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Activation of Type I Collagen Genes in Cultured Scleroderma Fibroblasts

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Fibroblasts cultured from affected skin areas of five patients with cutaneous scleroderma were found to produce increased amounts of collagen when compared with nonaffected control cells. Total RNA was isolated from the cultures and analyzed for its level of proα1(I)collagen mRNA by hybridization of RNA blots with a cloned cDNA probe. The levels of proα1(I)collagen mRNAs relative to total RNA were two- to sixfold higher in the samples from affected cells, accounting for the increased synthesis of type I collagen. Cytoplasmic dot hybridizations were performed to measure the cellular content of proα1(I)collagen mRNA: up to ninefold increases in the level of this mRNA per cell were found. Upon subculturing, scleroderma fibroblasts were found to reduce gradually the increased synthesis of collagen to the level of nonaffected controls by the tenth passage. The levels of type I collagen mRNAs were also reduced, but more slowly. The results suggest that in scleroderma fibroblasts the genes for type I collagen are activated at procollagen mRNA level or that they are more stable and that the activating factors are lost during prolonged cell culture because cells from affected areas lose their activated state.

Key words: collagen synthesis, collagen mRNA, gene expression, cell culture, scleroderma, fibroblast

The two major collagen types in normal skin are types I and III. These fibrillar collagens are synthesized by skin fibroblasts as procollagens. Type I procollagen is made up of two proα1(I)-chains and one different but homologous proα2(I)-chain. Type III collagen consists of three identical proα(III)-chains. Small amounts of other collagen types have also been detected in skin [1].

Overproduction of collagen is a characteristic feature in a number of well-known human fibrotic diseases, eg, lung fibrosis, liver cirrhosis, keloids, and scleroses [2]. Both systemic and localized scleroderma represent human fibrotic diseases characterized by excessive accumulation of collagen in the affected skin areas. A number of investigations have suggested this accumulation is due to activation of skin fibroblasts, as cells cultured from sclerodermatous areas produce increased amounts of collagen [3–6] but show no change in their production of collagenase [4]. Histological and chemical studies on affected skin areas have indicated that the accumulating

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collagen is of types I and III with no significant alteration in their ratio [7]. Fibroblasts cultured from sclerodermatous lesions also have been shown to maintain the normal adult ratio of types I and III collagens [4].

Fibroblasts control their rate of collagen synthesis at various levels of gene expression. Several studies have shown control at the level of procollagen mRNA synthesis [8–10]. Additional control mechanisms have been described at the translational level both through variable efficiencies of procollagen mRNA utilization [11,12] and by a feedback inhibitory mechanism mediated by the aminoterminal propeptides of type I procollagen [13,14]. Further control is possible at the level of extensive posttranslational modifications, while intracellular degradation may account for breakdown of up to 60% of newly synthesized collagen [15].

Alterations in these regulatory mechanisms leading to overproduction of collagen in scleroderma fibroblasts are poorly understood. A disturbance in the propeptide-mediated feedback mechanism has been suggested for scleroderma fibroblasts [16]. In a more recent study employing cell-free translation of RNAs from cultured scleroderma cells, increased levels of translatable type I procollagen mRNAs were detected, suggesting activation of collagen genes at the transcriptional level [17]. We have extended these studies and have used a direct hybridization assay to determine the molecular level and control mechanisms involved in the activation of type I collagen genes in scleroderma. Preliminary reports of this study have been published elsewhere [18,19].

MATERIALS AND METHODS

Skin Biopsies and Cell Cultures

Excision or punch biopsies were performed under local anesthesia from affected and closely related or symmetrically located nonaffected skin areas of five scleroderma patients listed in Table I. In addition, two control patients were studied (Table I). All scleroderma patients suffered from a localized disease (morphea) in an active, progressive stage. Patient TET was biopsied twice: first when his disease was in a very active stage [6,19], and for the second time when the disease was in a relatively inactive stage (cell line TET-U). The disease of patient MP was diagnosed as the

TABLE I. Clinical Data of Patients

Culture code	Sex	Age (years)	Clinical findings
KLU	F	11	Duration 3 years, progressive
TN	M	34	Duration 0.5 year, progressive
MP	M	48	Duration 1 year, progressive, Lichen sclerosus–type scleroderma
TET	M	51	Duration 1 year, progressive
TET-U	M	55	Duration 5 years, mildly active
SK	F	59	Duration 1 year, progressive
TH	F	13	Control patient
LP	M	35	Control patient

lichen sclerosus variant. All biopsies from the affected areas were taken near the progressive margin of the lesions.

Fibroblast lines were started from sliced biopsy specimens by the explantation method and cultured as described earlier [20] in Dulbecco's modified minimum essential medium (DMEM) supplemented with 10% fetal bovine serum. All the cultures were screened for mycoplasma with a DNA stain [21] and found to be mycoplasma free. Cells were cultured in 25-cm^2 tissue culture flasks and in 9- and 57-cm^2 petri dishes (Nunc, Roskilde, Denmark).

Measurement of Collagen Synthesis

All the experiments were performed by comparing fibroblast lines derived from affected skin areas of scleroderma patients with cells from nonaffected skin areas of the same patient and/or of normal controls. For the experiments each cell line was grown to confluence in six 57-cm^2 petri dish. One petri dish of affected and nonaffected (or control) cells was labeled for 24 hr with 100 μCi of [^{35}S]-methionine (SJ 204, Amersham International, Amersham, UK) in serum-free DMEM supplemented with freshly made ascorbate and β-aminopropionitrile (50 μg/ml each). Another pair of petri dishes was labeled similarily with [^{3}H]-proline (TRA 82, Amersham) but in a glutamine-free DMEM. The other four dishes of each cell line were incubated for 24 hr in serum-free DMEM containing ascorbate and β-aminopropionitrile. At the end of the 24-hr incubation the labeling media were collected and dialyzed exhaustively against 1 mM EDTA, pH 7.5, at 4°C. The corresponding cell layers were trypsinized and the cell numbers per plate were counted in a Bürker chamber. All the cells from the six plates were combined for total RNA isolation.

The dialyzed culture media were lyophilized and redissolved in electrophoresis sample buffer [22]. Aliquots of these samples corresponding to 10^6 fibroblasts were electrophoresed under both reducing and nonreducing conditions in 5–12% SDS-polyacrylamide gels [22] followed by fluorographic analysis [23]. Collagenous bands in the fluorograms were quantitated by densitometry. We have previously tested this method and found that it correlated well with those based on digestion with bacterial collagenase or on limited proteolysis with pepsin [6,19].

Measurement of Procollagen mRNA Levels

Total RNA was isolated from the same cells that were analyzed for collagen synthesis (approximately 2–5 $\times$ 10^7 cells per isolation) using the method of Rowe et al [24].

Duplicate samples of total RNAs were denatured with glyoxal and electrophoresed on 0.75% agarose gels [25]. After electrophoresis one set of samples was stained with ethidium bromide and photographed and the other transferred by blotting to nitrocellulose. Baked filters were prehybridized and hybridized at 42°C as described by Thomas [25]. Two cDNA clones were used as probes: pCAL1 and pCAL2 containing sequences complementary to chick proα1(I)- and proα2(I)-collagen mRNAs, respectively [26]. For hybridization the plasmids were nick-translated (reagent kit from BRL, Gaithersburg, MD) to specific activities of approximately 10^8 cpm/μg using [α-^{32}P]-dCTP (PB 10205, Amersham). Washed filters were exposed with Kodak X-Omat film using intensifying screens. The intensities of stained rRNAs and hybridization patterns on x-ray films were measured by densitometry. The

amounts of procollagen mRNAs were always corrected for variations in the rRNA concentrations.

In some experiments total RNAs were dotted onto nitrocellulose filters without fractination. After baking, the filters were hybridized, washed, and exposed as above. Densitometry was used to measure the intensity of hybridization.

Cytoplasmic Dot Hybridizations

Procollagen mRNA levels in affected and nonaffected fibroblasts in culture were also studied employing the cytoplasmic dot hybridization technique [27]. Fibroblast cultures were grown in 9-cm^2 petri dishes to confluence in DMEM with 10% fetal bovine serum and 50 μg/ml ascorbate. The cells were released with trypsin, counted in a Bürker chamber, and processed for dotting. Aliquots corresponding to 2–5 $\times$ 10^4 cells were dotted on nitrocellulose filters, which were baked, hybridized, and analyzed as above.

RESULTS

All the experiments were designed in such a way that affected cultures were compared with the respective nonaffected controls at the same time. Care was taken to monitor the cell density to study both cultures in the same growth phase. The same cultures were analyzed for the rate of collagen synthesis and relative levels of proα1(I)collagen mRNA. Two representative analyses of affected and nonaffected cell lines from patients TET-U and MP are shown in Figures 1 and 2.

Figure 1C shows that collagen synthesis by affected TET-U fibroblasts was increased by a factor of 2.5 over the nonaffected control cells when studied in the third passage. Affected TET-U cells represent the lowest level of activation in our series. The difference in collagen synthesis had disappeared in the sixth passage. The amount of collagen produced by the cells in the tenth passage did not differ either. An overall reduction in collagen and other protein synthesis was seen in affected and nonaffected cells when the passage number increased.

Measurement of proα1(I)collagen mRNA levels in isolated RNA from the same cells revealed a corresponding increase in the relative amount of this mRNA in affected fibroblasts in the third subculture (Fig. 1B). A similar increase was detected in the sixth passage cells from an affected area. In the tenth subculture the mRNA levels had dropped in both samples, in nonaffected cells below the detection limit of the hybridization. When corrected for variations in rRNA levels (Fig. 1A) the amounts of proα1(I)collagen mRNA in affected and nonaffected cells were reduced with the increasing passage number, but the levels in affected cells clearly remained higher.

In Figure 2 a similar analysis of affected and nonaffected fibroblasts of patient MP (with a lichen sclerosus variant of the disease) reveals a more stable increase both in collagen synthesis and in relative mRNA levels in affected cells for at least the ten subcultures studied. Patient MP represents a high level of activation of collagen synthesis in our series.

All cell lines listed in Table I have been analyzed in the third passage. In each case an increase was found in fibroblasts from affected areas in type I collagen synthesis and in the levels of proα1(I)collagen mRNA. The combined results of these experiments are listed in Table II.

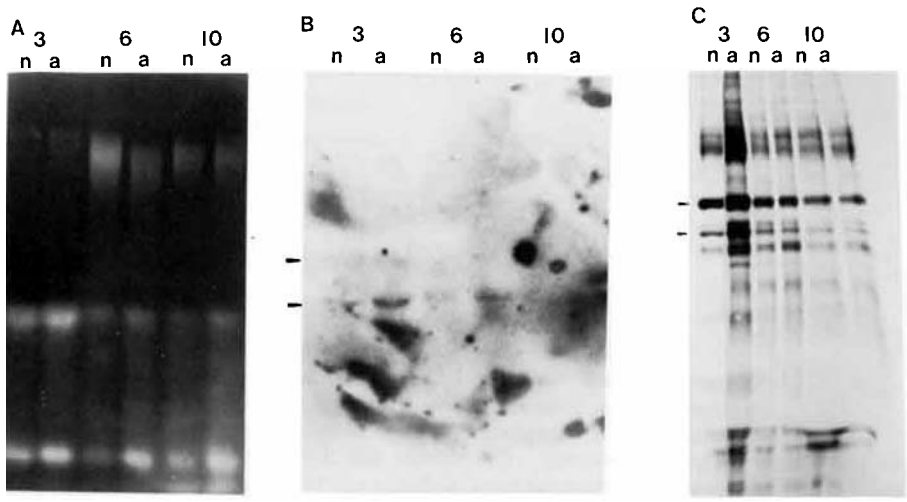

Fig. 1. Comparison of collagen synthesis rates and levels of proαI(I)collagen mRNAs in affected (a) and nonaffected (n) TET-U cells in the third, sixth, and tenth subcultures as indicated above the lanes. For each sample total RNA was isolated and aliquots of 12.5 μg were electrophoresed on 0.75% agarose gel as described in the text. A) Gel stained with ethidium bromide; the bands from the top are contaminating chromosomal DNA, 28 rRNA, and 18 S rRNA. Identically fractionated RNA samples were transferred by blotting to nitrocellulose, hybridized with [^{32}P]-labeled proα1(I)collagen-specific cDNA clone pCAL1 and autoradiographed (see text for details). B) Hybridization pattern; the arrowheads mark the larger and smaller mRNA species for proα1(I)collagen. The same cultures were labeled with [^{35}S]-methionine and the culture medium proteins were fractionated on a 5–12% SDS-polyacrylamide gel under reducing conditions. C) Fluorogram of the gel with the two major collagenous bands marked with arrows: upper band contains proα(I)- and proα1(III)-chains and lower bandα2(I)-chains.

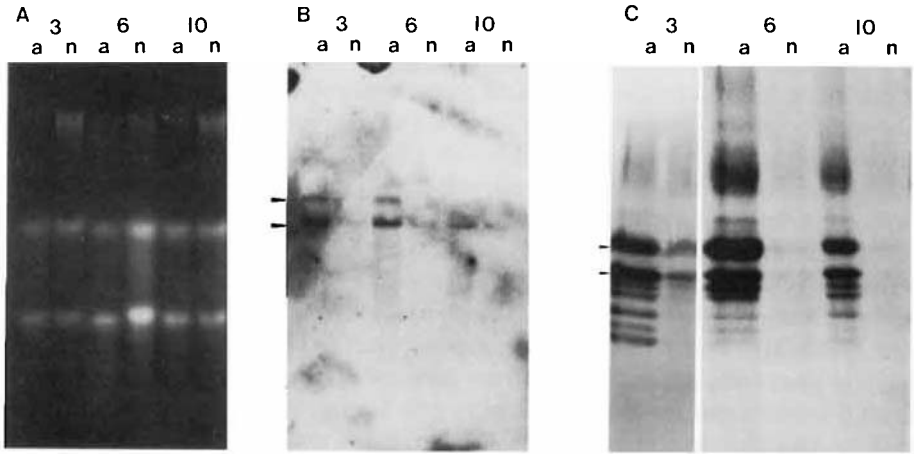

Fig. 2. Comparison of collagen synthesis rates and levels of proα1(I)collagen mRNAs in affected (a) and nonaffected (n) MP cells in the third, sixth, and tenth subcultures as indicated above the lanes. The cultures were analyzed and the results are shown as in Figure 1 with (A) showing the staining of rRNA and (B) the hybridization pattern with pCAL1 probe. The fluorogram shown in (C) is from fractionated [^{3}H]-proline-labeled culture medium proteins.

TABLE II. Summary of the Comparisons Between Affected and Nonaffected Scleroderma Cell Lines Analyzed in Third Subculture.*

Cell line	Collagen synthesis	Proα(I)collagen mRNA levels	
		Per rRNA	Per cell
KLU	3.3	1.9	2.1
TN	3.6	2.1	3.0
MP	10.3	6.0	6.9
TET	7.5	6.0	ND
TET-U	2.2	2.5	ND
SK	5.3	ND	9.0

*Collagen synthesis was estimated by densitometric analyses of fluorograms. The relative mRNA levels were measured by densitometry of RNA blots or RNA dots and corrected for variations in rRNA concentration. Cytoplasmic dot hybridizations were performed to measure the proα(I)collagen mRNA content per cell. The results are given as ratios of affected/nonaffected of the values obtained and represent the average of two to four measurements. ND, not determined.

As the RNA blots were prepared with isolated RNA, the hybridizations revealed only the relative amounts of proα1(I)collagen mRNA. To measure actual mRNA levels in a given number of cells, cytoplasmic dots were prepared from the cell lines in the third passage. Figure 3 shows the combined results of such experiments. The results of densitometric analyses of the autoradiographs are given in Table II. The results show that affected cells contain proportionately more proα1(I)collagen mRNA than suggested by measurements of relative mRNA levels.

DISCUSSION

Several studies have shown that cell lines started from affected skin areas of scleroderma patients produce increased amounts of collagen and other proteins [3–6]. The results of the present study (Table II) are in good agreement with previous investigations which have revealed up to 10–20fold increases in the synthesis of collagen and other proteins by scleroderma fibroblasts. To determne the level of gene expression involved in this increase we have measured mRNA levels of type I collagen in these fibroblast lines. Hybridization experiments clearly showed an increase in proα1(I)collagen mRNA collagen mRNA levels (relative to total RNA) corresponding reasonably well to the increased rate of type I collagen synthesis (Figs. 1, 2; Table II). This finding is in agreement with the results of Graves et al [17]: using in vitro translation of RNAs from cultured scleroderma fibroblasts they found about twofold higher levels of translatable type I collagen mRNAs in these cells. Both of these approaches measure mRNA levels relative to a given amount of total RNA and do not take into account possible alterations in total cellular RNA in the disease. Therefore, we extended these studies to direct determination of RNA levels in a known number of cells by cytoplasmic dot hybridization [27]. The results (Fig. 3; Table II) show that type I collagen mRNA levels in scleroderma fibroblasts are increased even more than suggested by hybridization to RNA blots. This result

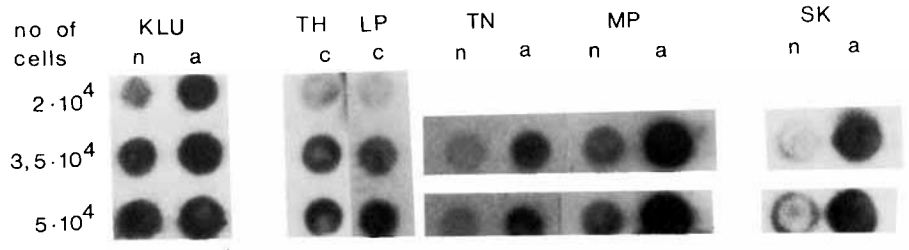

Fig. 3. Cytoplasmic dot hybridization patterns of affected (a) and nonaffected (n) scleroderma fibroblasts and control (c) cells in the third passage. The cells were counted and processed as described in the text. Aliquots corresponding to 2–5 × 10^4 cells were dotted on nitrocellulose. The filters were hybridized with pCAL1 probe and autoradiographed.

strongly suggests that the total content of RNA in scleroderma fibroblasts is increased and could explain why in addition to overproduction of collagen increased synthesis of other proteins has also been detected both in this study (Fig. 2) and in earlier investigations [5,6].

It is not surprising to find that increased levels of type I collagen mRNAs are found in cells producing greatly increased amounts of type I collagen. A correlation between mRNA levels and collagen production has been detected in fibroblasts during development [8,9] and in experimental systems such as Rous sarcoma virus infection [10]. It remains to be shown whether an increase in the transcription rate or an increased stability (prolonged half-life) of these mRNAs is responsible for the higher mRNA levels. Clearly fibrosis in scleroderma does not seem to result from selectively activated translation of type I collagen mRNAs, as has been shown for bleomycin-induced lung fibrosis [12].

There are a number of possible explanations why the correlation between the mRNA levels and type I collagen synthesis varies. We have previously shown that pCAL1 hybridizes specifically to the two main mRNA species for human proα1(I)collagen under the stringency conditions used [19,28] but we cannot exclude the possibility that the probe cross-hybridizes to some extent also the proα(III)collagen mRNA. Since the ratio of types I and III collagen calculated from reduced and nonreduced gels (data not shown) remains relatively unaltered the possible cross-hybridization should not create more than minor errors. Hybridization of probe pCAL2 to human proα2(I)collagen mRNA is weaker but our preliminary results agree with those obtained with pCAL1. The cell cycle is known to affect both the rates of protein (collagen) synthesis [29] and procollagen mRNA utilization [11] in cultured fibroblasts. Although care was taken to study the cells at the same cell density, minor changes in the growth phase could affect our results.

We have earlier reported that scleroderma fibroblasts in cell culture start to reduce their increased rate of collagen synthesis [6]. In the present study all the four scleroderma morphea cell lines exhibited a distinct reduction in type I collagen synthesis to normal values when the cultures were maintained for up to 10 passages (Fig. 1). The cells from an affected skin area of patient MP (lichen sclerosus) form an exception: they continued overproduction of type I collagen through the 10 subcultures studied (Fig. 2). Other investigators have also reported variable reduction in the increased synthesis rates of collagen in scleroderma fibroblasts [3]. The

mechanism behind this reduction was approached by isolating total RNA from fibroblasts in third, sixth, and tenth passages and by analyzing these for the proα(I)collagen mRNA levels. In all the cases studied it became apparent that the relative mRNA levels were not reduced to the same extent as the production of type I collagen. This suggests that additional mechanisms at translational or posttranslational levels could also play a role in the control of type I collagen synthesis in cell culture.

Our current findings should bring new clues to the etiopathogenic mechanisms in scleroderma. Clearly the fibroblasts are in an activated state: up to 6–10 fold higher levels of type I collagen mRNA are found in affected cells during early subcultures. The activation must affect either the rate of transcription of type I collagen genes or the stabilities of the corresponding mRNAs. The fact that the activated state is gradually reduced in culture both at protein and at mRNA levels suggests that the activating factor(s) responsible is lost during cell culture. Both sera from scleroderma patients and products of inflammatory cells in the lesion sites have been shown to stimulate fibroblasts [30]. A subpopulation of high-collagen–producing fibroblasts has been suggested to be the target of this activation [31]. Our results cannot clarify this issue, but cell cultures provide a test system where these factors can be tested for their mechanism of action at variour levels of gene expression.

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REFERENCES

1. Smith LT, Holbrook KA, Byers PH: J Invest Dermatol 79:93s, 1982.
2. Kulonen E: In Kulonen E, Pikkarainen J (eds): "Biology of Fibroblasts." London: Academic Press, 1973, pp 3–7.
3. LeRoy EC: J Clin Invest 54:880, 1974.
4. Uitto J, Bauer EA, Eisen AZ: J Clin Invest 64:921, 1979.
5. Fleischmjer R, Perlish JS, Krieg T, Timpl R: J Invest Dermatol 76:400, 1981.
6. Vuorio TK, Kähäri VM, Lehtonen A, Vuorio EI: Scan J Rheumatol 13:229, 1984.
7. Lovell CR, Nicholls AC, Duance VC, Bailey AJ: Br J Dermatol 100:359, 1979.
8. Tolstoshev P, Haber R, Trapnell BC, Crystal RG: J Biol Chem 256:9672, 1981.
9. Vuorio E, Sandell L, Kravis D, Sheffield VC, Vuorio T, Dorfman A, Upholt WB: Nucleic Acids Res 10:1175, 1982.
10. Sobel ME, Yamamoto T, de Crombrugghe B, Pastan I: Biochemistry 20:2678, 1981.
11. Tolstoshev P, Berg RA, Rennard SI, Bradley KH, Trapnel BC, Crystal RG: J Biol Chem 256:3135, 1981.
12. Sterling KM, Harris MJ, Mitchell JJ, Cutroneo KR: J Biol Chem 258:14438, 1983.
13. Wiestner M, Krieg T, Hörlein D, Glanville RW, Fietzek P, Müller PK: J Biol Chem 254:7016, 1979.
14. McPherson JM, Hörlein D, Abbott-Brown D, Bornstein P: J Biol Chem 257:8557, 1982.
15. Rennard SI, Stier LE, Crystal RG: J Invest Dermatol 79:77s, 1982.
16. Krieg T, Hörlein D, Wiestner M, Müller PK: Arch Dermatol Res 263:171, 1978.
17. Graves PN, Weiss IK, Perlish JS, Fleischmjer R: J Invest Dermatol 80:130, 1983.
18. Vuorio EI, Vuorio TK: J Cell Biochem Suppl 8B: 283, 1984.
19. Kähäri VM, Vuorio T, Näntö-Salonen K, Vuorio E: Biochim Biophys Acta 781:183, 1984.
20. Vuorio E: Scand J Clin Lab Invest Suppl 37, 37:1–72, 1977.

21. Chen TR: Exp Cell Res 104:255, 1977.
22. O'Farrell P: J Biol Chem 250:4007, 1975.
23. Laskey RA, Mills AD: Eur J Biochem 56:335, 1975.
24. Rowe DW, Moen RC, Davidson JM, Byers PH, Bornstein P, Palmiter RD: Biochemistry 17:1581, 1978.
25. Thomas PS: Proc Natl Acad Sci USA 77:5201, 1980.
26. Vuorio EI, Schaefer IM, Vuorio TK, Dorfman A, Upholt WB: Acta Chem Scand B 38:237, 1984.
27. White BA, Bancroft FC: J Biol Chem 257:8569, 1982.
28. Vuorio T, Vuorio E, Lehtinen P, Upholt WB, Dorfman A: Coll Relat Res 3:69, 1983.
29. Aumailley M, Krieg T, Razaka G, Müller PK, Bricaud H: Biochem J 206:505, 1982.
30. LeRoy EC: J Invest Dermatol 79:87s, 1982.
31. Botstein GR, Shierer GK, LeRoy EC: Arthritis Rheum 25:189, 1982.