Regulation of Collagen Gene Expression in Cutaneous Diseases With Dermal Fibrosis: Evidence for Pretranslational Control*

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Dermal fibrosis, characterized by collagen accumulation, is the hallmark of several cutaneous diseases. To examine the mechanisms of collagen deposition in fibrotic skin diseases, fibroblast cultures were established from the skin of patients with progressive systemic sclerosis, morphea, scleredema, familial cutaneous collagenoma, connective tissue nevi of the collagen type, or keloids; these patients served as prototypes of fibrotic skin diseases with varying clinical features and potentially different etiologic factors. Collagen production was assayed by the synthesis of [^{3}H]hydroxyproline, and types I and III procollagen messenger RNA (mRNA) levels were determined by dot blot hybridizations using human type I and type III procollagen-specific cDNA probes. The collagen production in fibroblast cultures from the fibrotic diseases was increased up to 6-fold over the controls, and a relatively good correlation between the collagen production and type I collagen mRNA levels was noted. The type I/III procollagen mRNA ratio in control fibroblast cultures was 5.9 ± 1.6 (mean ± SD). The corresponding ratio in keloid cell culture was markedly increased, while slightly decreased values were noted in the case of morphea and familial cutaneous collagenoma; the values in other cultures were within the normal range. The results suggest that procollagen production in fibroblast cultures derived from fibrotic skin diseases reflects elevated levels of the corresponding procollagen mRNA. The increased mRNA abundance, suggesting pretranslational control, may result from enhanced transcriptional activity of the corresponding gene or alternatively reflects increased stability of the mRNA molecule. *J Invest Dermatol 88:727–731, 1987*

Dermal fibrosis is the hallmark of several diseases affecting skin [2–4]. The cutaneous diseases with dermal fibrosis can be classified into several distinct categories on the basis of clinical features, associated findings, and inheritance (Table I). For example, the cutaneous fibrosis can be part of a generalized connective tissue involvement, as in progressive systemic sclerosis, or it can be strictly localized in the skin, as in morphea. The dermal fibrosis can result from an immunologic or metabolic aberration, or it can be chemically induced. Most fibrotic conditions are acquired, but dermal fibrosis is the major clinical feature in two heritable diseases, namely familial cutaneous collagenoma [5] and the shagreen patches in tuberous sclerosis [6] (Table I).

The cutaneous diseases with dermal fibrosis share several histopathologic features [7]. The dermis is usually thickened with the extracellular matrix of the connective tissue invading areas of the subcutaneous adipose tissue. The extracellular matrix is predominantly composed of collagen, which for example in the case of keloids comprises approximately 60% of the dry weight of the tissue [8]. Thus, there is a net increase in the amount of collagen in the lesional skin in fibrotic diseases. Furthermore, particularly in the early stages of the fibrotic process, there is a marked fibroblastic cell infiltrate.

The excessive accumulation of collagen in the fibrotic skin lesions can result from aberrations in collagen metabolism by several different mechanisms, as for example, by increased collagen production by fibroblasts, reduced degradation of collagen either intra- or extracellularly, or expansion of fibroblast subpopulations active in collagen production. Previous studies utilizing cell culture techniques have established that in some fibrotic diseases the skin fibroblasts are overproducing collagen. This has been shown to be true in cases of progressive systemic sclerosis [9–12], morphea [13], and keloids [8,14,15]. Also, in the case of scleroderma, recent studies have suggested that the levels of type I procollagen messenger RNA (mRNA) are similarly increased [16,17]. To explore the mechanisms leading to dermal fibrosis, we have examined fibroblasts derived from 6 patients, each with

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Abbreviations:
cDNA: complementary DNA
DMEM: Dulbecco's modified Eagle's medium
mRNA: messenger RNA
SDS: sodium dodecyl sulfate
SSC: 0.15 M NaCl, 0.015 M sodium citrate (pH 6.8)

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Table I. Classification of Cutaneous Diseases With Dermal Fibrosis

- Scleroderma
  - Systemic Scleroderma
    - Progressive systemic sclerosis
    - CREST syndrome
  - Localized Scleroderma (Morphea)
    - Circumscribed morphea
    - Linear morphea
    - Guttate morphea
    - Generalized morphea
- Scleroderma-like Skin Changes in Systemic Diseases
  - Inflammatory Connective Tissue Diseases
    - Mixed connective tissue disease
    - Features of scleroderma in lupus erythematosus and dermatomyositis
    - Eosinophilic fasciitis
  - Metabolic and Immunologic Disorders
    - Chronic graft-vs-host disease
    - Porphyrias
    - Phenylketonuria
    - Carcinoid syndrome
    - Scleredema with paraproteinemia
    - Juvenile-onset diabetes mellitus
    - Acromegaly
  - Premature Aging Syndromes
    - Werner's syndrome
- Chemically Induced Dermal Fibrosis
  - Drugs
    - Bleomycin
    - Pentazocine
  - Chemicals
    - Polyvinyl chloride
    - Silicates
    - Organic solvents
    - Contaminated rapeseed oil (toxic oil syndrome)
- Connective Tissue Hamartomas of the Collagen Type
  - Inherited
    - Familial cutaneous collagenoma
    - Shagreen patches in tuberous sclerosis
  - Acquired
    - Isolated collagenomas
    - Eruptive collagenomas
    - Keloids
    - Hypertrophic scars

a different type of fibrotic skin disease, for their collagen metabolism in vitro. These patients serve as prototypes representing different phenotypic forms of cutaneous fibrosis.

PATIENTS AND METHODS

Fibroblast cultures were established from the skin of 6 patients with dermal fibrosis. The clinical features of these patients are indicated in Table II. The primary cultures were established from biopsy specimens obtained from the lesional skin. Control cultures were established from age-matched normal subjects who underwent a surgical procedure for cosmetic reasons. The control cultures were established from the site-matched area of skin. The primary cultures were passed by trypsinization, and the secondary cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) under standard tissue culture conditions [20].

For collagen analyses, the fibroblast cultures in early visual confluency were incubated in DMEM supplemented with 20% fetal calf serum and 25 μg/ml of ascorbic acid. After a 4-h preincubation, [^{3}H]proline (L-[2,3,4,5-^{3}H]proline, 109 Ci/mmol; Amersham) was added and the incubations were continued for 18 h. At the end of incubation, the medium was removed and mixed with protease inhibitors to give final concentrations of 10 mM Na_2EDTA, 10 mM N-ethylmaleimide, and 1 mM phenylmethylsulfonyl fluoride. The cell layer was rinsed with 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5, and the rinse was combined with the medium fraction. The cells were scraped with a rubber policeman into 50 mM Tris-HCl, pH 7.5, containing 1.0 M NaCl and the protease inhibitors indicated above. The cells were homogenized with a Teflon-glass homogenizer at 4°C. Aliquots of the cells and medium were dialyzed against distilled water, hydrolyzed at 120°C for 16 h, and assayed for [^{3}H]hydroxyproline [21]. Aliquots of the cell homogenates were also dialyzed against 1 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, and assayed for cell protein [22].

For assay of types I and III procollagen mRNA levels, poly(A)$^+$-RNA was isolated from the cells by oligo-dT cellulose chromatography, as indicated previously [8,23,24]. The recovery of the poly(A)$^+$-RNA was determined by a technique utilizing rabbit globin mRNA as an internal standard [8]. The collagen-specific mRNA levels were then determined by dot blot hybridizations utilizing well-characterized human proα1(I), proα2(I), and proα1(III) collagen-specific complementary DNA (cDNA) probes [25–27]. Hybridizations were performed by dotting aliquots of poly(A)$^+$-RNA in distilled water mixed with an equal volume of a solution containing 3 vol of 20× SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 6.8) and 2 vol of 37% formaldehyde [28]. The samples were heated at 65°C for 5 min and dotted on nitrocellulose filters using a vacuum manifold (Minifold II, Schleicher & Schuell). The nitrocellulose filters were air dried and heated at 78°C for 90 min in a vacuum oven. The filters were then prehybridized at 42°C in a solution containing 3× SSC, 50% formamide, 0.1% sodium dodecyl sulfate (SDS), 50 μg/ml denatured salmon sperm DNA, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin, and 0.02% Ficoll [28]. The hybridizations were carried out in the same solution containing 10 ng/ml of a collagen-specific cDNA, labeled by nick translation with α-^{32}P-labeled deoxyribonucleotides to a specific activity of approxi-

Table II. Clinical Features of the Patients With Dermal Fibrosis

Patient No.	Diagnosis	Age (years)/Sex	Duration of Disease	Clinical Features
1	Progressive systemic sclerosis	56/M	6 months	Rapidly progressing induration and thickening of the skin. Severe involvement of the lungs and esophagus. Patient died 1 year after onset of the disease.
2	Morphea	14/M	6 months	Indurated plaque on the trunk surrounded by inflammation. The lesion enlarging progressively.
3	Scleredema	49/F	1 month	Rapidly developing thickening and induration of the skin on the face and neck. A circulating paraprotein of IgM type was detected by serum immunoelectrophoresis [18].
4	Familial cutaneous collagenoma	42/M	26 years	Multiple asymptomatic dermal nodules on the upper trunk. Family history consistent with autosomal dominant inheritance [5].
5	Connective tissue nevi of the collagen type	14/F	13 years	Cerebriform connective tissue nevi over the plantar surfaces of both feet. No family history of a similar condition [19].
6	Keloid	21/F	6 months	Rapidly growing lesion on the earlobe.

mately 5 × 10^8 cpm/μg [29]. The hybridizations were carried out for 18 h at 42°C. The filters were washed successively in 500 ml of 2× SSC at 24°C, and then 1× SSC, 0.5× SSC + 0.1% SDS, and 0.25× SSC, all at 65°C, 10 min each. The stringency of the hybridizations and the washing conditions preclude any cross-hybridization between type I and type III procollagen mRNAs when type-specific probes are used, as detected by Northern blot analyses [30]. The filters were dried and exposed to x-ray films (Dupont, Cronex) in cassettes equipped with intensifying screens. The mRNA-[^{32}P]cDNA hybrids were visualized by autoradiography and quantitated by liquid scintillation counting. The results were corrected for the specific activity and length of the cDNA probes, as well as for chain composition of type I and III procollagens.

Statistical evaluations were performed by Aspin-Welch two-sample test of means.

RESULTS

Production of procollagen, measured as synthesis of [^{3}H]hydroxyproline in cultures incubated with [^{3}H]proline, was increased in all 6 cell lines studied (Table III). The highest increase, approximately 6-fold, was noted in fibroblast cultures established from patient no. 2 with morphea, while the values in other cell cultures were increased by 1.8- to 3.0-fold (Table III).

To examine the mechanisms of increased procollagen production in these cultures, the levels of types I and type III procollagen mRNAs were measured. The type I procollagen mRNA levels, determined by hybridizations with proα2(I) collagen cDNA, in the culture from fibrotic skin diseases were 16.7 ± 3.1 cpm/ng poly(A)$^+$-RNA (mean ± SEM), and the corresponding values in the control fibroblast cultures were 6.0 ± 0.3 cpm/ng. The ratio of proα1(I)/proα2(I) mRNAs in all cultures was approximately 2:1, and consequently, the assay of type I procollagen mRNA levels utilizing either proα1(I) or proα2(I) specific cDNA probe gave the same results after correction for chain composition of type I procollagen. A relatively good overall correlation between the collagen production and the type I procollagen mRNA levels was observed (Fig 1).

The ratio of type I/type III procollagen mRNAs was also examined by hybridizations with proα1(I) and proα1(III) collagen-specific cDNA probes. This ratio in control fibroblast cultures was 5.9 ± 1.6 (mean ± SD). A markedly increased type I/III procollagen mRNA ratio was measured in the keloid cell line studied (patient no. 6), whereas slightly reduced values were noted in cases of morphea (patient no. 2) and familial cutaneous collagenoma (patient no. 4) (Fig 2).

DISCUSSION

In this study, we have demonstrated that fibroblast cultures established from a variety of fibrotic skin diseases display enhanced collagen production in vitro. This observation suggests, there-

Table III. Collagen Production in Fibroblast Cultures Established From Patients With Cutaneous Fibrosis

Patient[a] No.	[^{3}H]Hydroxyproline (dpm/μg protein)[b]	(% of control)
1	385.3 ± 24.9	181.3
2	1351.3 ± 359.8	635.9
3	639.3 ± 146.3	300.8
4	561.2 ± 143.1	264.1
5	475.8 ± 93.8	223.9
6	465.2 ± 8.1	218.9
Controls[c]	212.5 ± 80.7	100.0

[a] The numbers refer to patients in Table II.

[b] The values are expressed as dpm per μg cell protein, mean ± SD of 3 parallel flasks. The values in patients 1–6 are statistically different from the controls, $p < 0.05$, as calculated by Aspin-Welch two-sample test of means.

[c] The controls represent normal fibroblast cultures which were established from the site-matched skin of 5 age-matched control subjects and incubated parallel to the affected cell cultures under identical tissue culture conditions.

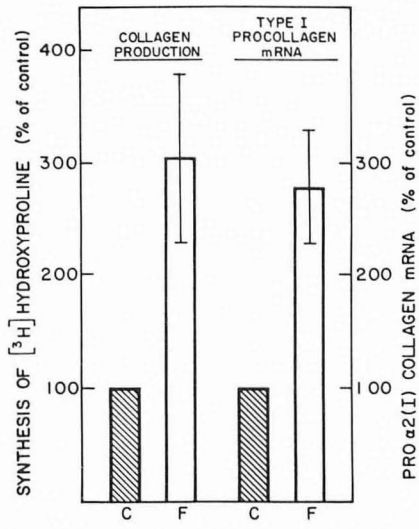

Figure 1. Correlation between collagen production and type I procollagen mRNA levels in fibroblast cultures established from patients with cutaneous fibroses. Collagen production was assayed by determination of newly synthesized [^{3}H]hydroxyproline, expressed as dpm per μg cell protein (see Table III). Type I procollagen mRNA levels were determined by hybridizations with proα2(I) collagen-specific cDNA probe, and the values were expressed as cpm per ng poly(A)$^+$-RNA (see *Results*). The values in fibroblast cultures from patients with cutaneous fibrosis (*F*) are expressed as percent (mean ± SEM) of the corresponding controls (*C*) which represent 6 paired normal fibroblast cultures examined under identical culture conditions.

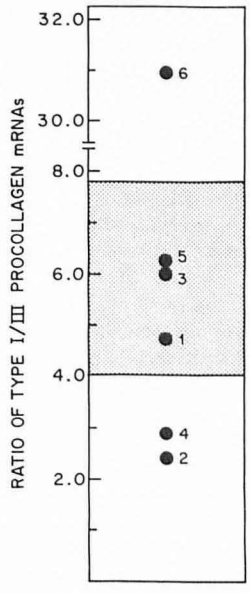

Figure 2. The ratio of type I/III procollagen mRNAs in fibroblast cultures established from patients with cutaneous fibrosis or from site-matched skin of age-matched control subjects. The cultures were incubated in a parallel manner under identical tissue culture conditions and examined at early visual confluency. Poly(A)$^+$-RNA was isolated by oligo-dT cellulose chromatography and dotted on nitrocellulose filters in serial dilutions. Parallel filters were hybridized with human proα1(I) and proα1(III) collagen cDNA probes. The limits of mean ± SD of 5 control cultures are indicated by the *hatched area*. The numbers by the values obtained with fibrotic skin diseases (*solid circles*) refer to patients in Table II. The individual values are the means of 2 separate experiments, each representing 6 parallel determinations with a variation of <18% (SD).

fore, that the deposition of collagen, which can be demonstrated by histopathologic examination of the affected skin, results from increased synthesis of this protein in vivo. These observations are in agreement with previous demonstrations that the collagen production by fibroblast cultures established from patients with progressive systemic sclerosis [9–12], morphea [13], and keloids [8,14,15] is increased.

Collagen synthesis by human skin fibroblasts is a process involving several distinct intracellular steps [31,32]. First, the expression of collagen gene is initiated by transcription of the information in DNA to a corresponding mRNA precursor molecule which, in addition to the coding sequences (exons), contains noncoding sequences (introns) [32]. The precursor mRNA undergoes several posttranscriptional modifications, including removal of the introns by splicing. Functional mRNA molecules then serve as template for the synthesis of procollagen polypeptides. These polypeptides, pro-α chains, are extensively modified by co- and posttranslational reactions which are enzymatically mediated [33]. Upon completion of the intracellular events, the procollagen molecules are secreted into the extracellular space where they undergo further modifications, including the removal of the precursor-specific extensions [31]. Thus, the production of the extracellular collagen molecules could be controlled at several different levels of biosynthesis, either intracellularly or extracellularly.

In this study, we demonstrated that the increased procollagen production by cultured fibroblasts was accompanied by a parallel increase in type I procollagen mRNA levels. This observation suggests then that procollagen production in fibroblast cultures established from fibrotic skin diseases reflects elevated levels of the corresponding procollagen mRNA. The increased mRNA levels may result from enhanced transcriptional activity of the corresponding genes [34], or alternatively, increased stability associated with longer half-life of the collagen mRNAs could lead to accumulation of these molecules [35]. Nevertheless, the demonstration of a correlation between type I procollagen mRNA levels on one hand and the production of the corresponding protein on the other, further supports previous suggestions [36,37] that a major control point in collagen production exists on the pretranslational level. These data also indicate that the differences in the rate of collagen production between the fibroblasts from the fibrotic diseases and the controls cannot be explained by altered regulation on the posttranscriptional level [38].

Recent studies have clearly established that collagens are a family of closely related yet genetically distinct proteins [39,40]. The two major types of procollagens synthesized by human skin fibroblasts in culture are type I and type III [41,42]. In this study, we examined the relative expression of type I and type III procollagen genes by measuring the corresponding mRNA levels. In control fibroblasts, the ratio of type I/III procollagen mRNAs was 5.9, a value which agrees with the ratio of type I/III procollagens synthesized by these cells in culture [42]. The ratio of type I/III procollagen mRNA was markedly elevated in patient no. 6 with keloids, suggesting that keloid fibroblasts may offer a unique model by which to study the independent regulation of gene expression of two genetically distinct procollagens [30]. In contrast to keloids, slightly reduced type I/III procollagen mRNA ratios were noted in the fibroblasts from patients with morphea or familial cutaneous collagenoma. It should be noted that type I/III procollagen mRNA ratios were measured in patients' fibroblast cultures which were incubated parallel to the control cell cultures under identical conditions. Thus, the altered collagen gene expression in fibrotic diseases may be selective in that one of the genetically distinct types of collagens is preferentially expressed.

In summary, enhanced procollagen production was demonstrated in fibroblast cultures established from several patients with fibrotic skin diseases. A close correlation between the procollagen production and type I procollagen mRNA levels was noted, suggesting regulation on the pretranslational level of procollagen gene expression. These observations further suggest that the accumulation of collagen in these conditions may result from enhanced synthesis of this protein in vivo. It should be noted that the diseases examined in this study were representative only of some of the distinct categories of dermal fibrosis. It is clear that many of the categories within the proposed classification of the cutaneous diseases with dermal fibrosis (see Table I) are heterogeneous both clinically and biochemically. Furthermore, previous biochemical studies on collagen biosynthesis in a large number of fibroblast cultures established from patients with keloids [8] or progressive systemic sclerosis [11] have revealed considerable heterogeneity. Thus, the cell lines studied here may not be exclusively representative of the phenotypic expression within each diagnostic category, and it is conceivable that in other cases with fibrotic cutaneous diseases, additional factors, such as translational and posttranslational control of protein synthesis or altered degradative pathway [19,43] may contribute to the excessive deposition of collagen in the skin.

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