs, hexosamine and sialic acid levels were significantly higher in scleroderma cell culture media compared with control media. In no culture was there a net decrease or "consumption" of glycoprotein.

Hexosamine is a component of both glycoprotein and glycosaminoglycan; it is considered to represent largely glycoprotein in these studies because of the

low levels of uronic acid observed (Table IV). Although not as striking as the differences in collagen synthesis, the increases noted in glycoprotein synthesis in scleroderma cell cultures raise the question of abnormalities of a heterogeneous group of connective tissue glycoproteins (acidic structural proteins) in scleroderma. Because of inadequate material, insoluble glycoprotein synthesis in the cell pellet was not studied.

Chondromucoprotein Synthesis.—Uronic acid was used as an indicator of the synthesis of the glycosaminoglycan moiety of chondromucoproteins. Although four of five culture pairs showed increases in the scleroderma culture, increases and differences in uronic acid production were small. The obvious interpretation that glycosaminoglycan synthesis is low could be in error if the cell cultures were producing primarily dermatan sulfate (chondroitin sulfate B), a

TABLE IV

Carbohydrate Assay

Experiment	Hexosamine		Uronic acid		Sialic acid	
	Control	Scleroderma	Control	Scleroderma	Control	Scleroderma
			µg/µg DNA*			
1	16	50	3.8	12.9	1.4	58.4
2	69	17	3.5	4.8	22.6	22.2
3	25	38	14.6	8.4	20.0	47.2
4	13	41	1.5	5.6	8.6	33.3
5	22	49	2.2	6.2	20.3	51.6
Mean ± SEM	29.0 ± 10	39.0 ± 6	5.1 ± 2.4	7.6 ± 1.5	14.6 ± 4.1	42.5 ± 6.5
P	NS‡		NS		<0.02	

* Net increase of each determination (i.e., media level subtracted).

‡ NS, not significant at the 5% level, i.e., $P > 0.05$.

glycosaminoglycan found in skin whose uronic acid gives approximately one-third the color value of other uronic acids with the Dische method (22). Isolation and identification of the glycosaminoglycans produced in these cultures will be necessary to answer this question. Insoluble chondromucoprotein was not measured because of the inadequate quantities of cell pellet material.

DISCUSSION

In this report scleroderma skin fibroblasts in vitro have been shown to synthesize increased amounts of collagen and glycoprotein compared with control skin fibroblasts. Thus scleroderma joins an increasing number of both heritable and acquired disorders in which abnormalities are demonstrable in cells propagated in vitro, which allows more precise study of the pathogenesis and, ultimately perhaps, of the etiology of the disease.

It is not surprising that cell cultures from subjects with a large number of inherited disorders have been shown to maintain the phenotypic biochemical

abnormality in the culture setting. These disorders include those in which a specific enzyme deficiency accounts for the abnormality (acatalasemia, galactosemia) and those in which the observed abnormality cannot be readily explained by a single enzyme deficiency (Hurler's syndrome, Hunter's syndrome, cystic fibrosis [8, 23]). It is less predictable that acquired diseases, such as scleroderma, would maintain demonstrable abnormalities in tissue culture conditions; nonetheless, a precedent exists for such a hypothesis. Castor has found that synovial fibroblasts from subjects with rheumatoid arthritis, considered to be an acquired disorder, show increased hyaluronate synthesis, increased glucose uptake, and decreased suppression by glucocorticoids (9, 10). These abnormalities persist after repeated subcultures, and can be induced in normal synovial fibroblasts by a connective tissue activating principle obtained from a variety of normal tissues. Experimental amyloidosis is another acquired disorder in which the abnormality persists in tissue and organ culture. In mice stimulated to develop amyloidosis by hyperimmunization or casein administration, splenic explants were observed to deposit amyloid after 2–4 wk in an organ culture setting. Radioautographic localization of labeled tryptophan and/or leucine incorporation into the amyloid fibers confirmed the morphologic and tinctorial suggestions that amyloid was produced in vitro (24, 25).

Although these studies in the acquired disorders rheumatoid arthritis and amyloidosis provide a precedent for the present observations of a persistent increase in collagen synthesis in scleroderma, they do not shed light on the mechanism of the abnormalities observed. Among the tenable possibilities are the selection of a population of fibroblasts committed to increased collagen and glycoprotein synthesis in scleroderma skin; the persistence of increased connective tissue synthesis after the original stimulus, possibly metabolic or nutrient, has been removed; or somatic mutation induced by unknown mechanisms. Most of the apparent hypotheses are amenable to direct experimental testing.

The present observations of increased collagen synthesis are based on elevated levels of hydroxyproline in the media. Although this criterion has been extensively used by Green and Goldberg (26) as evidence of newly synthesized collagen, alternate explanations involving the degradation of collagen and hydroxyproline are possible. Further studies of the role of specific mammalian collagenase in scleroderma are contemplated. However, collagenase does not affect the capacity to detect hydroxyproline, and variations in collagenase activity would not alter the data of the present report. Man has the demonstrated capacity to degrade hydroxyproline; a study of enzymatic mechanisms by Efron and associates (27), stemming from the observation of a child with extremely high levels of free hydroxyproline in urine and blood, demonstrated the degradative pathway from hydroxyproline to hydroxyglutamic acid. Whether such a mechanism is operative in the present culture system is unknown; this question can be pursued experimentally.

Although the over-all picture of collagen synthesis in scleroderma is confusing

in existing reports, recent morphologic and metabolic data support the contention that there is enhanced collagen synthesis in the skin of scleroderma subjects. Aside from the direct data of the present report, the morphologic studies of several laboratories provide the most compelling evidence of new collagen synthesis in scleroderma skin. Bahr (28) originally described an abnormal population of small-diameter collagen fibers in scleroderma in 1956. Subsequent independent studies by Fisher and Rodnan (29) and, more recently, by Rupec and Braun-Falco (30) have confirmed the presence of these small fibers; further, Hayes and Rodnan (31) have, by negative-staining ultrastructural techniques, observed a beaded filament morphology of these thin fibers that is characteristic of embryonic collagen. This observation, coupled with morphologic evidence of active protein synthesis in the scleroderma fibrocyte, led to the suggestion that collagen may well be synthesized in excess in scleroderma skin.

Metabolic confirmation of these morphologic observations is, aside from the present study, somewhat indirect. Two lines of indirect evidence by each of two laboratories suggest increased collagen synthesis in scleroderma. First, an enzyme unique to collagen synthesis, protocollagen proline hydroxylase (which converts peptide-bound proline into hydroxyproline), is enhanced in scleroderma skin. This observation was made originally by Uitto et al. (32) and, independently, by Keiser et al. (33). It is noteworthy that the latter group observed increased levels in skin from areas (parasacral) that could represent uninvolved skin. A metabolic defect in both uninvolved and involved skin of scleroderma subjects has important implications for an understanding of the disease. It will be important to know if the data of the present report can be duplicated in clinically and histologically normal skin from scleroderma subjects. The second line of indirect evidence that collagen synthesis is increased in scleroderma skin stems from observations of increased incorporation of labeled proline by skin biopsy material. These data are more variable, only some biopsies demonstrate increased uptake, and collagen is not the only protein into which proline is incorporated. Still, increased uptake has been observed by both Laitinen et al. (34) and Keiser and Sjoerdsma (35).

Extraction studies of the solubility of collagen from scleroderma skin have given conflicting results. Harris and Sjoerdsma (5) noted decreased concentrations of acid-soluble collagen in both clinically involved and clinically normal skin of subjects with scleroderma. Subsequent studies have shown normal or increased concentrations of neutral salt-soluble collagen in scleroderma skin biopsies when compared with skin samples from normal subjects (6, 36). The most recent of these studies demonstrated twofold increases in soluble skin collagen in scleroderma patients 40–70 yr of age; 2–3% of the total skin collagen of several patients was soluble in 0.45 M sodium chloride (6). The use of a high-speed mechanical homogenizer potentially capable of collagen denaturation is a possible explanation for the latter results. These data are nonetheless consistent

with increased collagen biosynthesis in the skin of patients with scleroderma. The data of the present report are the first direct demonstration of increased collagen synthesis in scleroderma skin.

The tentative conclusion of the present study that glycoprotein synthesis is increased in scleroderma skin fibroblast cultures is based on the observed increases in sialic acid levels and the suggestive increases in hexosamine, with no change in uronic acid levels. Some caution regarding the conclusion stems from the well-documented observation that iduronic acid, a constituent of dermatan sulfate, and its derivatives give as little as 20–30% of the color of an equivalent weight of glucuronic acid (22). The presence of unusual amounts of dermatan sulfate in the scleroderma cultures could mask differences in glycosaminoglycan synthesis; isolation of the polysaccharide moieties will be necessary to resolve this possible alternate explanation for the data of the present report.

It is possible that the observed differences in extracellular matrix are related to general phenomena of cells propagated in cell culture. From fundamental investigations of cells in culture, it is felt that continued cell passage selects those cells that revert to a less-differentiated (dedifferentiated) state (11, 37). Since collagen synthesis is considered to be a function of differentiated fibroblasts, the present data might be viewed as a more rapid dedifferentiation of normal fibroblasts, leaving scleroderma fibroblasts more differentiated and making more collagen. Were this the case, normal fibroblasts would be expected to produce more protein-polysaccharide, a characteristic of the dedifferentiated state. Since four of five normal cell cultures contained less protein-polysaccharide than the scleroderma cultures, a more rapid dedifferentiation of normal skin fibroblasts is not a likely explanation for the present observations (26).

In this context, it is not possible to state the precise metabolic meaning of the increases in soluble collagen noted in the present study. In general, the finding of increased soluble or extractable collagen has been most clearly linked to increased collagen synthesis (38), and the dense fibrosis of scleroderma skin provides support for this interpretation. Experiments designed to separate synthesis from degradation will be necessary to answer these questions. It will also be of value to characterize the macromolecular structure of both the collagen and the polysaccharide produced by these cell lines. Of particular interest is the recent awareness that fibroblasts from normal human skin produce unusual ratios of α-1 and α-2 collagen subunits; moreover, up to 80% of the soluble hydroxyproline is present in the form of a large molecular weight, protease-sensitive precursor of collagen α-chains, which may be important in the regulation of collagen biosynthesis (39). These findings by several laboratories have led to the concept of "procollagen" as a precursor of collagen with a nonhelical N-terminal portion that may function in the transport of collagen from cell to fiber. The application of this interesting concept to the study of scleroderma in cell culture is under way in this laboratory.

The present study provides direct evidence supporting the inference drawn from morphologic and collagen solubility studies that collagen synthesis is increased in scleroderma skin. Also, the present model, isolated cells in tissue culture, provides an optimally controlled setting for studying the mechanism of this increased collagen synthesis in the hope of controlling the presently irreversible fibrosis of scleroderma. Preliminary studies suggest that the abnormality of collagen synthesis persists in scleroderma fibroblasts after four or more subculture passages.

After many negative investigations concerning collagen in scleroderma skin, including hydroxyproline concentration, collagen amino acid composition, and X-ray diffraction (40–42), it is encouraging that several different types of approach lead to the conclusion that collagen synthesis is increased in this disorder.

SUMMARY

Skin fibroblasts from subjects with scleroderma and control subjects were grown in tissue culture to compare the characteristics of connective tissue metabolism. A striking increase in soluble collagen (media hydroxyproline) was observed in eight of nine scleroderma cultures when they were compared with identically handled control cultures matched for the age and sex of the donor and the anatomic site of the donor skin. Glycoprotein content as estimated by hexosamine and sialic acid was also significantly increased in the scleroderma cultures. Estimations of protein-polysaccharide content by uronic acid determinations were low in all cultures and not significantly increased in scleroderma cultures.

This report demonstrates the feasibility of using fibroblast cell cultures to study chronic rheumatic and connective tissue disorders. The initial results suggest a net increase in collagen and glycoprotein synthesis in scleroderma fibroblast cultures. The implications of an abnormality of connective tissue metabolism by skin fibroblasts propagated in vitro in the acquired disorder scleroderma are discussed.

REFERENCES

1. Rodnan, G. P. 1971. Progressive systemic sclerosis (diffuse scleroderma). *In* Immunological Diseases. M. Samter, editor. Little, Brown & Co. Inc., Boston, Mass. 2nd edition. **2:**1052.
2. Heptinstall, R. H. 1966. *In* Pathology of the Kidney. Little, Brown & Co. Inc., Boston, Mass. 1st edition. 554.

3. Fleischmajer, R., V. Damiano, and A. Nedwich. 1971. Scleroderma and the sub-
cutaneous tissue. *Science (Washington).* **171:**1019.

4. Black, M. M., E. Bottoms, and S. Shuster. 1970. Skin collagen content and
thickness in systemic sclerosis. *Brit. J. Dermatol.* **83:**552.

5. Harris, E. D., Jr., and A. Sjoerdsma. 1966. Collagen profile in various clinical
conditions. *Lancet.* **2:**707.

6. Uitto, J., K. Øhlenscheäger, and I. Lorenzen. 1971. Solubility of skin collagen in
normal human subjects and in patients with generalised scleroderma. *Clin.
Chim. Acta.* **31:**13.

7. Fleischmajer, R., and S. Krol. 1967. Chemical analysis of the dermis in sclero-
derma. *Proc. Soc. Exp. Biol. Med.* **126:**252.

8. Krooth, R. S., G. A. Darlington, and A. A. Velazquez. 1968. The genetics of cul-
tured mammalian cells. *Annu. Rev. Genet.* **2:**141.

9. Castor, C. W., and F. F. Fries. 1961. Composition and function of human synovial
connective tissue cells measured in vitro. *J. Lab. Clin. Med.* **57:**394.

10. Castor, C. W. 1971. Connective tissue activation. II. Abnormalities of cultured
rheumatoid synovial cells. *Arthritis Rheum.* **14:**55.

11. Robbins, E., E. M. Levine, and H. Eagle. 1970. Morphologic changes accom-
panying senescence of cultured human diploid cells. *J. Exp. Med.* **131:**1211.

12. Prockop, D. J., and S. Udenfriend. 1960. A specific method for the analysis of
hydroxyproline in tissue and urine. *Anal. Biochem.* **1:**228.

13. LeRoy, E. C., A. Kaplan, S. Udenfriend, and A. Sjoerdsma. 1964. A hydroxy-
proline-containing, collagen-like protein in plasma and a procedure for its
assay. *J. Biol. Chem.* **239:**3350.

14. Burton, K. 1956. Study of the conditions and mechanism of the diphenylamine
reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.*
62:315.

15. Spiro, R. G. 1966. Characterization of carbohydrate units of glycoproteins.
Methods Enzymol. **8:**28.

16. Boas, N. F. 1953. Method for the determination of hexosamines in tissue. *J.
Biol. Chem.* **204:**553.

17. Swann, D. A., and E. A. Balazs. 1966. Determination of the hexosamine content
of macromolecules with manual and automated techniques using the p-di-
methylaminobenzaldehyde reaction. *Biochim. Biophys. Acta.* **130:**112.

18. Dische, Z. 1947. A new specific color reaction of hexuronic acids. *J. Biol. Chem.*
167:189.

19. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* **234:**
1971.

20. Paul, J. 1970. *In* Cell and Tissue Culture. The Williams & Wilkins Company,
Baltimore, Md. 4th edition. 350.

21. Leslie, I. 1955. The nucleic acid content of tissues and cells. *In* The Nucleic Acids.
E. Chargaff and J. N. Davidson, editors. Academic Press, Inc., New York.
2:9.

22. Davidson, E. A. 1966. Analysis of sugars found in mucopolysaccharides. *Methods
Enzymol.* **3:**52.

23. Krooth, R. S. 1969. Genetics of cultured somatic cells. *Med. Clin. N. Amer.* **53:**
795.

24. Cohen, A. S., E. Gross, and I. Shirahama. 1965. The light and electron microscopic

autoradiographic demonstration of local amyloid formation in spleen explants. *Amer. J. Pathol.* **47**:1079.

25. Sorenson, G. D., and W. A. Bari. 1968. Murine amyloid deposits and cellular relationships. *In* Amyloidosis. E. Mandema, L. Ruinen, J. H. Scholten, and A. S. Cohen, editors. Excerpta Medica Foundation, Amsterdam. 65.

26. Green, H., and B. Goldberg. 1965. Synthesis of collagen by mammalian cell lines of fibroblastic and nonfibroblastic origin. *Proc. Nat. Acad. Sci. U.S.A.* **53**:1360.

27. Efron, M. L., E. M. Bixby, and C. V. Pryles. 1965. Hydroxyprolinemia. II. A rare metabolic disease due to a deficiency of the enzyme "hydroxyproline oxidase." *N. Engl. J. Med.* **272**:1299.

28. Bahr, G. F. 1956. Elektronenoptische Untersuchungen bei Sklerodermie Zugleich ein Beitrag zur Kenntnis des Bindegewebes der Haut. *Aerztl. Forsch.* **10**:255.

29. Fisher, E. R., and G. P. Rodnan. 1960. Pathologic observations concerning the cutaneous lesion of progressive systemic sclerosis: electron microscopic, histochemical, and immunohistochemical study. *Arthritis Rheum.* **3**:536.

30. Rupec, M., and O. Braun-Falco. 1964. Elektronemikroskopische untersuchungen über das verhalten der kollagenfibrillen der haut bei sklerodermie. *Arch. Klin. Exp. Dermatol.* **218**:543.

31. Hayes, R. L., and G. P. Rodnan. 1971. The ultrastructure of skin in progressive systemic sclerosis (scleroderma). *Amer. J. Pathol.* **63**:433.

32. Uitto, J., J. Halme, M. Hannuksela, P. Peltokalio, and K. I. Kivirikko. 1969. Protocollagen proline hydroxylase activity in the skin of normal human subjects and of patients with scleroderma. *Scand. J. Clin. Lab. Invest.* **23**:241.

33. Keiser, H. R., H. D. Stein, and A. Sjoerdsma. 1971. Increased protocollagen proline hydroxylase activity in sclerodermatous skin. *Arch. Dermatol.* **104**:57.

34. Laitinen, O., J. Uitto, M. Hannuksela, and K. K. Mustakallio. 1969. Solubility and turnover of collagen in collagen diseases. *Ann. Clin. Res.* **1**:64.

35. Keiser, H. R., and A. Sjoerdsma. 1969. Direct measurement of the rate of collagen synthesis in skin. *Clin. Chim. Acta.* **23**:341.

36. Korting, G. W., H. Holzmann, and K. Kuhn. 1964. Biochemische Bindegewebsanalysen bei progressiver sklerodermie. *Klin. Wochenschr.* **42**:247.

37. Hayflick, L., and P. S. Moorhead. 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**:585.

38. Jackson, D. S., and J. P. Bentley. 1960. On the significance of the extractable collagens. *J. Biophys. Biochem. Cytol.* **7**:37.

39. Layman, D. L., E. B. McGoodwin, and G. R. Martin. 1971. The nature of the collagen synthesized by cultured human fibroblasts. *Proc. Nat. Acad. Sci. U.S.A.* **68**:545.

40. Nelder, K. H., J. D. Jones, and R. K. Winkelmann. 1966. Scleroderma: dermal amino acid composition with particular reference to hydroxyproline. *Proc. Soc. Exp. Biol. Med.* **122**:39.

41. Fleischmajer, R. 1964. The collagen in scleroderma. *Arch. Dermatol.* **89**:437.

42. O'Leary, P. A., H. Montgomery, and W. E. Ragsdale. 1957. Dermatohistopathology of various types of scleroderma. *Arch. Dermatol.* **75**:78.