INCREASED SENSITIVITY OF SCLERODERMA FIBROBLASTS IN CULTURE TO STIMULATION OF PROTEIN AND COLLAGEN SYNTHESIS BY SERUM

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Summary Serum effects on ^{14}C-proline incorporation and ^{14}C-hydroxyproline synthesis by normal and scleroderma fibroblasts in culture were studied. Serum resulted in 97% and 212% increases in ^{14}C-proline incorporation in two lines of scleroderma fibroblasts while the increase in normal fibroblasts was only 53%. Effects on collagen synthesis were more pronounced. Addition of serum resulted in 124% and 445% increments in ^{14}C-hydroxyproline synthesis in the scleroderma fibroblasts but only a 43% increment in the normal fibroblasts. The results indicate that cultured scleroderma fibroblasts have increased sensitivity to biosynthetic stimulation by serum and this mechanism may be of pathogenetic importance in the excessive collagen accumulation characteristic of the disease.

Scleroderma, also known as progressive systemic sclerosis, is a connective tissue disease in which excessive collagen deposition is found in the skin and internal organs (1). The mechanisms responsible for the collagen accumulation in sclerodermatous tissues are not entirely known,but several studies have suggested that increased synthesis by scleroderma fibroblasts may be one factor (2,3). Since the studies of Bankowski and Mitchell (4) showed that addition of serum to normal human skin fibroblasts in culture resulted in increased biosynthetic activity of these cells, we decided to examine the possibility that scleroderma fibroblasts may be more sensitive to serum stimulation of collagen synthesis than normal fibroblasts. To this end, we undertook studies to compare the effects of serum on ^{14}C-proline incorporation and collagen biosynthesis in normal and scleroderma fibroblasts. We found that serum stimulation of collagen hydroxyproline synthesis was 4 to 10-fold greater in the scleroderma fibroblasts than in normal cultures. Our data indicate that the scleroderma fibroblasts are more sensitive to stimulation of synthetic activity by serum than normal fibroblasts, and it is sug-

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TABLE I. Effects of Serum on the Incorporation of ^{14}C -Proline

Sample	Serum Added	Media	Cell Layers	Total	% Change
		dpm x 10^{-3}			
Normal	None	139.3	25.2	164.5	
	10%	209.0	42.3	251.3	53
Scleroderma I	None	73.6	38.9	112.5	
	10%	223.4	106.7	350.7	212
Scleroderma II	None	80.0	25.0	105.0	
	10%	164.7	42.1	206.8	97

All values represent average of 3 dishes

gested that this may be one of the mechanisms responsible for the collagen accumulation characteristic of the disease.

Materials and Methods. Fibroblast cultures were derived from punch biopsies obtained from normal and scleroderma human skin by standard tissue culture techniques as described previously (5,6).

Approximately 50,000 cells from cultures in early passages were seeded into 100 mm Falcon culture dishes containing 10 ml of Eagles MEM, 10% FCS and 2 X BME vitamins. The cells were kept at 37° C in a moist atmosphere containing 95% air, 5% CO_2 and the medium was changed 3 times weekly until a stationary growth phase was established.

When the cultures reached confluency, fresh medium containing 50 µg/ml ascorbic acid and 5 µCi of ^{14}C-proline (240 µCi/µM, New England Nuclear) was added to each dish. The dishes were divided into two sets, one receiving in addition 10% FCS, whereas the other did not receive the serum supplement.

Each experiment was performed in triplicate. All incubations were terminated 24 hours after the addition of the radioactive precursor.

The medium was removed and the cells washed twice with phosphate-buffered saline. The cells were dispersed from the dishes with 0.25% trypsin, sedimented at 600 g X 5 min and resuspended in 3 ml of Hank's medium. An aliquot was taken to determine cell numbers using a hemocytometer or a Coulter Counter Model B, and the remainder was homogenized with a motor driven teflon pestle homogenizer. The homogenized cells and the media were dialyzed against several changes of 0.5 M HAc to remove all un-incorporated label. After dialysis, aliquots were hydrolyzed in 6 N HCl and total ^{14}C-proline incorporated and ^{14}C-hydroxyproline content of the samples were determined by the method of Juva and Prockop (7).

The labeled macromolecules from the media were examined by molecular sieve chromatography in SDS*-Agarose columns. For this, aliquots of media were heated

SDS* = sodium dodecyl sulfate

TABLE II. Effects of Serum on the Synthesis of ^{14}C -Hydroxyproline

Sample	Serum Added	Media	Cell Layers	Total	% Change
		dpm x 10^{-3}			
Normal	None	38.07	1.38	39.44	
	10%	53.23	3.19	56.43	43
Scleroderma I	None	11.13	2.38	13.51	
	10%	62.10	11.52	73.62	445
Scleroderma II	None	14.18	1.01	15.19	
	10%	31.90	2.19	34.10	124

All values represent average of 3 dishes

at 100° C for 10 minutes in a solution containing 1% SDS, 1% mercapto-ethanol, 0.1 M Na_2PO_4 at pH 7.4. The samples were then dialyzed and chromatographed on a 6% agarose (Bio Gel A-5M, 200-400 mesh, Bio Rad) column as described previously (8). Fractions of 2 ml were collected and total ^{14}C-proline and ^{14}C-hydroxyproline were determined in each fraction.

Results. Table I shows the total incorporation of ^{14}C-proline into media and cell layer proteins by normal and scleroderma fibroblast cultures in presence and absence of serum. Serum stimulated the incorporation of ^{14}C-proline in normal and scleroderma cultures. However, the degree of stimulation was greater in scleroderma cultures (97 and 212%) than in normals (53%). When non-dialyzable radioactive hydroxyproline synthesized by the cultures was examined (Table II), serum stimulation of scleroderma fibroblasts was even more pronounced. In normal cultures, there was a 43% increase in ^{14}C-hydroxyproline synthesized in presence of serum, while in the scleroderma cultures, there were 124 and 445% increments under similar conditions. The extent of stimulation by serum was approximately of the same magnitude for media and cell layers in all cultures examined (Tables I and II). Comparison of the results shown in Tables I and II indicates that in the presence of serum, the increase

TABLE III. Effects of Serum on the Degree of Hydroxylation (%)[a]

Sample	Serum Added	Cells	Media	Total	Purified Procollagen[b]
Normal	None	5.46	27.3	24.0	29.7
	10%	7.57	25.4	22.5	37.6
Scleroderma I	None	6.1	15.13	11.86	20.7
	10%	11.5	27.7	23.04	31.7
Scleroderma II	None	4.10	17.7	14.5	20.7
	10%	5.26	19.4	16.5	35.4

All values represent average of 3 dishes

a $\frac{^{14}C \text{ Hydroxyproline}}{^{14}C \text{ Proline}} \times 100$

b Purified Procollagen was separated on a SDS-agarose column as described under figures 1 and 2

in ^{14}C-hydroxyproline content of the samples was greater than the increase in total ^{14}C-proline incorporation. These results can be due to either increased synthesis of collagen polypeptides or to increased hydroxylation of proline residues in collagen molecules. In order to clarify these findings, the degree of hydroxylation of proline was calculated in these cultures. Table III shows the effects of serum on the degree of hydroxylation of cells and media in the various cultures. It is apparent that when serum was added to scleroderma cultures there was an increase in the degree of hydroxylation of the newly synthesized polypeptides. In contrast, the addition of serum to normal cultures resulted only in a very minor increase. Furthermore, the effect in the scleroderma cultures was clearly apparent in cell layers and media, whereas, the minor effect observed in the normal cultures was confined to the cell layers. When the degree of hydroxylation of chromatogra-

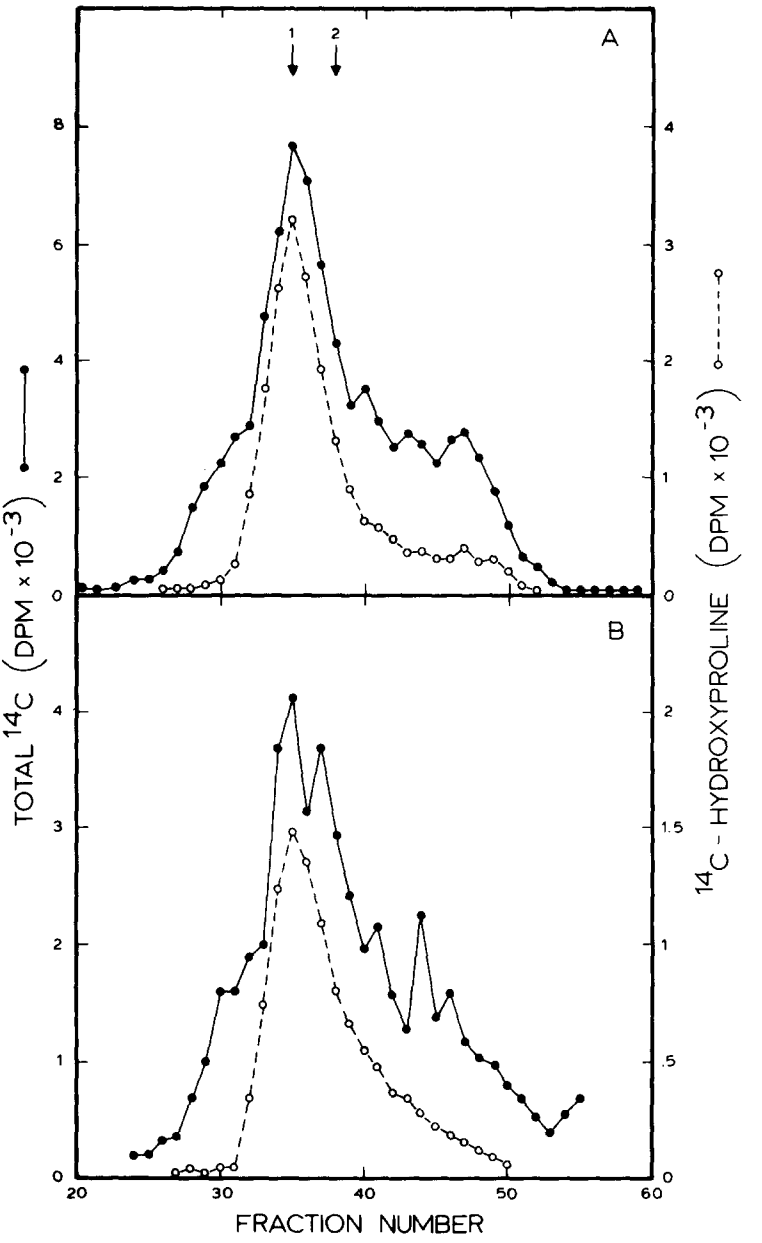

Figure 1. Gel filtration on SDS-agarose of reduced ^{14}C-proteins from culture media of normal fibroblasts in presence (A) and absence (B) of serum. Elution position of reduced procollagen (1) and tendon collagen α-chains (2) are indicated. Culture and chromatography conditions were as described in materials and methods.

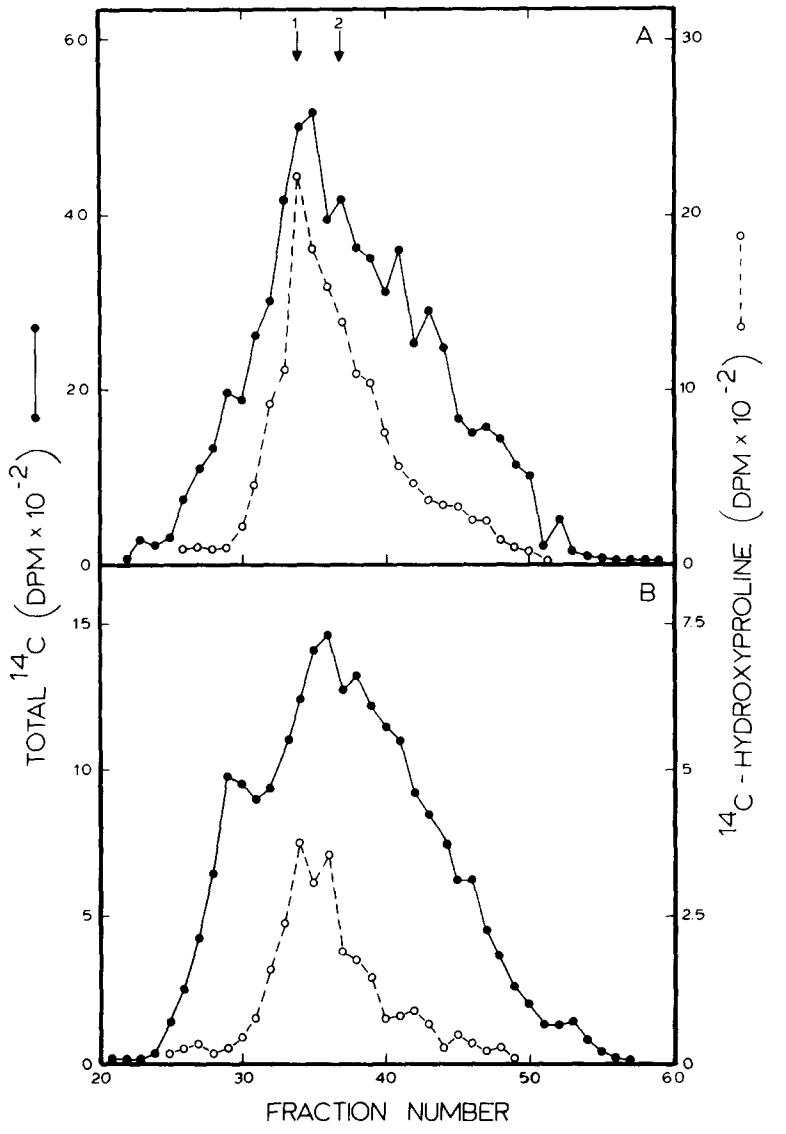

Figure 2. Gel filtration on SDS-agarose of reduced ^{14}C-proteins from culture media of scleroderma fibroblasts in presence (A) and absence (B) of serum. The conditions are exactly as described under figure 1.

phically purified procollagen from the media of the various cultures was determined, it was found that in the absence of serum the molecule was underhydroxylated in all cultures examined. The extent of underhydroxylation, however, was more striking in the scleroderma (20.7%) than in the normal cul-

tures (29.7%). When serum was added, the degree of hydroxylation of the procollagen increased in all cases, but the increase was much greater in the two scleroderma cultures.

In order to determine if addition of serum resulted in any qualitative changes in the newly synthesized proteins, the labeled media proteins were examined by gel-filtration on SDS-agarose columns after reduction of disulfide bonds (9). Chromatograms shown in Fig. 1 indicate that serum did not affect the pattern of elution of labeled proteins in the normal cultures. In contrast, differences were noted in the chromatographic patterns in the scleroderma fibroblasts (Fig. 2). In the absence of serum, approximately 30% of the total radioactivity eluted in a peak with an apparent m.w. greater than 200,000 daltons (Fractions 24-31). This peak was of non-collagenous nature since it did not contain ^{14}C-hydroxyproline. The relative proportion of these large molecular weight polypeptides decreased markedly in cultures labeled in the presence of 10% F.C.S. In addition, in the scleroderma cultures labeled in the absence of serum, greater amounts of radioactivity appeared in the region of elution of small molecular weight peptides (Fraction 40-55) and probably represented degradation products.

Discussion. The results presented here clearly show that scleroderma skin fibroblasts in culture exhibit greater serum requirement than normal skin fibroblasts for optimal synthesis of various proteins including collagen. All the experiments were performed in confluent stationary cultures and the addition of serum did not result in increased cell numbers during the short period of labeling (not shown). It therefore appears that the role of serum is due to the presence of factors other than those necessary for proliferation of cells in culture.

Previously, Bankowski and Mitchell had observed that addition of serum to the culture media of normal skin fibroblasts resulted in increases in ^{3}H-proline incorporation and collagen synthesis(5). Similarly, we found that addition of serum to normal skin fibroblasts resulted in increases in ^{14}C-proline

incorporation and ^{14}C-hydroxyproline synthesis. The response of scleroderma fibroblasts to serum addition, however, was very striking, since the increase in collagen biosynthesis in these cells was from 3 to 10-fold greater than in normal cells.

The effects of serum on scleroderma fibroblasts were not confined to quantitative changes. We also found that the biosynthetic patterns of scleroderma fibroblasts were markedly influenced by the addition of serum to the culture medium. In the absence of serum, the scleroderma fibroblasts synthesized a large molecular weight protein containing polypeptides of approximately 200,000 daltons after reduction of di-sulfide bonds. Synthesis of this non-hydroxyproline containing protein decreased markedly when the scleroderma cultures were supplemented with serum. Although the nature of this protein is unknown, it may represent a biosynthetic product peculiar to scleroderma cells since it was not detected in the normal cultures under any conditions.

Another important difference found between normal and scleroderma fibroblasts was that the collagen synthesized in the scleroderma cultures was underhydroxylated even in the presence of serum. This observation is more remarkable since all cultures were supplemented with ascorbic acid during labeling period (10).

These results suggest that scleroderma fibroblasts have more stringent metabolic requirements than normal skin fibroblasts since even ascorbate and serum supplements were not sufficient to support their full biosynthetic activity. In this context, it is important to note that establishment of scleroderma cells in culture from primary explants requires special tissue culture conditions such as high serum concentration (30%)(11) and very rich media (2). The mechanisms responsible for the stringent metabolic requirements of the scleroderma cultures demonstrated here are not known. Since these cultures were originally obtained by establishing primary explants in media containing 30% serum, it is conceivable that selection of a cell population with high

serum requirements may have resulted and may be responsible for the increased sensitivity of their biosynthetic activity to serum.

Alternatively, it is possible that in these cells the intracellular concentrations of co-factors or other substances may not be sufficient to sustain their optimal synthetic and metabolic activity and that addition of serum may have corrected these deficiencies.

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