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Biochemical Composition of the Connective Tissue in Keloids and Analysis of Collagen Metabolism in Keloid Fibroblast Cultures*

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Keloids are histologically characterized by an abundance of the extracellular matrix of connective tissue. In the present study, we examined the connective tissue composition of keloids, and analyzed the details of collagen metabolism utilizing fibroblast cultures established from keloid tissue. Quantitative connective tissue analyses indicated that collagen was the predominant extracellular matrix component in keloids. The ratio of genetically distinct collagens type I/III was significantly increased, as compared to normal human skin. Collagen biosynthesis was measured in fibroblast cultures by the formation of radioactive hydroxyproline: 5 of 9 keloid cell cultures studied demonstrated increased procollagen production in comparison to age-, sex-, and passage-matched control skin fibroblast lines, while the remaining 4 cell lines were within the control range. Keloid fibroblast cultures which were high collagen producers also demonstrated elevated prolyl hydroxylase activity. The mechanisms of increased procollagen production in fibroblast cultures were first examined by assaying the abundance of type I procollagen-specific mRNA utilizing dot blot hybridizations with a proα2(I)-chain-specific cDNA. The type I procollagen mRNA levels were significantly increased in 4 keloid fibroblast lines, and a good correlation between the mRNA levels and the rate of procollagen production in the same cultures was noted. These observations suggest regulation of the collagen gene expression on the transcriptional level. The catabolic pathway of collagen metabolism in fibroblast cultures was examined by determining the degradation of newly synthesized procollagen polypeptides through assay of radioactive hydroxyproline in small-molecular-weight peptide fragments. In 3 keloid cell cultures, the degradation of newly synthesized collagen polypeptides was below the range of normal controls. These findings suggest that a reduced degradation of newly synthesized polypeptides might contribute to the accumulation of procollagen in some keloid fibroblast cultures. The results of this study suggest two possible mechanisms for deposition of collagen in keloid lesions in vivo: first, the growth of the lesions may result from a localized loss of control of the extracellular matrix production by fibroblasts; secondly, reduced degradation of the newly synthesized procollagen polypeptides may contribute to collagen deposition in some keloids.

Keloids are firm dermal nodules with predilection sites on the upper back, shoulders, ear lobes, and anterior portion of the chest (for review on keloids, see [1–3]). Most frequently, the lesions occur in patients 10–30 years of age, but the onset may be at any age. The development of keloids is often preceded by trauma at the site of the lesion. Keloids are common in blacks and Chinese, but the lesions have been reported in most races. The incidence of keloids is unknown; however, examination of certain African populations has suggested an incidence of approximately 6%. In 10–20% of the European cases, a positive family history of keloids has been obtained, and in some cases, the pedigree suggests an autosomal dominant inheritance.

Histopathology of keloids suggests that the lesions are rich in the extracellular matrix of connective tissue [4]. In early stages of development, a marked fibroblast infiltrate may be present, while at later stages, the lesions are characterized by deposition of dense collagenous meshwork. These observations suggest then that keloids result from a localized loss of control in the regulation of extracellular matrix production. However, the exact mechanisms of keloid formation are currently unknown, and previous studies utilizing cultured fibroblasts have yielded inconclusive results [2].

In the present study, we have analyzed the biochemical composition of keloids with a special emphasis on connective tissue. Furthermore, we have examined the metabolism of collagen, the major fibrillar component of keloids, in fibroblast cultures established from the lesions.

MATERIALS AND METHODS

Clinical Material

Tissue specimens from 9 patients with keloids were subjected to biochemical analyses. The keloid tissue was surgically removed under local anesthesia after obtaining informed consent. The patients were black females with ages varying from 19–64 years. The lesions were located on the earlobe and they were diagnosed as keloids on the basis of their clinical appearance, persistence for several years, expansion beyond the limits of the original trauma, and histopathology. The histopathology of the lesions showed areas with thick, compact and hyalinized collagen fibers in a whirl-like or nodular pattern, while in some areas the collagen fibers showed a parallel wavy orientation. In some lesions, areas of fibroblastic cell infiltrates were seen. Control

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Abbreviations:
DMEM: Dulbecco's modified Eagle's medium
EDTA: ethylenediamine tetraacetate
NEM: N-ethylmaleimide
PMSF: phenylmethylsulfonyl fluoride
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SCC: 0.15 M NaCl in 15 mM sodium citrate, pH 6.8

skin was obtained from sex- and age-matched patients who underwent a surgical procedure for cosmetic reasons.

Part of the tissue was fixed for histology in 10% formaldehyde and processed routinely for hematoxylin-eosin and Verhoeff-van Gieson stain. A small piece of tissue was also fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and examined by transmission electron microscopy. Another tissue specimen was immediately weighed for wet weight determination, and then lyophilized to obtain a constant dry weight. The difference between the wet and dry weights was used as an estimate of water content of the tissue. A piece of tissue was also used to establish fibroblast cultures (see below).

Connective Tissue Analyses

Tissue specimens with a known dry weight were homogenized in 0.5 M acetic acid with a Polytron homogenizer at 4°C. The homogenates were extracted for 16 h at 4°C and centrifuged at 30,000 *g* for 30 min at 4°C. Aliquots of the supernatant and the pellet were hydrolyzed in 6 M HCl for 18 h at 120°C. The hydrolysates were evaporated and assayed for hydroxyproline [5] and desmosine [6] as an index of collagen and elastin, respectively. Another aliquot of the acetic acid-soluble and insoluble material was subjected without acid hydrolysis to uronic acid determination [7] as an index of glycosaminoglycans.

For determination of genetically distinct collagen types, tissue specimens were homogenized in 0.5 M acetic acid, and submitted to limited proteolysis by pepsin (Worthington, 2 × crystallized), in a final concentration of 300 μg pepsin per ml. The samples were incubated for 3 h at 24°C, followed by 16 h at 4°C. The pepsin-solubilized material was recovered by centrifugation for 60 min at 30,000 *g* at 4°C, and the insoluble material was subjected to further pepsinization as above. The supernatants containing the pepsin-solubilized material were combined, and protease inhibitors were added to a final concentration of 5 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM N-ethylmaleimide (NEM), and 50 μg/ml iodoacetamide. The pH of the samples was adjusted to 8.5 by the addition of 1 M Tris, and the samples were incubated for 60 min at 4°C to inactivate pepsin. The samples were then dialyzed against 0.4 M NaCl, 50 mM Tris-HCl, pH 7.5, containing the proteinase inhibitors. Collagen was precipitated by the addition of NaCl to the final concentration of 4.4 M, and the precipitate was collected by centrifugation. The pellet was extracted in 50 mM Tris-HCl, pH 7.5, and the solubilized collagen was successively precipitated by increasing the NaCl concentration to 1.7, 2.6, and 4.4 M. Between precipitations, the insoluble material was collected by centrifugation at 30,000 *g* for 60 min at 4°C. The pellets were dissolved in 50 mM Tris-HCl, pH 7.5, and aliquots were hydrolyzed in 6 M HCl for assay of hydroxyproline [5]. Another aliquot was treated for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described elsewhere [8].

SDS-PAGE was performed using 8% polyacrylamide gels [9], with or without delayed reduction with 2-mercaptoethanol [10]. The collagen polypeptides were visualized by staining with Coomassie Brilliant Blue and quantitated with an automatic computing densitometer (ACD 18, Gelman Instrument).

Fibroblast Cultures

Primary cultures of fibroblasts were established from keloids and control skin by growing the cells on plastic flasks (Costar) in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal calf serum. The primary cultures were passed by trypsinization, and the secondary cultures were maintained in the same medium. The cell cultures were subjected to biochemical analyses in passages 3–9. To study collagen synthesis, cell cultures in early visual confluency were preincubated for 60 min in DMEM containing 20% dialyzed fetal calf serum, 50 μg/ml ascorbic acid, and 20 μg/ml β-aminopropionitrile [11]. After preincubation, radioactive proline (L-[2,3,4,5-^{3}H]proline, sp act 109 Ci/mmole; Amersham) was added, and the incubations were continued for 20 h. At the end of the incubation, medium was removed and the protease inhibitors in final concentrations of 20 mM EDTA, 1 mM PMSF, and 10 mM NEM were added. The cells were sonicated in 50 mM Tris-HCl, pH 7.5, containing 0.4 M NaCl and the protease inhibitors in the same concentrations as above. Aliquots of the medium and cell fractions were dialyzed against distilled water, hydrolyzed in 6 M HCl at 120°C for 16 h, and assayed for [^{3}H]hydroxyproline by a radiochemical assay [12]. In some experiments, the [^{3}H]hydroxyproline in dialyzable form was assayed by dialyzing the samples first against 10 volumes of distilled water, and the dialysates were hydrolyzed and assayed for [^{3}H]hydroxyproline as above. Aliquots of the cell sonicates were dialyzed against 1 mM Tris-HCl, pH 7.5, containing 50 mM NaCl, and assayed for protein [13] and DNA [14].

For assay of the genetically distinct collagens synthesized by the fibroblasts in culture, the cells were labeled with radioactive proline for 4 h as above. Cell and medium fractions were subjected to limited pepsin proteolysis [15], and examined by SDS-polyacrylamide slab gel electrophoresis [9], followed by fluorography [16].

Assay of Type I Procollagen mRNA levels

For isolation of mRNA, the cells were scraped into 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 1% SDS. The samples were digested with 100 μg/ml proteinase K (Boehringer) for 60 min at 37°C. Poly(A)$^+$-RNA was then isolated by oligo-dT-cellulose column chromatography, as described elsewhere [17]. The concentration of RNA was determined by absorbance at 260 nm. The recovery of the poly(A)$^+$-RNA was estimated by employing rabbit globin mRNA (BRL) as an internal standard. In this procedure, 100 ng rabbit globin mRNA is added to each cell culture at the beginning of the extraction procedure. The recovery of globin mRNA is then estimated by dot blot hybridization of the poly(A)$^+$-RNA preparations with rabbit globin specific 4.4 kilobase genomic DNA fragment [18] (kindly provided by Dr. T. Maniatis, Harvard University). The recovery of the rabbit globin mRNA, based on densitometric scanning of the dots containing the isolated poly(A)$^+$-RNA and compared with parallel dots containing known amounts of pure globin mRNA (BRL), is taken as a recovery of the total poly(A)$^+$-RNA.

The dot blot hybridizations were performed by dotting 50-μl aliquots of the poly(A)$^+$-RNA, mixed with an equal volume of solution containing 3 vol of 20 × SSC (0.15 M NaCl in 15 mM sodium citrate, pH 6.8) and 2 vol of 37% formaldehyde [19,20], on the 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 prehybridized at 42°C in a solution containing 4 × SSC, 50% formamide, 0.1% SDS, 50 μg/ml denatured salmon sperm DNA, 0.02% polyvinylpyrolidone, 0.02% bovine serum albumin, and 0.02% ficoll. The hybridizations were carried out in the same solution containing either human proα2(I) collagen sequence-specific cDNA [21] or rabbit globin-specific genomic DNA probe [22] labeled by nick translation with α-^{32}P-labeled nucleotides to a specific activity of approximately 5×10^8 cpm/μg. After hybridizations at 42°C for 48 h, the filters were washed twice in 500 ml of 2 × SSC at 24°C, and then in 1 × SSC, 1 × SSC + 0.1% SDS, 0.5 × SSC + 0.1% SDS, and 0.2 × SSC, respectively, all at 50°C. The dry filters were exposed to x-ray films (Kodak) in cassettes equipped with intensifying screens. The levels of mRNA were quantitated by either scanning the radioautograms at 700 nm using an automatic computing densitometer (ACD 18, Gelman Instruments) or by counting strips of nitrocellulose filters corresponding to the radioactive mRNA-[^{32}P]DNA hybrids.

Enzyme Assays

For assay of collagenase activity, fibroblasts were cultured in serum-free DMEM for 6 h. The medium was collected and aliquots were subjected to brief trypsin proteolysis [23] using 0.1–10 μg trypsin per ml for 10 min at 25°C. Trypsin was then inactivated by the addition of soybean trypsin inhibitor, 50 μg/ml, and collagenase activity was assayed by incubating samples with radioactive type I collagen, as described previously [24]. The collagenase activity was expressed as degradation of ^{3}H-labeled collagen, dpm × h^{-1}/mg DNA.

For assay of prolyl hydroxylase activity, the cells were incubated for 1 h in medium containing 50 μg/ml ascorbic acid. After incubation, the medium was removed and cells were homogenized in 0.2 M Tris-HCl, pH 7.5, containing 0.2 M NaCl, and 0.01% Triton X-100. The cells were homogenized with a Teflon-glass homogenizer, extracted for 60 min at 4°C, and then centrifuged for 30 min at 30,000 *g*. Aliquots of the supernatant were used for assay of prolyl hydroxylase by incubation with radioactive unhydroxylated collagen, as described elsewhere [25].

Statistical Analyses

The significance of the differences was calculated by Aspin-Welch two-sample test of means.

RESULTS

Connective Tissue Analyses of Keloids

Histopathology and electron microscopy of the keloid tissues analyzed in this study suggested that the lesions consisted predominantly of the fibrous extracellular matrix of connective

tissue. To accurately quantitate the connective tissue components, keloid specimens were subjected to biochemical analyses. The water content was significantly higher in keloids than in normal control skin, the values being 78.4 ± 2.8 and 64.0 ± 6.8%, respectively ($p < 0.005$). Assay of hydroxyproline as an index of collagen revealed no difference in the concentration of this imino acid between the keloids and normal skin, when expressed per mg dry weight of tissue (Fig 1). The concentrations of elastin and glycosaminoglycans, determined by desmosine and uronic acid analyses, were significantly increased in keloids, but these components represented only a minor fraction of the tissue (Fig 1). Estimation of the relative concentrations of collagen, based on hydroxyproline assay, indicated that this protein was the predominant component of the keloids, comprising approximately 60% of the dry weight of tissue.

Since collagen was the major connective tissue component of keloids, its composition with respect to genetically distinct collagen types was examined in further detail. For this purpose, collagen was isolated by limited pepsin proteolysis, which solubilized more than 95% of the total collagen both in keloids and in normal skin. Initial analyses of the solubilized collagen by SDS-PAGE revealed the presence of α1(I) and α2(I) chains of type I collagen as the predominant collagenous polypeptides (Fig 2*A*). However, peptides representing type III and type V collagen α-chains were also noted. In order to quantitate the relative concentrations of the genetically distinct collagens, the solubilized polypeptides were fractionated with successive precipitations with 1.7, 2.6, and 4.4 M NaCl; these precipitations enrich the fractions in type III, type I, and type V collagens, respectively [26] (Fig 2*A,B*). The relative contents of various collagen types were then quantitated on the basis of the total amount of hydroxyproline in each fraction and the relative distribution of α2(I), α1(III), and α1(V) chains as markers of type I, III, and V collagens, respectively. The results indicated that the relative concentration of type I collagen was significantly higher in keloids than in control skin (94.7 ± 2.4 vs 77.0 ± 1.2% of the total collagen; $p < 0.001$) and the concentration of type III collagen was correspondingly decreased (Fig 3). No difference in type V collagen concentrations between keloids and the controls was noted.

The solubility of collagen in 0.5 N acetic acid was also

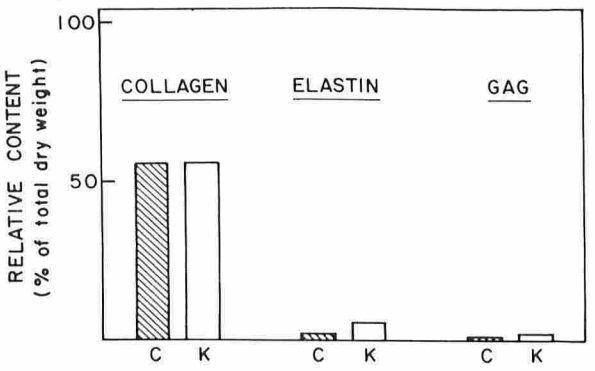

FIG 1. Relative concentrations of collagen, elastin, and glycosaminoglycans (GAG), determined by assays of hydroxyproline, desmosine, and uronic acid, respectively, in keloids (*K*) and normal skin (*C*). The values are mean of 8 keloids and 3 control specimens.

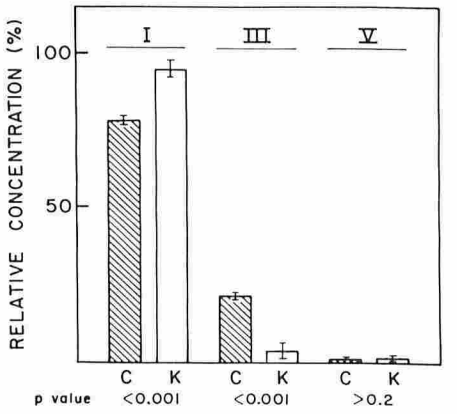

FIG 3. Relative concentrations of collagen types I, III, and V in keloid tissues and in normal human skin. Collagen was solubilized by pepsin proteolysis, and the relative concentrations of genetically distinct collagens were determined on the basis of separation of α-chains by SDS-PAGE as shown in Fig 3, and quantitation of hydroxyproline in various NaCl precipitations. The values are mean ± SD, expressed as percent of the total collagen in the tissues. The statistical significance between the keloid and control tissues, calculated by Aspin-Welch two-sample test of means, are indicated in the figure. *C* = normal human skin, *K* = keloid tissue.

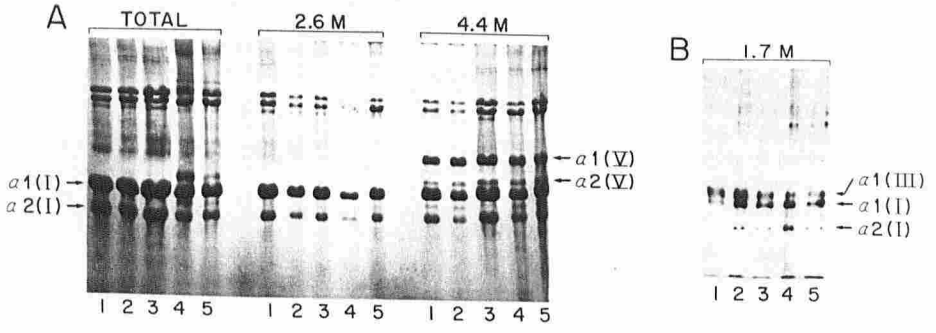

FIG 2. Isolation of genetically distinct collagens in keloid tissues. Collagen in keloids and in normal human skin was solubilized by pepsin proteolysis, as indicated in *Materials and Methods*. The solubilized collagen molecules were fractionated by successive precipitations with 1.7, 2.6, and 4.4 M NaCl; these fractions are enriched in type III, I, and V collagens, respectively. The collagen was then examined by SDS-PAGE, followed by visualization of the polypeptides with Coomassie Brilliant Blue. *A*, Electrophoresis of solubilized total collagen before differential salt precipitations, as well as in 2.6 and 4.4 M NaCl precipitation fractions. *B*, Electrophoresis of collagens precipitable with 1.7 M NaCl. The electrophoresis was performed with delayed reduction of the disulfide bonds by 2-mercaptoethanol. The migration positions of α1(I) and α2(I) chains of type I collagen, α1(V) and α2(V) of type V collagen, and α1(III) of type III collagen are indicated by the *arrows*. *Lanes 1–3* are keloid specimens and *lanes 4 and 5* represent normal skin.

TABLE I. *Collagen production in human skin fibroblast cultures established from keloids and normal human skin*[a]

Cell line	[^{3}H]Hydroxyproline (dpm × 10^{-3}/μg DNA)[b]	Total ^{3}H incorporation (dpm × 10^{-4}/ μg DNA)[b]	[^{3}H]Prolyl hydroxylation (%)[c]
Experiment I			
Keloid 1	11.9 ± 3.0*	3.7 ± 1.1	32.2
Keloid 2	6.2 ± 0.6*	3.2 ± 0.2	19.4
Keloid 3	2.6 ± 0.4	1.4 ± 0.2	18.6
Control 1	3.6 ± 0.7	1.3 ± 0.1	27.7
Control 2	2.2 ± 0.3	1.1 ± 0.1	20.0
Control 3	2.7 ± 0.4	1.2 ± 0.2	22.5
Experiment II			
Keloid 4	15.0 ± 0.1*	5.0 ± 0.4	30.0
Keloid 5	11.0 ± 0.9*	4.3 ± 0.5	25.6
Keloid 6	4.6 ± 1.3	3.2 ± 0.4	14.4
Keloid 7	4.7 ± 0.7	3.1 ± 0.5	15.2
Control 4	4.6 ± 0.6	3.2 ± 0.4	14.4
Experiment III			
Keloid 8	10.1 ± 0.7*	4.1 ± 0.8	24.4
Control 5	5.1 ± 0.6	2.8 ± 0.4	18.2
Control 6	6.5 ± 1.2	3.1 ± 0.9	21.0
Experiment IV			
Keloid 9	3.6 ± 0.4	2.7 ± 0.2	13.3
Control 7	3.0 ± 0.1	2.7 ± 0.1	11.1

[a] Confluent fibroblast cultures were incubated with [^{3}H]proline under conditions optimized for procollagen synthesis, and the collagen production was assayed by the synthesis of nondialyzable [^{3}H]hydroxyproline.

[b] The values are mean ± SD of 3 parallel cultures. The values indicated by an *asterisk* are statistically different from the controls ($p < 0.005$).

[c] Calculated as 100 × [^{3}H]hydroxyproline/total ^{3}H incorporation.

determined; the solubility of collagen is known to be increased in situations associated with increased rate of collagen synthesis [27]. In control skin specimens, 3.6 ± 2.3% (mean ± SD) of collagen was acid soluble. In 6 keloid specimens the values were in the same range as that noted in the controls, while in 2 of the 8 keloid specimens there was a marked increase in collagen solubility.

Fibroblast Culture Studies

To examine the mechanisms of collagen accumulation in keloid tissues, fibroblast cultures were established from 9 keloid specimens; they were then compared with age-, sex-, and passage-matched control skin fibroblast cultures established from normal human skin. For determination of collagen production, the cells were incubated with [^{3}H]proline and assayed first for the synthesis of [^{3}H]hydroxyproline in nondialyzable macromolecules. Assay of [^{3}H]hydroxyproline revealed that in 4 different experiments, 4 keloid cell lines (nos. 1, 2, 4, and 5) demonstrated values which were more than 2-fold higher than the corresponding mean values of the age-matched and passage-matched controls included in each experiment (Table I). Keloid cell line 8 showed approximately 1.7-fold increased collagen production, as compared to 2 control cell lines. The remaining 4 keloid cell lines demonstrated collagen production rates which were within the control limits (Table I).

The collagen production in keloid fibroblast cultures was also examined by determining the degree of prolyl hydroxylation in newly synthesized proteins, expressed as 100 × [^{3}H]hydroxyproline/total ^{3}H radioactivity in the nondialyzable form. The highest values were noted in 2 keloid cultures (nos. 1 and 4, Table I); these cell lines also demonstrated the highest rate of procollagen production. The mean values for [^{3}H]prolyl hydroxylation in all keloid cell lines and the controls were 21.5 ± 6.9 and 19.3 ± 5.4% (mean ±SD), respectively.

In further studies, the activity of prolyl hydroxylase was measured in 4 keloid cell lines (nos. 4, 5, 6, and 8) and compared with the activity in age- and passage-matched control cultures. Two of the keloid fibroblast lines (nos. 8 and 4), which also demonstrated relatively high procollagen production rates, had elevated levels of prolyl hydroxylase activity.

To examine the types of procollagen synthesized by the keloid fibroblasts, the cells were labeled with radioactive proline, the medium and cell fractions were subjected to limited pepsin proteolysis, and the pepsin-resistant collagenous polypeptides were examined by SDS-PAGE (Fig 4). Fluorograms of the collagen α-chains in the medium fraction of the control fibroblast cultures revealed the presence of α1(I) and α2(I) chains of type I collagen; also, α1(III) chains of type III collagen were apparent (Fig 4). In addition to these polypeptides, a band representing α1(V) of type V collagen was noted in the keloid fibroblast culture medium, but not in the media of control cell cultures (Fig 4). Analyses of the cell fractions from the same fibroblast cultures revealed the presence of α1(V) chains both in keloid and control cultures, in addition to type I collagen polypeptides. Thus, the keloid fibroblast cultures synthesize and secrete type I, III, and V collagens.

Assay of Type I Procollagen mRNA Levels

Previous indications in the literature have suggested that the control of collagen gene expression in cells exists primarily on the transcriptional level [28–30]. To test this hypothesis, we assayed the abundance of type I procollagen mRNA in 4 keloid fibroblast lines by dot blot hybridizations. In these experiments, hybridizations of mRNA with ^{32}P-labeled human α2(I) collagen sequence-specific cDNA allowed the estimation of the relative abundance of type I collagen mRNA (Fig 5). The recovery of poly(A)$^+$-RNA was estimated by adding rabbit β-globin mRNA into cell cultures prior to mRNA isolation, and the procollagen mRNA levels were adjusted for the recovery of β-globin mRNA.

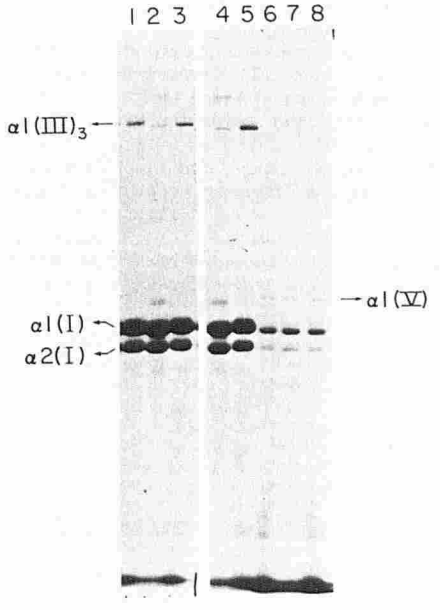

FIG 4. Synthesis of type I, III, and V collagens by cultured fibroblasts established from keloid tissue or from normal human skin. The cell cultures were labeled with [^{3}H]proline, and the newly synthesized proteins in cell and medium fractions were subjected to limited pepsin proteolysis, as described in *Materials and Methods*. The α-chains were electrophoresed on 8% SDS-polyacrylamide gels, and the radioactive polypeptides were visualized by fluorography. The positions of α1(I) and α2(I) chains of type I collagen, $\alpha1(III)_3$ of type III collagen, and α1(V) of type V collagen are indicated by *arrows*. The samples were as follows: *lanes 1, 3, and 5*: medium proteins from control fibroblast cultures; *lanes 2 and 4*: medium proteins from keloid cell cultures; *lanes 6 and 8*: cell fraction proteins from control cultures; *lane 7*: cell fraction proteins from a keloid cell culture.

The results indicated that the mean abundance of α2(I) collagen-specific mRNA in the 4 keloid and control cells was 2.97 ± 0.55 and 1.72 ± 0.35 cpm/ng poly(A)$^+$-RNA, respectively ($p < 0.05$). The mean value in keloid cell lines was thus 173% of the control. The mean collagen production in the same keloid cell cultures was 220% of the controls. Thus, in these keloid cell lines, the mRNA abundance roughly paralleled the rate of collagen production.

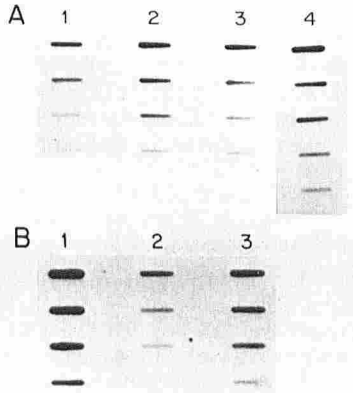

FIG 5. Determination of type I procollagen mRNA abundance in keloid and normal human skin fibroblasts. Poly(A)$^+$-RNA was isolated from the cells by oligo-dT cellulose chromatography. For estimation of the recovery of poly(A)$^+$-RNA, 100 ng of rabbit globin mRNA was added to the cultures prior to isolation procedure. Poly(A)$^+$-RNA in varying concentrations was then dotted on the nitrocellulose filters and hybridized with a rabbit β-globin-specific genomic DNA (*panel A*) or with a human α2(I) collagen-specific cDNA (*panel B*), both labeled with ^{32}P-labeled nucleotides by nick translation. The RNA-[^{32}P]DNA hybrids were visualized by autoradiograms and quantitated by scanning the bands with a densitometer. The recovery of the poly(A)$^+$-RNA was calculated from the recovery of added rabbit globin mRNA, as compared with known amounts of standard mRNA dotted directly on the nitrocellulose filters in concentrations from 100 ng to 6.25 ng in 1:2 dilutions (*panel A, lane 4*). *Lanes 1 and 3* represent assay from keloid cell lines 5 and 4, and *lane 2* represents control cell line 4 (Table I).

TABLE II. *Intracellular degradation of newly synthesized procollagen in keloid and control fibroblast cultures*[a]

Cell line	Total [^{3}H] hydroxyproline (dpm × 10^{-3}/μg DNA)[b]	[^{3}H]Hydroxyproline in dialyzable form (dpm × 10^{-3}/μg DNA)[c]	Collagen degradation (%)[d]
Experiment I			
Keloid 4	16.1 ± 0.1	0.6 ± 0.0	5.0
Keloid 5	11.8 ± 1.0	0.6 ± 0.2	5.1
Keloid 6	5.5 ± 1.5	0.8 ± 0.1	14.5
Keloid 7	5.7 ± 1.0	0.9 ± 0.3	15.8
Control 4	5.6 ± 0.7	0.9 ± 0.1	16.1
Experiment II			
Keloid 8	10.8 ± 0.9	0.7 ± 0.1	6.5
Control 5	5.7 ± 0.7	0.5 ± 0.1	8.8
Control 6	7.1 ± 1.1	0.5 ± 0.1	7.0
Experiment III			
Keloid 9	4.2 ± 0.5	0.5 ± 0.0	11.9
Control 7	3.9 ± 0.2	0.6 ± 0.0	15.4

[a] Confluent fibroblast cultures were incubated with [^{3}H]proline and the synthesis of [^{3}H]hydroxyproline both in dialyzable and nondialyzable form was assayed.

[b] Total [^{3}H]hydroxyproline both in dialyzable and nondialyzable form; mean ± SD of 3 parallel determinations.

[c] Dialyzable [^{3}H]hydroxyproline; mean ± SD of 3 parallel determinations.

[d] Calculated from the dialyzable [^{3}H]hydroxyproline as percent of the total [^{3}H]hydroxyproline.

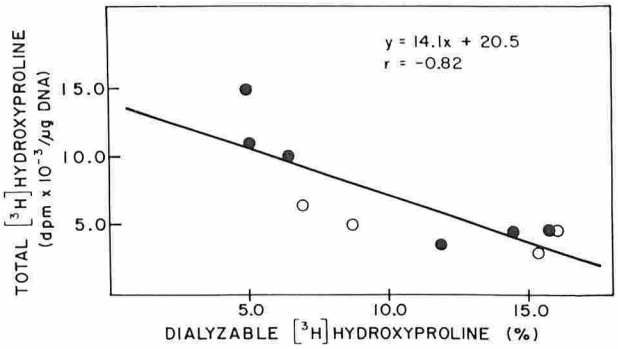

FIG 6. The correlation between the total synthesis of [^{3}H]hydroxyproline and the degradation of newly synthesized procollagen polypeptides, as measured by [^{3}H]hydroxyproline in dialyzable form, in keloid and control fibroblast cultures. The cells were labeled with [^{3}H]proline, and the total radioactive hydroxyproline (dialyzable + nondialyzable) was determined as described in *Materials and Methods*. Linear regression analyses of the data indicated the line of best fit, y = (14.1)x + 20.5, with a correlation coefficient (r) −0.82. The individual data points are means of 3 parallel determinations. Keloid cell cultures (●), control fibroblast cultures (○).

Collagen Degradation

Previous studies have demonstrated that a significant fraction of procollagen synthesized in cultured cells is rapidly degraded into dialyzable peptides which are not accounted for when [^{3}H]hydroxyproline is assayed in nondialyzable form. We, therefore, measured [^{3}H]hydroxyproline both in dialyzable and nondialyzable form in 6 keloid and 4 control fibroblast cultures after a 20-h incubation with radioactive proline. In control fibroblast cultures, the percent of dialyzable [^{3}H]hydroxyproline varied from 7.0–16.1% of the total [^{3}H]hydroxyproline (Table II). In 3 keloid cell lines (nos. 4 and 8), which demonstrated high collagen production, the dialyzable [^{3}H]hydroxyproline was less than 7.0% (Table II). On the average, the fraction of newly synthesized collagen, which was in dialyzable fragments, in all keloid fibroblast lines was 9.8 ± 4.9% (mean ± SD), while the corresponding values in control cultures were 11.8 ± 4.6%; these values are not statistically different. However, in the total material, including the data on 6 keloid and 4 control cell lines, an inverse correlation between the rate of total collagen production and the percent of collagen degraded could be observed (Fig 6). Thus, in some keloid fibroblast cultures, the high procollagen production could be explained, in part, by reduced degradation of the newly synthesized procollagen polypeptides. Since the in vivo accumulation of collagen in keloids could also be explained by reduced removal of the extracellular collagen fibers, the production of proteolytically activatable collagenase, as an indication of the degradative pathway, was assayed in fibroblast cultures. The collagenase activity in 9 keloid fibroblast cultures was not statistically different from that noted in 6 age- and passage-matched control cultures; the mean ± SD of the collagenase activity in keloid and control cultures was 11.4 ± 3.9 and 9.8 ± 1.7 × 10^{-4} cpm radioactive collagen degraded × h^{-1} per mg cell protein.

DISCUSSION

Detailed analyses of collagen in the keloid tissues demonstrated that the composition of the genetically distinct collagens was altered, as compared to normal skin. Specifically, the concentration of type I collagen was significantly increased while that of type III collagen was reduced. These results differ from those in previous reports suggesting that the relative amounts of type III collagen may be increased in scar tissues [31,32]. This difference may reflect differences in the pathologic specimens, and increased type III/I collagen ratio may well be found in hypertrophic scars but not in keloids.

To examine the collagen metabolism under well-defined in vitro conditions, fibroblast cultures were established from keloid tissue. Analyses of collagen synthesis in these cultures indicated that 5 of 9 keloid cell lines produced increased amounts while 4 of 9 produced normal amounts of procollagen. In accordance with this observation, the activity of prolyl hydroxylase was increased in cell lines demonstrating increased collagen synthetic rates. Furthermore, the solubility of collagen was increased in 2 out of 8 keloids studied. These observations are in good agreement with previous demonstrations that collagen synthesis is increased in some but not all keloids [33–36]. At the same time, our results extend previous studies showing that keloid fibroblasts in culture synthesize collagen approximately 2 times more than normal skin fibroblasts [37]. Thus, these results suggest that keloids can be classified as either high or normal producers of collagen.

The reasons for the fact that some keloid cell lines demonstrated normal rates of collagen production are not clear. However, it has been previously demonstrated that human skin fibroblasts are a heterogenous population of cells which can exhibit up to 20-fold differences in their collagen synthesis rates, and such heterogeneity is conserved over multiple population doublings [38,39]. Thus, in some keloid cell cultures, the fibroblasts with increased collagen production may predominate, while other cultures may consist primarily of cells with relatively normal collagen production rates. The relative proportions of cells in any culture characterized either as normal- or overproducer of collagen may depend on the selection of the cells during initiation of the cultures, or may reflect heterogeneity in the disease state at the time the cultures were established. Nevertheless, the data on the overproduction of collagen by cultured cells raise the possibility that keloids represent a process of selection of fibroblasts programmed to produce increased amounts of collagen.

Previous observations in the literature have suggested that a major control point in collagen production exists on the transcriptional level [28–30]. In our study, analyses of type I collagen-specific mRNA levels demonstrated an increased abundance in keloid cultures which were collagen overproducers, and a relatively good correlation between the mRNA abundance and the collagen production was observed. These observations suggest that the increased collagen synthesis in these cultures results from elevated levels of the corresponding mRNA.

Previous studies have indicated that keloid fibroblasts demonstrate normal growth characteristics [37], but that they may be susceptible to modulation by environmental factors in a manner different from that in control fibroblasts [37,40–43]. It is possible, therefore, that keloid cells represent a population that is committed to increased collagen production. Although the concentration of collagen in terms of mg hydroxyproline per dry weight of tissue was not different between keloid tissue and normal skin, the bulk of the keloid lesions result from deposition of connective tissue, predominantly collagen. Thus, there may be a localized loss of control in regulation of extracellular matrix production, and this alteration appears to occur on the transcriptional level of collagen gene expression. Since the ratio of genetically distinct collagens of type I and type III was altered in keloid tissue in comparison to normal skin, such a loss of control may be selective. Alternatively, the deposition of collagen in keloids could result from reduced removal of collagen fibers from the extracellular space. The latter possibility appears unlikely, however, since the activity of collagenase in keloid fibroblast cultures is either normal, as shown here, or increased [44] in comparison to control cells.

It was of interest to note that degradation of newly synthesized collagen was relatively low in fibroblast cultures that demonstrated increased collagen production. Thus, reduced degradation of the newly synthesized procollagen polypeptides might contribute to the increased collagen production in some keloid fibroblast cultures. Such modulation of procollagen production at the level of intracellular degradation of newly synthesized polypeptides has been previously demonstrated in several experimental situations [45–47].

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