SOLUBILITY OF COLLAGEN FROM NORMAL AND SCLERODERMA FIBROBLASTS IN CULTURE

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Summary. Cultured skin fibroblasts from scleroderma and normal patients were incubated with [^{14}C] proline to compare synthesis and extractability of collagen. Although total [^{14}C] proline incorporation and [^{14}C] hydroxyproline formation were similar in the two cultures, radioactive collagen associated with cell layers of scleroderma was more salt extractable. Addition of β-aminopropionitrile increased the percentage of soluble collagen in normal fibroblasts but had no effect on scleroderma cultures. Increasing the period of incubation increased the radioactivity of the insoluble collagen fraction in normal fibroblasts; however, in scleroderma cultures, there was no such increase.

Scleroderma is a connective tissue disease characterized by excessive collagen deposition in the skin and internal organs. We have used tissue culture techniques to obtain dermal fibroblasts from the skin of scleroderma patients in order to determine the pathogenesis of this systemic disorder. Human dermal fibroblast cultures have been established as a useful tool for the study of heritable connective tissue disorders (1,2) as well as for general studies of collagen (3,4). They have contributed to the identification and characterization of procollagen, a high molecular weight precursor form of collagen (3). Fibroblasts in culture have also been used for the study of some acquired diseases: rheumatoid arthritis (5) and systemic sclerosis (6,7).

In the present investigation, we have measured the salt solubility of collagen in normal and scleroderma-derived skin fibroblasts in culture after incubation with [^{14}C] proline. Our studies indicate that there is a possible defect in the *in vitro* maturation of collagen synthesized by the scleroderma fibroblasts.

Materials and Methods. Fibroblasts derived from punch biopsies of normal and scleroderma human skin by standard tissue culture techniques

were grown in plastic culture flasks in Eagle's Minimum Essential Medium supplemented with 2 x BME vitamins and 10% fetal bovine serum. Equal numbers of cells were seeded in 100 mm petri dishes (Falcon) and grown to confluency. All cells were fed three times weekly with the above medium and incubated in an atmosphere of 5% CO_2-95% air at 37°C until they reached the stationary phase. Each dish received 2.5 μCi of uniformly labeled [^{14}C] proline; in addition, half of the cultures received 100 μg of BAPN[1]. In the first experiment, the cells were sacrificed 24 hours after the addition of the radioactive precursor. In the second experiment, dishes were incubated for an additional 24 hours in fresh medium containing 2.5 μCi of [^{14}C] proline. In all experiments, the medium was saved, the cell layers were washed twice with phosphate buffered saline, harvested with trypsin (0.25%) and brought into suspension with Hank's medium. Aliquots were taken for protein and cell number determinations. The cells were then pelleted and resuspended in 5 ml of 1M NaCl in 0.05M tris (pH 7.4) and extracted for 2 days at 4°C. The samples were centrifuged at 10,000 x g for fifteen minutes. The salt soluble fraction, the pellet (insoluble fraction) and the medium were all dialyzed exhaustively in water and aliquots were taken for measurement of total radioactivity. Aliquots were also hydrolyzed in 6N HCl at 110°C for 18 hours in order to determine [^{14}C] hydroxyproline content (8). Radioactivity was measured in a Beckman LS 100 liquid scintillation spectrometer.

Results. The amount of nondialyzable radioactive hydroxyproline synthesized by the cultures is shown in Table I. Over 70% of the hydroxyproline synthesized was in the medium in both normal and scleroderma fibroblasts. Seventy-eight per cent of the peptide-bound hydroxyproline in the cell layer of normal cultures was extractable with cold neutral salt solution, the remaining (22%) being precipitated as crosslinked collagen fibers in between the fibroblasts. However, the scleroderma collagen was more soluble under similar conditions of extraction with the result that insoluble, crosslinked collagen represented only 14% of the total collagen synthesized by the cell layers. In the presence of BAPN, over 90% of cell layer [^{14}C]

[1] β-aminopropionitrile

Table I. [^{14}C]hydroxyproline synthesis by normal and scleroderma fibroblasts in culture.

	Normal		Scleroderma	
	CPM		CPM	
Cell layer collagen				
1M NaCl extract	8,362[a]	(78%)[b]	4,213	(86%)
pellet	2,427	(22%)	684	(14%)
Medium	24,793		18,463	
+BAPN				
Cell layer collagen				
1M NaCl extract	8,497	(93%)	4,494	(87%)
pellet	553	(7%)	620	(13%)
Medium	21,897		16,122	

[a] average of 4 dishes
[b] percentage of cell layer collagen

hydroxyproline could be extracted from normal cultures, while BAPN had no influence on the distribution of collagen between extractable and insoluble collagen of scleroderma fibroblasts. These preliminary data indicate a defect in the in vitro maturation of scleroderma collagen and/or the presence of atypical crosslinking in scleroderma not sensitive to BAPN. In an effort to clarify these findings, the transfer of radioactivity [^{14}C] hydroxyproline from soluble to insoluble collagen fractions was followed with time. Two more scleroderma and one normal dermal fibroblast cultures in confluency were incubated as before for 24 hours with [^{14}C] proline. One-half of all cultures were harvested at this time and the remaining dishes received fresh medium containing an additional 2.5 μCi of [^{14}C] and the incubation continued for another 24 hours. Table II shows the percentage distribution of radioactive hydroxyproline in salt soluble and insoluble collagen fractions in the cell layers of the fibroblasts in culture. In the normal culture, there was an increase in radioactive hydroxyproline in the insoluble collagen with time, from 18 to 29%, indicating a transfer of activity from soluble to insoluble fractions which is to be expected in the normal in vitro maturation of collagen.

Table II. [^{14}C]hydroxyproline synthesis in the cell layer of fibroblasts in normal and scleroderma cultures.

	24 hours		48 hours	
Normal	CPM		CPM	
1M NaCl extract	3850[a]	(82%)[b]	6236	(71%)
pellet	860	(18%)	2559	(29%)
Scleroderma I				
1M NaCl extract	4471	(91%)	6874	(91%)
pellet	416	(9%)	683	(9%)
Scleroderma II				
1M NaCl extract	6190	(85%)	6042	(86%)
pellet	1109	(15%)	926	(14%)

[a] average of 4 dishes
[b] percentage of cell layer collagen

However, in both scleroderma cultures, the amount of [^{14}C] hydroxyproline in the insoluble fraction remained the same between 24 and 48 hours.

Discussion. The behavior of cell-associated collagen reveals three interesting differences between normal and scleroderma cultures as shown in this report. These are: (a) scleroderma cultures synthesized collagen which was more extractable in 1M NaCl at 4^{o}C (b) BAPN increased the solubility of collagen from normal fibroblasts but not from scleroderma fibroblasts and (c) incubation with additional [^{14}C] proline for another 24 hours increased the amount of radioactivity in the insoluble collagen fraction of the normal culture with no such increase in the scleroderma collagen. Since solubility of collagen is related to the degree of crosslinking, there probably is a defect in collagen maturation and crosslinking in these fibroblasts as compared to normal fibroblasts.

Solubility of collagen is influenced by a number of factors such as ascorbic acid (9) , pH (10) and cell density. Nigra et al. (10) have shown that collagen was more salt extractable when culture pH was lowered from 8 to 6.8. They further suggested that this phenomenon may be related to to lysyl oxidase activity-the enzyme responsible for the 1st step of cross-

link biosynthesis: the conversion of lysine to allysine.

It has been shown that a considerable portion of the peptide-bound hydroxyproline synthesized by human dermal fibroblasts in culture is released into the medium. A major portion of the medium hydroxyproline is procollagen (3). As judged by the distribution of radioactive hydroxyproline in the medium and the cell layers (Table I) there seems to be no difference between normal and scleroderma fibroblasts in their ability to make procollagen or in converting it into collagen. We have repeatedly observed that the amount of radioactivity is the same for both the cell layer and medium (incorporation of [^{14}C] proline and synthesis of [^{14}C] hydroxyproline) for both scleroderma and normal fibroblasts (7,11).

In order to pinpoint the reasons for high solubility of scleroderma collagen and the defect that might exist in its maturation, additional work is needed. This would include isolation and characterization of collagen, measurement of the levels of lysyl oxidase and determination of the pattern of reducible crosslinks after reduction with radioactive sodium borohydride in both normal and scleroderma cultures under our conditions.

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