Collagen Metabolism in Cutis Laxa Fibroblasts: Increased Collagenase Gene Expression Associated with Unaltered Expression of Type I and Type III Collagen

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Collagen metabolism was studied in cutis laxa by analyzing collagen and collagenase gene expression in three dermal fibroblast strains from patients with congenital cutis laxa and comparing them with fibroblasts obtained from age-matched healthy subjects. Normal collagen synthetic activity was observed in the cutis laxa fibroblasts. An increased level of collagenase mRNA and unaltered levels of α_1(I) and α_1(III) collagen mRNA were found in all cutis laxa cell strains by dot blot hybridization. Reduced levels of elastin mRNA were also detected in these strains. However, no qualitative differences in these mRNA transcripts were detected between the control and cutis laxa fibroblasts by Northern blot analysis. Collagenase activity in fibroblast culture supernatants was then measured using fluorescein isothiocyanate (FITC)-labeled type I collagen. Increased collagenolytic activity in cutis laxa fibroblast culture supernatants was also found. These data suggest that increased collagenase expression of fibroblasts is related to the structural abnormality of dermal connective tissue in cutis laxa. *J Invest Dermatol 97:483–487, 1991*

Cutis laxa is a rare disease that is clinically diagnosed from cutaneous findings of loose and sagging skin, with reduced resilience and elasticity [1,2]. Therefore, it has been strongly suggested that there are histologic and biochemical abnormalities in the elastic fibers in cutis laxa. Intensive investigations of the elastic fibers in cutis laxa have been carried out, and structural abnormalities have been found using morphologic techniques [3–5]. Frequently, there is a paucity of the elastic structures, and, in many cases, the existing elastic fibers are fragmented. Biochemically, recent papers have reported that elastin mRNA levels are reduced in several cutis laxa cell strains [6,7].

With regard to collagen fibers, occasional irregularities in collagen morphology have been detected in cutis laxa skin [5]. Small and separated collagen bundles and fibrils of varied diameters and aggregated fibrils have been reported in cutis laxa skin [5]. However, collagen metabolism in cutis laxa has not been sufficiently studied and is poorly understood. Increased synthesis of collagen VI has been observed in fibroblasts from one affected individual [8]. Studies of the synthesis of collagens I and III in cutis laxa fibroblasts have been also performed using only one cutis laxa fibroblast strain [8,9]. To achieve a better understanding of collagen metabolism in cutis laxa we investigated the gene expression of not only collagen but also collagenase, a metalloprotease with the unique ability to initiate collagen degradation, in skin fibroblast cultures established from three patients with cutis laxa. Here, we report the presence of an increased level of collagenase expression accompanying unaltered collagen production and unaltered levels of type I and type III collagen mRNA in cutis laxa fibroblasts.

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Abbreviations:
cDNA: complementary deoxyribonucleic acid
DMEM: Dulbecco's modified Eagle's medium
DTT: dithiothreitol
FBS: fetal bovine serum
FITC: fluorescein isothiocyanate
mRNA: messenger ribonucleic acid
NCP: non-collagen protein
PBS: phosphate-buffered saline
PDL: population doubling level
SDS: sodium dodecyl sulfate
5 × SSC: 0.75 M NaCl and 0.075 M sodium citrate
TCA: trichroloacetic acid

MATERIALS AND METHODS

Fibroblast Cultures Three dermal fibroblast strains derived from patients with congenital cutis laxa were used. Two strains (CL1, CL2) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). One strain (CL3) was obtained from the abdominal skin of a two-year-old girl with a diagnosis of cutis laxa. The clinical diagnosis was confirmed by a skin biopsy that revealed a paucity of dermal elastic fibers. Control strains (N1, N2, N3) were obtained from the abdominal skin of age-matched healthy subjects. Primary cultures of the dermal fibroblasts were established by routine methods [10], and cells were maintained in 25-cm^2 plastic flasks with 5 ml of DMEM containing 10% fetal bovine serum (FBS). The medium was changed every third day, and routine subcultivation was performed at a 1:2 split ratio. All cells were utilized for each study at a five or six population doubling level (PDL). The fibroblast strains used in this study are listed in Table I.

Measurement of Collagenous and Non-Collagenous Protein Synthesis Both cutis laxa and age-matched normal fibroblasts were seeded onto 12-well multiplate dishes. After the cells grew to confluency, the medium was refed with serum-free DMEM con-

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Table I. Fibroblast Strains Used in This Study

Fibroblast Strains	Age	Sex[a]	PDL
CL1 (GM01353)	12 days	M	5
CL2 (GM02768)	4 months	F	6
CL3	2 years	F	5
N1	9 months	M	5
N2	1 year	F	6
N3	2 years	F	5

[a] M, male; F, female.

taining 0.1 mM ascorbic acid, 0.1 mM β-aminopropionitrile, and 10 μCi/ml L-[2,3-^{3}H] proline (specific activity 34.8 Ci/mmol, NEN Chemicals, Boston, MA). Incorporation into collagen and non-collagen protein (NCP) was measured using purified bacterial collagenase as described previously [10] with minor modifications. Briefly, the combined medium plus cell fractions was sonicated and precipitated in 10% trichloroacetic acid (TCA) containing 10 mM proline. After washing thoroughly in a solution of 5% TCA with 10 mM proline, the precipitate was dissolved in 0.2 N NaOH, neutralized, and digested with collagenase form III (Advance Biofactures Corporation, Lynbrook, NY). Radioactivity in the collagenase digest was considered as collagenous protein synthesis. The collagenase resistant fraction was precipitated in 5% TCA containing 0.25% tannic acid and then boiled in 0.5% SDS/5 mM DTT for 5 min. The radioactivity was considered as NCP synthesis. The relative rate of collagen production was calculated with the assumption that collagen has an imino acid content 5.4 times higher than that of other proteins [11].

Analysis of mRNA Levels Levels of mRNA were measured as previously described [8]. Cells were placed in 150 × 10 mm dishes, and when they achieved confluency the cells were washed twice with cold phosphate-buffered saline (PBS) and homogenized in 5 M guanidine thiocyanate containing 0.75% of 2-mercaptoethanol. Total RNA was isolated by centrifugation over a cushion of 5.7 M cesium chloride. Constant amounts of RNA (2 μg/lane) were separated by electrophoresis on 1% agarose/formaldehyde gels and transferred to a nitrocellulose filter for Northern hybridization by standard procedures [12]. For dot blot analysis, RNA (1–0.25 μg) was denatured in a buffer containing formaldehyde for 15 min at 65°C and applied to nitrocellulose filters with a vacuum dot blot template. Filters were then baked for 2 h at 80°C in a vacuum and prehybridized for 3 h at 42°C. Specific hybridization was carried out for 24 h at 42°C as described elsewhere [13] with ^{32}P-labeled cDNA probes. The filters were washed twice in 2 × SSC, 0.1% SDS at room temperature and twice in 0.1 × SSC, 0.1% SDS at 62°C, air dried, and exposed to x-ray films at −80°C for various periods of time. The radioactivity was determined by scanning autoradiograms with a densitometer.

cDNA Probes Plasmids Hf-677 with a 1.5-kb insert specific for the C-terminal end of the α_1(I) chain [14] and pIII-33 (a gift from Dr. R. Crystal, National Institutes of Health) with a 0.9-kb insert specific for the α_1(III) chain [15] were used as the probes for collagen I and III mRNAs, respectively. Plasmid K4 with a 0.7-kb insert specific for human collagenase [16] and plasmid pHFA-1 with a 0.5-kb insert specific for human β-actin [17] have been previously described. The 1.0-kb cDNA probe for elastin was obtained by polymerase chain reaction (PCR) amplification with primers derived from exons 10 and 18 of human elastin [18].

Assay of Collagenase Activity Cells inoculated onto 35 × 10-mm plastic dishes were assayed at confluency. The culture medium was changed to serum-free DMEM and incubated in a CO_2 incubator for 48 h at 37°C. After the incubation, the medium was removed, 1 M Tris-HCl (pH 7.5) and 1 M $CaCl_2$ were added to a final concentration of 0.05 M Tris-HCl (pH 7.5) and 0.01 M $CaCl_2$, and stored at −80°C for the assay. Five hundred microliters of the cultured medium of fibroblasts was first treated with 20 μg of TPCK-trypsin (Worthington Biochem, Freehold, NJ) for 10 min at 37°C to activate latent collagenase in the medium and then the excess trypsin was inactivated by adding 90 μg of soybean trypsin inhibitor (Sigma, St. Louis, MO). Collagenase activity was assayed by the solution method [19,20], using FITC-labeled type I collagen as a substrate and ethanol instead of dioxane as a precipitant of the undigested collagen substrate. Freshly prepared serum-free DMEM was treated similarly and used as a blank. The enzyme activity was estimated by measuring the fluorescence intensity of the supernatant at 495 nm (excitation) and 520 nm (emission). One unit of collagenase activity is defined as the amount of enzyme degrading 1 μg of collagen per minute under the conditions employed.

The significance of differences in collagenase activity between normal and cutis laxa fibroblasts was tested using the Rank-Sum test [21] and the level of significance was set at $p < 0.05$.

RESULTS

Collagen Synthesis by Cutis Laxa Fibroblasts Collagen synthesis was determined in three strains of both cutis laxa and normal control fibroblasts. The results are shown in Table II. Collagen synthesis was expressed as the rate of collagen production to total protein production, which was calculated by the formula described in *Materials and Methods*. The mean values of collagen synthesis in the cutis laxa fibroblasts and control fibroblasts were 6.96 and 7.16, respectively. There was no significant difference in collagen synthesis between cutis laxa and age-matched control fibroblasts.

Steady-State Levels of Collagen, Collagenase, and Other Protein mRNA of Cutis Laxa Fibroblasts To determine the expression of not only elastin, the mRNA levels of which in some cases of cutis laxa are known to be decreased, but also collagen and collagenase at mRNA levels, we measured the mRNA levels of various proteins using specific cDNA probes. The total RNA from normal and cutis laxa fibroblast cultures were analyzed by dot blot analysis (Fig 1). The data obtained by densitometry are summarized in Table III. Although there was no difference in the values of β-actin, α_1(I)collagen, and α_1(III)collagen mRNA between normal and cutis laxa fibroblasts, cutis laxa fibroblasts showed about a 2–4-times decrease (twofold in CL2, threefold in CL1, and fourfold in CL3) in elastin mRNA and about a 2–4-times increase (twofold in CL2, threefold in CL3, and fourfold in CL1) in collagenase mRNA as compared with normal fibroblasts.

Total RNA was also examined qualitatively by Northern blot analysis. No qualitative differences were detected in the mRNA transcripts of α_1(I)collagen (4.8 and 5.8 kb), α_1(III)collagen (4.8 and 5.4 kb), collagenase (2.5 kb), elastin (3.5 kb), and β-actin (2.0 kb) between the control and cutis laxa fibroblasts (Fig 2).

Collagenase Activity in Cutis Laxa Fibroblasts To determine whether the increased level of collagenase gene expression was reflected in the collagenase activity in cutis laxa fibroblasts, we performed an assay for collagenase activity using culture medium. The

Table II. Collagen Synthesis (%) by Cutis Laxa Fibroblasts

	Cell Strain	Collagen Synthesis (%)[a]	p Value
Cutis laxa	CL1 (n = 8)	6.6 ± 0.3	NS[b]
	CL2 (n = 4)	6.4 ± 0.3	NS[b]
	CL3 (n = 8)	7.6 ± 0.2	NS[c]
	Mean ± SEM	6.96 ± 0.2	NS[c]
Controls	N1 (n = 8)	7.5 ± 0.1	
	N2 (n = 8)	6.4 ± 0.3	
	N3 (n = 8)	7.6 ± 0.1	
	Mean ± SEM	7.16 ± 0.16	

[a] Collagen synthesis is expressed by the relative rate of collagen production as described in *Materials and Methods*. Values are expressed as mean ± SEM.
[b] NS, not significant to the mean of controls at 5% level (Student t test).
[c] NS, not significant to the mean of controls at 1% level (Student t test).

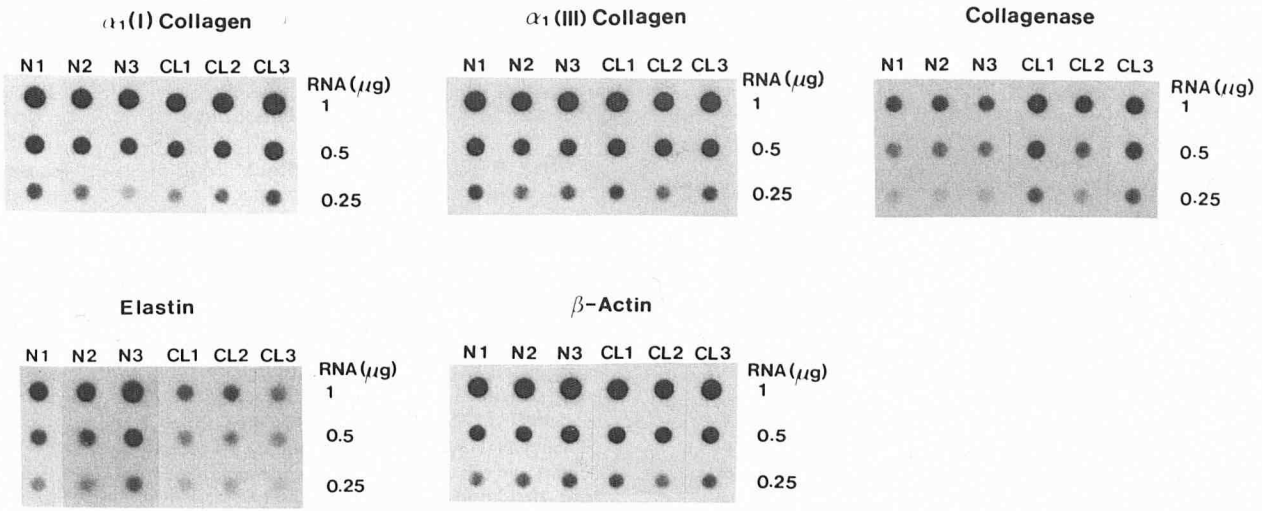

Figure 1. Blot quantification of mRNA of dermal fibroblasts derived from cutis laxa patients (CL1, CL2, CL3) and age-matched normal controls (N1, N2, N3). Serial dilutions of total RNA (1, 0.5, 0.25 μg) were dotted onto a nitrocellulose filter, baked, and hybridized with cDNA probes of α_1(I), α_1(III)collagen, collagenase, elastin, and β-actin.

culture medium from normal and cutis laxa fibroblasts was treated with trypsin to activate latent collagenase, and then collagenase activity was assayed. The collagenase activity of the medium from cutis laxa fibroblasts was about 2 times in CL2 and 3 times in CL1 and CL3 higher than the average of that obtained from control fibroblasts (Fig 3).

DISCUSSION

Morphologic studies of skin biopsies from individuals with congenital forms of cutis laxa frequently reveal alterations in the quantity and morphology of elastic fibers [7]. However, structural abnormalities have been described in not only elastic fibers but also collagen fibers. Small and separated bundles and collagen fibrils of varied diameters and loosely aggregated collagen fibrils have been observed in the skin of some patients with cutis laxa [5,9]. It is, therefore, conceivable that the synthesis or degradation of type I and type III collagen, major components of the extracellular matrix, might be aberrant in the skin of a patient with this disorder. There has been one report [8] of no abnormality in the synthetic activity of collagens I and III in one cutis laxa strain and another [9] in which increased collagen synthesis was observed in one cutis laxa strain. In this study, the collagen synthetic activity of three strains from patients with congenital cutis laxa was examined. None of these strains were found to have abnormal collagen synthetic activity and/or abnormal type I and type III collagen mRNA levels.

Interstitial collagens are degraded by the neutral proteinase, collagenase, that is synthesized as a latent enzyme by human fibroblasts and other types of cells [22]. Previous experiments have demonstrated that cultured human skin fibroblasts synthesize collagenase [23], and collagenase gene expression can be analyzed by hybridizations with homologous human collagenase cDNAs [24,25]. We found an increased steady-state level of collagenase mRNA in cutis laxa fibroblasts and increased collagenase activity in the culture supernatant of these fibroblasts. It is suggested that increased collagenase activity is modulated mainly at the transcriptional level. Our data suggest increased collagenase expression of fibroblasts is related in some way to the structural abnormality of dermal connective tissue in cutis laxa.

The expression of collagenase was found to be induced by several cytokines such as interleukin 1 [26,27], interferon-γ [28], and tumor necrosis factor [28,29]; several growth factors such as epidermal growth factor [30] and platelet-derived growth factor [30,31]; and the tumor-promoting phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate [16]. In addition, it has recently been reported that the phorbol-ester induction of the collagenase gene is mediated by an inducible enhancer element located in the 5′-flanking region [32]. These cytokines, some growth factors and a chemical have been shown to cause divergent regulation of collagen and collagenase gene expression [25]. Therefore, the pattern of regulation of collagen and collagenase in cutis laxa fibroblasts under basal conditions differs from the pattern found in normal fibroblasts stimulated by cytokines, growth factors or a chemical.

In this study, reduced elastin gene expression in three cutis laxa fibroblast strains was detected. This observation was consistent with previous reports. Olsen et al [6] reported that all the cutis laxa cell strains they examined showed a reduced level of elastin gene expression, and Sephel et al [7] indicated that tropoelastin production was reduced in three of six cutis laxa cell strains suggesting heterogeneity of elastin expression in cutis laxa fibroblast strains. Our data indicate that fibroblasts from patients with cutis laxa having reduced elastin expression are associated with increased collagenase expression.

Table III. mRNA Levels Specific for α_1(I)Collagen, α_1(III)Collagen, Collagenase, Elastin, β-Actin of Cutis Laxa Fibroblasts[a]

	mRNA Analyzed				
Cells	α_1(I)Collagen (%)	α_1(III)Collagen (%)	Collagenase (%)	Elastin (%)	β-Actin (%)
Controls	100	100	100	100	100
CL1	92	98	389	32	96
CL2	99	100	188	48	94
CL3	104	103	325	23	96

[a] The dot blot filter was exposed to x-ray film and the intensity of hybridization was quantitated densitometrically. Values were expressed as the percent compared to the average of three normal controls. Values given are the average of two independent experiments. All values are normalized for the same amount of RNA.

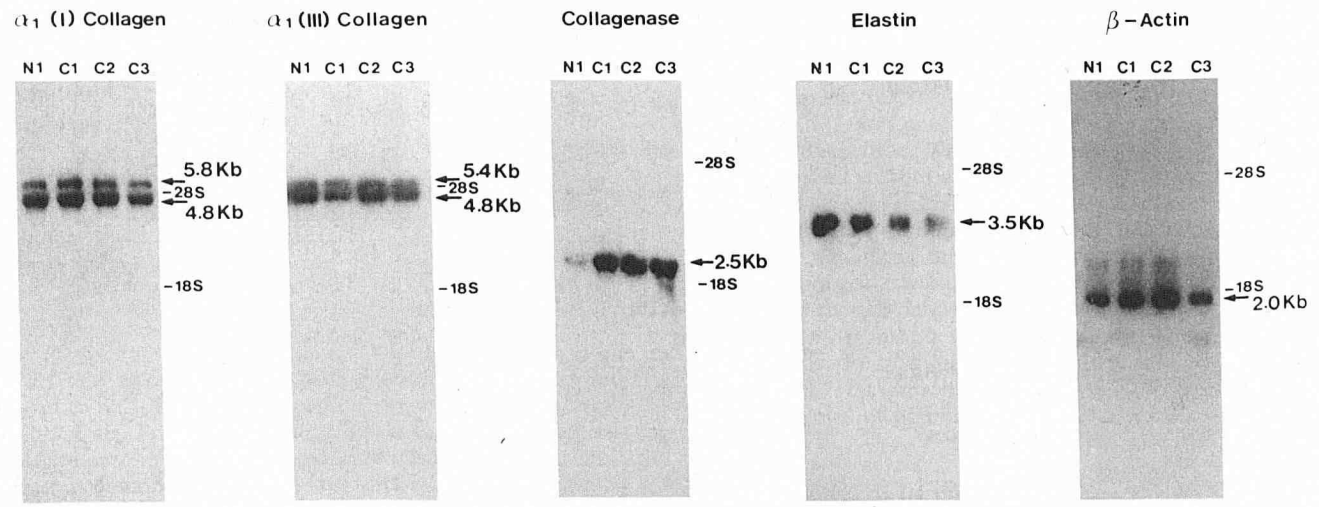

Figure 2. Northern blot detection of α_1(I), α_1(III)collagen, collagenase, elastin, and β-actin specific mRNA obtained from dermal fibroblasts derived from cutis laxa patients (C1, C2, C3) and a normal control (N1). Total RNA (2 μg each) was electrophoresed and transferred to nitrocellulose, and mRNA were hybridized to labeled cDNA probes. The positions of 28 S and 18 S RNA are indicated by *bars*.

To understand the mechanism of increased collagenase expression in fibroblasts from patients with cutis laxa, it is very important to study the conditions causing the increased expression of collagenase accompanying reduced elastin expression. We could find only one case. There have been reports that collagenase and collagenase mRNA production were enhanced in late passage human fibroblasts [33] and that elastin gene expression was reduced in fibroblasts from the skin of an old individual [34]. Cutis laxa fibroblasts, in which a selective increase in collagenase and a decrease in elastin gene expression is observed, appear to offer a unique model for the study of mutual control in the metabolism of different components of connective tissue.

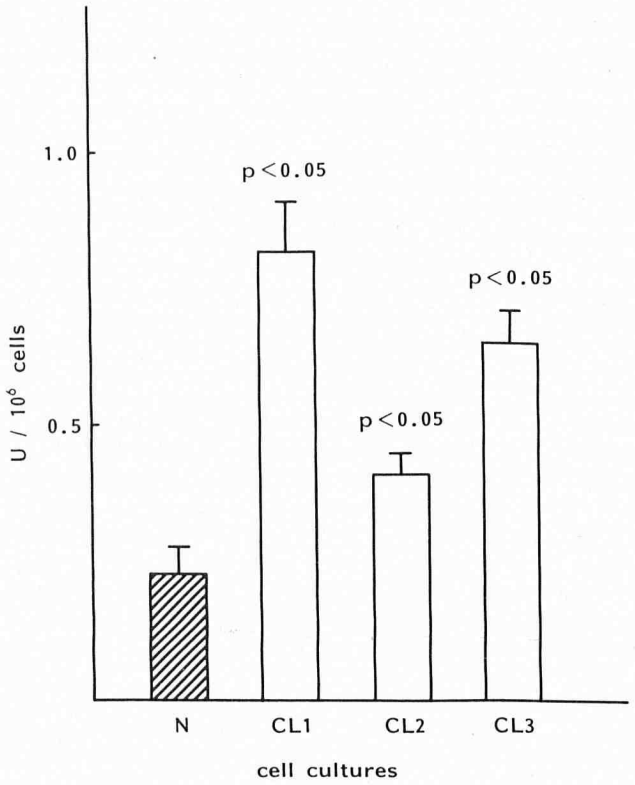

Figure 3. Collagenase activity in cutis laxa fibroblast cultures. The culture medium of fibroblasts derived from cutis laxa patients (CL1, CL2, CL3) and normal controls was obtained from 48-h cultures with serum-free DMEM. Each determination was done with four dishes, and the values given are the mean ± SEM. N, average of collagenase activity of three control fibroblast strains.

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