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Collagen Synthesis in Scleroderma: Selection of Fibroblast Populations During Subcultures

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Summary. In progressive systemic scleroderma, excessive deposition of collagen leads to fibrosis of several tissues including the skin. It has been found that different populations of fibroblasts are present in scleroderma skin; these can be obtained by establishing cell cultures from different layers of the involved skin. Excessive overproduction of collagen was noted in primary cultures of cells obtained from deeper layers of the skin of patients in an early stage of the disease, whereas control fibroblasts did not manifest significant variations dependent on the layers of skin used to initiate the cultures. The synthesis of type-I and -III collagen was found to be altered concomitantly. The production of collagen and collagenous proteins was then followed during subcultivations of overproducing fibroblasts. In many cell strains, increased synthesis of collagen and/or noncollagenous proteins had already been lost after the first subcultivation, whereas overproduction was stable in others. However, after five passages, most of the cultures showed normal collagen synthesis, which probably indicates a loss of phenotype due to successive subcultures or overgrowth by another population of fibroblasts.

Key words: Collagen – Fibroblast populations – Scleroderma

Introduction

Progressive systemic scleroderma (PSS) is a generalized disorder of the connective tissue, with its main manifestations being in the skin, lungs, oesophagus and kidneys (for a review, see [18]). In these organs, the extensive depostition of connective-tissue components leads to the development of sclerosis [4]. Several mechanisms have been postulated that may modulate the regulation of connective-tissue components by fibroblasts, and therefore play a role in the pathogenesis of scleroderma [7]. Detailed studies have previously been performed to identify an alteration in collagen metabolism in cultured scleroderma fibroblasts [16]. Several groups have reported an increased production of connective-tissue proteins [2, 8, 13, 16], whereas others have been unable to corroborate these data [15]. Recently, some of these discrepancies have been explained by the discovery that heterogenous populations of fibroblasts are present in scleroderma skin [6]. By dissecting individual layers of skin, fibroblast cultures can be established, which reveal an excessive (five- to tenfold) overproduction of collagen and fibronectin. These overproducers are mainly found when deeper layers of the skin (reticular-fat layer) are used to initiate cell cultures, whereas fibroblasts obtained from the papillary layer show a normal production of both macromolecules [6]. In order to preserve the phenotype of the cells and to prevent overgrowth of other populations of fibroblasts, all studies demonstrating excessive overproducing fibroblast populations were performed in primary cultures.

Here we report data obtained by investigating several subcultures of primary cultures that were orginally found to be characterized by excessive overproduction of collagen.

Materials and Methods

Skin Biopsy

Skin biopsies were obtained from four patients with scleroderma and from age-matched controls. Three of the patients had PSS, and one had circumscribed scleroderma (morphea; Table 1). Biopsies were obtained under local anaesthesia from the forearm, and one specimen was taken from the leg. Routine histology was carried out for each biopsy.

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Table 1. Clinical characterization of the patients

Patient	Sex	Race	Age (years)	Duration of disease (years)	Site of biopsy	Diagnosis
1	Female	White	46	1.5	Forearm	PSS
2	Female	White	23	1.0	Leg	CS
3	Male	White	47	1.0	Hand/forearm	PSS
4	Female	White	29	5.0	Forearm	PSS

PSS, progessive systemic scleroderma; CS, circumscribed scleroderma

Tissue Culture

Skin samples were sectioned at three different levels, thus yielding three specimens with a thickness of about 1 mm representing the papillary, reticular and reticular-subcutaneous layers and subcutaneous tissue. Primary cultures were established in culture dishes (60 × 15 mm) in Ham's F10 medium containing glutamine and supplemented with 10% fetal calf serum, 400 I = U/ml penicillin, 50 µg/ml streptomycin, 5 µg/ml chlortetracycline hydrochloride and 25 I = U/ml mycostatin. Cells were subcultured through passage five according to established procedures [8].

Radioactive Labelling

Prior to labelling, confluent cultures were preincubated for 24 h in Ham's F10 medium containing penicillin (400 I = U/ml) and 10% fetal calf serum. The cultures were then labelled for 24 h by incubation in Ham's F10 medium supplemented with 50 µg/ml sodium ascorbate, 50 µg/ml beta-aminopropionitrile, 400 I = U/ml penicillin and 5 µCi/ml ^{3}H proline.

Measurement of Collagen and Non-Collagen Proteins

The synthesis of collagen and non-collagenous proteins was determined by measuring the amount of protein-bound hydroxyproline and proline present after incubation with tritiated proline. The combined medium and cell layer were dialysed against dilute acetic acid, lyophilized and then hydrolized (6 *N* HCl, 110° C, 24 h). The samples were further chromatographed on an automated amino-acid analyser. The effluent volume was collected in a fraction collector, and the radioactivity of the fractions was measured in a scintillation counter. The amount of collagenous and non-collagenous proteins was calculated as described previously [17].

Characterization of Collagen Types

The combined medium and cell layer were treated with pepsin (6 h, 18° C) as described previously [9, 12]. The collagen chains were then separated by slab gel electrophoresis [11] and quantified by densitometric scanning of the gels processed for fluorography [1].

Measurement of Proteins

The amount of protein in the cell layer was measured according to the method of Lowry et al. [14].

Results

All biopsies were taken from patients in an active stage of the disease, and histologically, they revealed a perivascular lymphocytic infiltrate and thickened collagen bundles. Three patients were suffering from progressive sclerosis, whereas one had circumscribed scleroderma (Table 1). In all instances the biopsies were taken from the involved area. The separation of the subcutaneous fat, the reticular layer and the papillary layer was carried out using a dissecting microscope. In primary culture, no morphological difference was noted between fibroblasts grown from sclerodermatous skin and those grown from controls.

Table 2. Collagen production in primary cultures of fibroblasts derived from scleroderma patients and controls[a]

Dermal layer	Patients			
	1	2	3	4
Papillary	23	54	41	16
Reticular	9	80	21	77
Subcutaneous fat	33	46	159	99

Mean of controls ($n = 6$), 31 ± 28

[a] Counts per minute of hydroxyproline per 10^{-2} mg protein

Control cells derived from the reticular layer tended to be more active with respect to collagen synthesis, but no statistically significant difference was noted between cultures grown from different layers. The mean value of all estimates was 31 ± 28 cpm hydroxyproline per 10^{-2} mg protein. In contrast, considerable variation was found when scleroderma fibroblasts were analysed (Table 2) Whereas most of the cells grown from the papillary layer of the involved area showed normal collagen synthesis, pronounced overproduction was found in fibroblast cultures derived from the reticular- and subcutaneous-fat layers. In order to determine whether this altered phenotype expressed in primary culture was stable, duplicates of the overproducing cell cultures were passaged and investigated after several subcultivations.

Control fibroblasts derived from different dermal layers did not differ from one another after the first passage and showed no variation in their synthetic capacities after four passages (not shown). Aliquots of medium and cell layer were also used for the determination of collagen Types I and III (not shown). No

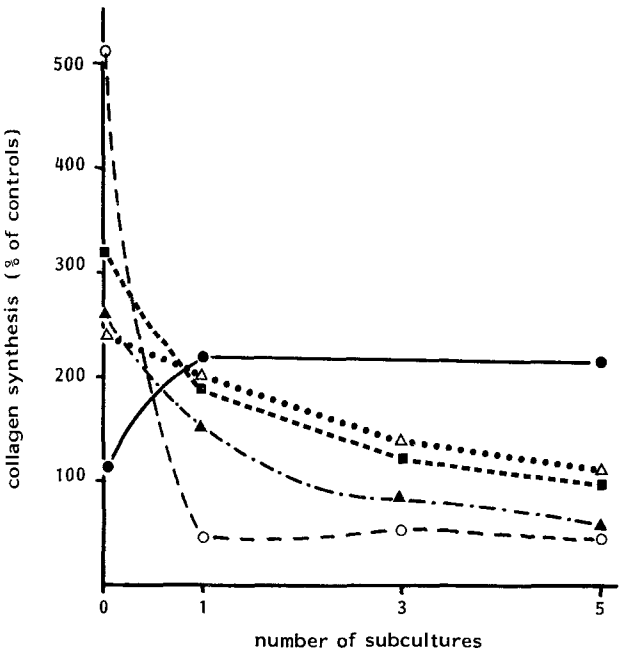

Fig 1. Influence of subcultivation on collagen synthesis in five scleroderma fibroblasts characterized by overproduction of collagen in primary culture. The synthesis of collagen was determined by measuring the amount of protein-bound hydroxyproline (see Materials and Methods). Each *point* represents total collagen synthesis by different scleroderma fibroblast strains; this is expressed as a percentage of collagen synthesis in control fibroblasts (100%)

difference was noted with respect to the dermal layer or the number of cell passages.

After the first subcultivation increased collagen synthesis was a characteristic feature of four out of five fibroblast strains obtained from sclerodermatous skin. However, when these cultures were studied after five passages, overproduction of collagen and/or non-collagenous proteins was found in only a few cell strains. In most, the amount of collagen synthesized dropped to the range of the controls. Overproduction of collagen was often accompanied by an elevation of total-protein synthesis; however, in some cases, an increased percentage of collagen vs total-protein production was found even when the total amount of collagen synthesized was unaltered (Table 3). In other samples (Patient 1, reticular layer), the production of non-collagenous proteins was even more pronounced than the synthesis of collagen. This resulted in a decrease in the percentage of collagen vs total proteins.

Whereas most of the investigated cell strains revealed their highest values for collagen synthesis during early subcultures, in one sample (Patient 1, subcutaneous fat) an increased percentage of collagen synthesis vs total proteins was found only after the fifth passage.

Table 3. Collagen synthesis in scleroderma fibroblasts and dependence on subcultures[a]

Dermal layer	Subculture	Patient			
		1	2	3	4
Papillary layer	1	9.3	15.0	12.5	5.4
	5	13.7	17.9	11.7	5.8
Reticular layer	1	4.1	18.1	25.3	11.8
	5	13.8	12.8	10.9	10.8
Subcutaneous fat	1	7.7	17.2	9.4	13.0
	5	17.2	7.5	8.9	10.6

Controls (subcultures $1-5$; $n = 18$), 10.4 ± 3.0

[a] Percentage of collagen vs total-protein synthesis

In order to investigate whether, in some cell strains, the altered collagen synthesis involved both collagen types, Type-I and -III collagen were separated, and the ratio was calculated. No difference in this ratio was noted in all of the cultures investigated (not shown).

Discussion

Fibroblast cultures derived from PSS or circumscribed scleroderma have been used as models in order to obtain a better understanding of the fibrotic processes [3, 10, 13, 16]. In the present study, biopsies were taken from patients with PSS or morphea in the early stage of the disease where, using histological criteria, the main involement was observed in the deeper layers of the dermis. Fibroblasts were grown from anatomically distinct areas of the skin in order to determine whether an alteration in collagen metabolism could be correlated with the histological signs of fibrosis. Although, as reported previously [6], reticular fibroblasts tended to show a more active synthesis, no significant variation was noted in control cultures obtained from different levels of the dermis. Furthermore, the synthesis of collagen and non-collagenous proteins remained similar after several subcultures. This suggests that, with respect to these parameters, uniform populations of fibroblasts can be obtained by outgrowth from different levels of normal skin.

In contrast, cells grown from different layers of scleroderma skin showed a large variation in their synthesis of collagen and non-collagenous proteins when studied in primary cultures. Most of the cultures showing excessive production of Type-I and -III collagen were derived from the reticular dermis and the subcutaneous fat, thus corroborating earlier data [6, 10]. This finding suggests a correlation between protein metabolism and fibrotic areas as determined by regular histology in PSS and circumscribed scleroderma [5].

In order to obtain information as to whether excessively increased production of collagen may be a stable feature of affected cell strains grown from individual layers of the dermis, those cells which had initially produced large amounts of collagen were subcultured. In contrast to cells which had been grown from whole biopsies, it was assumed that the selection of the affected area for outgrowth would result in a uniform population of affected cells. However, after subculturing, the biosynthetic capacities of individual cell strains showed great variability with regard to both collagen synthesis and the production of non-collagenous proteins.

In some subcultures, the total production of collagen was not different to that in controls; however, an altered ratio of collagen to non-collagenous proteins pointed to an alteration of the control of protein and collagen synthesis. In most of the investigated cultures, the loss of excessive collagen production was noted after one to three passages, whereas in a few cultures only we could find evidence of the maintenance of the initially altered phenotype. However, in some instances, trypsinization resulted in the selection of fibroblasts characterized by a disturbed regulation of collagen production, although primary cultures of these cells were found to be similar to controls. These findings provide further evidence for the metabolic heterogeneity of fibroblast populations derived from scleroderma skin [6]. They also clearly show that even selection of the affected area for establishing outgrowth cultures does not guarantee that a uniform population of affected cells will be obtained. Our data are able to explain discrepant reports of increased or normal collagen synthesis in fibroblasts obtained from scleroderma patients [13, 15, 16], and they emphasize the influence of alteration of the phenotype of cells or selection of fibroblast populations in in vitro metabolic studies.

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