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Research Article

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Modulation of Procollagen Gene Expression by Retinoids

Inhibition of Collagen Production by Retinoic Acid Accompanied by Reduced Type I Procollagen Messenger Ribonucleic Acid Levels in Human Skin Fibroblast Cultures

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Abstract

Recent clinical observations have suggested that retinoids, which are in frequent use in dermatology, can affect the connective tissue metabolism in skin and other tissues. In this study, we examined the effects of several retinoids on the metabolism of collagen by human skin fibroblasts in culture. Incubation of cultured fibroblasts with all-*trans*-retinoic acid or 13-*cis*-retinoic acid, in 10^{-5} M or higher concentrations, markedly reduced the procollagen production, as measured by synthesis of radioactive hydroxyproline. The effect was selective in that little, if any, inhibition was noted in the incorporation of [^{3}H]leucine into the noncollagenous proteins, when the cells were incubated with the retinoids in 10^{-5} M concentration. Similar reduction in procollagen production was noted with retinol and retinal, whereas an aromatic analogue of retinoic acid ethyl ester (RO-10-9359) resulted in a slight increase in procollagen production in these cultures. The reduction in procollagen production by all-*trans*-retinoic acid was accompanied by a similar reduction in proα2(I) of type I procollagen specific messenger RNA (mRNA), as detected by dot blot and Northern blot hybridizations. Hybridizations with human fibronectin and β-actin specific DNA probes indicated that the levels of the corresponding mRNAs were not affected by the retinoids, further suggesting selectivity in the inhibition of procollagen gene expression. Further control experiments indicated that all-*trans*-retinoic acid, under the culture conditions employed, did not affect the posttranslational hydroxylation of prolyl residues, the mannosylation of newly synthesized procollagen, the specific radioactivity of the intracellular prolyl-transfer RNA pool, or DNA replication. All-*trans*-retinoic acid also elicited a reduction in trypsin-activatable collagenase, but not in the activity of prolyl hydroxylase or an elastaselike neutral protease in the fibroblast cultures. Incubation of three fibroblast lines established from human keloids with all-*trans*-retinoic acid or 13-*cis*-retinoic acid also resulted in a marked reduction in procollagen production. The results, therefore, suggest that further development of retinoids might provide a novel means of modulating collagen gene expression in patients with various diseases affecting the connective tissues.

Introduction

Retinoids are a class of compounds consisting of four isoprenoid units joined in a head-to-tail manner. All retinoids may be formally derived from a monocyclic parent compound containing five carbon–carbon double bonds and a functional group at the terminus of the acyclic portion (1). Recently, several retinoids, such as all-*trans*-retinoic acid (retinoic acid [RA][1]), 13-*cis*-RA, and etretinate (a synthetic aromatic analogue of RA) have gained considerable use in dermatology for treatment of a variety of hyperproliferative epidermal disorders (2, 3). One of the side effects of the retinoid therapy is excessive fragility of the skin (4). Although the skin fragility has been suggested to be of the epidermal origin (4), it is also possible that the connective tissue matrix of the dermis might be affected by the treatment. A recent report has also suggested that retinoids may be useful for treatment of keloids and hypertrophic scars, two cutaneous disorders characterized by excessive deposition of collagen (5).

In the present study, we have examined a variety of retinoids for their effects on connective tissue metabolism in human skin fibroblast cultures established from control skin or from keloids. The results indicate that various retinoids demonstrate differential modulation of collagen gene expression, but all-*trans*- and 13-*cis*-RA clearly inhibit collagen production in an apparently selective manner.

Methods

Retinoids. All-*trans*-RA, all-*trans*-retinal, retinyl acetate (all-*trans*-retinol acetate), and retinol were purchased from Sigma Chemical Co., St. Louis, MO. 13-*cis*-retinoic acid and RO-10-9359 (trimethylmethoxyphenyl RA ethylester; etretinate) were obtained from Hoffmann-La Roche, Nutley, NJ.

Fibroblast cultures. Human skin fibroblast cultures were initiated from punch biopsies or from skin obtained from surgical procedures after obtaining informed consent. Also, fibroblast cultures were initiated from keloid specimens obtained at plastic surgery. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing Hepes buffer, pH 7.6, 200 U/ml of penicillin, 200 μg/ml of streptomycin, and 20% fetal calf serum. Confluent primary cultures were trypsinized and subcultured in 75-cm^2 flasks (Falcon Plastics, Oxnard, CA) at 37°C. The cells were studied in passages 3–7.

Collagen assays. For assay of collagen synthesis, confluent monolayer cultures of fibroblasts were rinsed with DMEM and then placed in fresh serum-free medium containing 25 μg/ml ascorbic acid (Fisher Scientific Co., Pittsburgh, PA) and a retinoid dissolved in absolute ethanol or dimethyl sulfoxide (DMSO); the control cultures received medium containing the corresponding final concentrations of the solvent alone. After a 60-min preincubation, the cell cultures were labeled with [^{3}H]proline (2,3-^{3}H proline; Amersham Corp., Arlington Heights, IL) (40–80 μCi/flask) for 6–8 h at 37°C.

At the end of the labeling period, the medium was removed, cooled

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1. *Abbreviations used in this paper:* DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; RA, retinoic acid; SAPNA, succinyl-L-(alanyl)$_3$-paranitroanilide; SSC, 0.15 M NaCl in 15 mM sodium citrate, pH 6.8.

at 4°C, and protease inhibitors were added to give the following final concentrations: 20 mM Na_2EDTA, 10 mM *N*-ethylmaleimide, and 1 mM phenylmethylsulfonyl fluoride. The cell layer was scraped with a rubber policeman at 4°C into solution containing 0.4 M NaCl, 0.01 M Tris-HCl, pH 7.5, and the same protease inhibitors; the cells were then sonicated at 50 Hz for 30 s. To quantitate the synthesis of [^{3}H]hydroxyproline and the total ^{3}H-radioactivity in cultures labeled with [^{3}H]proline, aliquots of medium and homogenized cell fractions were dialyzed against running tap water, hydrolyzed in 6 M HCl in sealed tubes at 120°C for 24 h, and assayed for [^{3}H]hydroxyproline and total ^{3}H-radioactivity by using a specific radiochemical method (6).

To determine the relative ratio of [^{3}H]hydroxyproline to [^{3}H]proline in collagen α-chains after RA treatment, the cells were labeled with [^{3}H]proline (100 μCi/flask) as above. The media were removed into tubes containing protease inhibitors (see above), dialyzed against 0.5 N acetic acid at 4°C for 24 h, and digested with pepsin (Sigma Chemical Co., twice crystallized), 300 μg/ml, for 6 h at 22°C. The collagenous proteins were precipitated by adding 1.8 M NaCl, and the precipitates were recovered by centrifugation. Precipitates were dialyzed and hydrolyzed and [^{3}H]hydroxyproline and [^{3}H]proline were separated by an amino acid analyzer (Beckman 120B, Beckman Instruments, Inc., Fullerton, CA).

For assay of the specific radioactivity of prolyl-transfer RNA (tRNA), the cell layer of the fibroblast cultures, after incubation with 100 μCi of [^{3}H]proline in the presence of all-*trans*-RA, was scraped into 4 ml of solution containing 10 mM Tris, pH 7.5, 5 mM Na_2EDTA, 1% sodium dodecyl sulfate and 60 μg/ml of proteinase K (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Nucleic acids were extracted with 3 ml of a mixture of phenol/chloroform/isoamylalcohol (2:2:0.04). The phenol layer was reextracted with 3 ml of solution containing 10 mM Tris, pH 7.5, 5 mM Na_2EDTA, and 1% SDS. The amount of [^{3}H]proline that was bound to prolyl-tRNA was determined by incubation of the isolated total nucleic acid preparation with 2 M NaOH for 20 min at 37°C. The incubation was terminated by the addition of 10% ice-cold trichloroacetic acid (TCA), and the samples were collected on Millipore glassfiber filters (Millipore Corp., Bedford, MA). Aliquots of the nucleic acid preparations were simultaneously processed without deacylation by NaOH. The radioactivity retained on the filters was determined using a Beckman LS 7500 liquid scintillation counter (Beckman Instruments, Inc.), after extraction of the filters with 10 ml of liquid scintillation fluid. The difference in the radioactivity between the alkali-treated and nontreated preparations of each sample was taken as a measure of the incorporation of [^{3}H]proline into prolyl-tRNA, and the values were expressed per cellular DNA in the same preparation to correct for changes in cell number.

Assay of type I procollagen messenger RNA (mRNA). For mRNA level determinations, two RNA isolation procedures were utilized. First, for isolation of poly(A)-RNA, fibroblast cultures, after 8 h of incubation with all-*trans*-RA, were scraped into 3.0 ml of 10 mM Tris-HCl, pH 7.4, containing 1.0 mM Na_2EDTA and 1% SDS. Proteinase K, 100 μg/ml, was added and the samples were incubated for 120 min at 65°C. NaCl, in a final concentration of 0.4 M, was then added and poly(A)-RNA was isolated by a chromatography on an oligo-dT-cellulose column (7). For assay of type I procollagen mRNA levels, aliquots of the poly(A)-RNA samples were mixed with an equal volume of solution containing 3 vol of 20 × 0.15 M NaCl in 15 mM sodium citrate, pH 6.8 (SSC) and 2 vol of 37% formaldehyde (8). Aliquots of 100 μl were dot blotted on the nitrocellulose filters using a vacuum manifold (model SRC-96, Schleicher & Schuell, Inc., Keene, NH). Aliquots of the poly(A)-RNA were also subjected to Northern blot analyses by electrophoresis on 1.0% agarose gels under denaturing conditions (9). The RNA was then transferred to nitrocellulose filters, air dried, and heated at 78°C for 90 min in a vacuum oven. The RNA samples bound to the nitrocellulose filters were hybridized with a human proα2(I) complementary DNA (cDNA) (Hf 32) (10), labeled with ^{32}P-nucleotides by nick translation to a specific activity of 4 × 10^8 cpm/μg.

The second method used for preparation of RNA from fibroblasts consisted of isolation of the total RNA as described previously (11), with minor modifications. In this procedure, the fibroblasts were scraped and homogenized in buffer solution containing 4 M guanidium thiocyanate, 5 mM sodium citrate, 0.5% sarkosyl, 0.1 M 2-mercaptoethanol, and 0.1% Antifoam A (Sigma Chemical Co.). The homogenate was layered on top of a 2.5 ml cushion of 5.7 M CsCl and centrifuged at 35,000 rpm for 16 h at 15°C using a Beckman SW 40.1 rotor (Beckman Instruments, Inc.). The pellet containing RNA was washed with 70% ethanol by centrifugations at 18,000 *g* for 30 min at −20°C. The final pellet was dissolved in sterilized H_2O and the RNA concentration was determined by absorbance at 260 nm.

Varying amounts of total RNA were dotted on the nitrocellulose filter and hybridized with ^{32}P-labeled proα2(I)-collagen specific cDNA probe, as described above. For comparison, parallel filters were hybridized with human fibronectin and β-actin specific cDNA probes labeled with ^{32}P by nick translation. The fibronectin probe is a 1.3-kilobase (kb) cDNA (FN-771) complementary to the 3′-end of the human fibronectin mRNA. The recombinant plasmid containing the human β-actin cDNA (pHF A-1) (12) was kindly provided by Dr. L. H. Kedes, Stanford University School of Medicine.

The nitrocellulose filters were first prehybridized for 16 h 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, 25 μl/cm^2. Hybridization was carried out in the same solution, 15 μl/cm^2, containing ^{32}P-labeled DNA probes, at 42°C for 48 h. The filters were washed first twice in 500 ml of 2× SSC, at 24°C, and then twice each with 1× SSC, 1× SSC plus 0.1% SDS, 0.5× SSC plus 0.1% SDS, and 0.2× SSC, all at 68°C. The dried filters were then exposed to x-ray films. The level of mRNA was quantitated by scanning the dot blots at 700 nm using an automatic computing densitometer (Gelman ACD 18, Gelman Sciences, Inc., Ann Arbor, MI).

For estimation of the recovery of the proα2(I)-specific mRNA, globin mRNA, which is not present in human skin fibroblasts, was added as an internal standard (13). The details of this technique will be published elsewhere (Uitto, J., and D. J. Prockop, manuscript in preparation). Briefly, 100 ng of purified rabbit globin mRNA (Bethesda Research Laboratories, Inc., Gaithersburg, MD) was mixed with the fibroblasts in culture at the beginning of the extraction procedure. The recovery of globin mRNA was estimated by hybridization of the poly(A)-RNA preparations in dot blots with a rabbit globin-specific 4.4-kb genomic DNA fragment (kindly provided by Dr. Maniatis, Harvard University) (14). The calculated recovery of the globin mRNA, based on densitometric scanning of the dots containing the isolated sample and the parallel dots with known amount of globin mRNA, was taken as a recovery of the total poly(A)-RNA.

Assay of the synthesis of noncollagenous proteins. For assay of the synthesis of noncollagenous proteins, fibroblasts were plated on 24-well tissue culture plates. At early visual confluency, the cultures were placed in serum-free medium containing all-*trans*-RA or 13-*cis*-RA in 10^{-5} M concentration, or ethanol alone, as described above under *Collagen assays.* After a 60-min preincubation, [^{3}H]leucine, 30 μCi/well was added, and the incubations were continued for 6 h. At the end of the incubation, protease inhibitors were added (see above), and the cells were homogenized at 4°C by sonication. Aliquots of the cell plus medium fraction were dialyzed against 50 mM Tris-HCl, pH 7.5, containing 0.4 M NaCl and 1 mM Na_2EDTA. One-half of each sample was then incubated with chromatographically purified (15) bacterial collagenase (Sigma Chemical Co., type VII), 100 μg/ml, in the presence of 10 mM $CaCl_2$, 10 mM N-ethylmaleimide, and 0.3 mM phenylmethylsulfonyl fluoride for 180 min at 25°C. The other half of each sample was incubated under identical conditions, except without collagenase and added $CaCl_2$. An equal volume of 20% TCA was added, the samples were incubated for 30 min at 4°C, and the precipitates were collected and washed with 10% TCA on glass fiber filters (Millipore Corp., AP 1502500), using a vacuum manifold. The

radioactivity on the filters was counted in a Beckman Instruments LS 7500 liquid scintillation counter. The incorporation of [^{3}H]leucine into noncollagenous proteins was determined in samples digested with bacterial collagenase, and the incorporation of ^{3}H-radioactivity into collagenous polypeptides was calculated from the difference between the radioactivity in TCA-precipitable fraction in the samples not digested with bacterial collagenase minus the radioactivity in the samples subjected to enzyme digestion.

Assay of collagenase activity. For assay of collagenase activity, cells were cultured in 75-cm^2 flasks. The medium was removed and the cells were rinsed twice with serum-free DMEM. Thereafter, 7 ml of fresh DMEM containing all-*trans*-RA or ethanol was added, and the cultures were incubated for 6 h. The medium was then collected and assayed for collagenase activity. For collagenase assay, the enzyme preparations were activated by a brief trypsin proteolysis (16) (10 μg/ml for 10 min at 25°C), trypsin was inactivated by soybean trypsin inhibitor, and collagenase activity was assayed by incubating samples with ^{3}H-labeled type I collagen, as described previously (17). In a standard incubation, 40-μl aliquots of medium were incubated in 50 mM Tris-HCl, pH 7.6, containing 10 mM $CaCl_2$, 0.15 M NaCl, and 20 μg/ml bovine serum albumin, in a final volume of 100 μl, for 4 h at 37°C. The reaction was stopped by the addition of 40 mM Na_2EDTA and the samples were treated by a mixture of trypsin and α-chymotrypsin, employing conditions under which the undegraded collagen substrate resists proteolysis, while the collagen degradation products are digested to TCA-soluble peptides (17). The enzyme activity was expressed as degradation of ^{3}H-labeled collagen, disintegrations per minute × hour^{-1}/milligram of DNA.

Other assays. Elastaselike neutral protease activity was assayed by incubating 50-μl aliquots of the medium with a synthetic substrate, succinyl-L-(alanyl)$_3$-paranitroanilide (SAPNA) in 50 mM Tris-HCl, pH 7.8, for varying time periods up to 24 h (18). The reaction was monitored by the change in the absorbance at 410 nm. The enzyme activity was expressed as hydrolysis of SAPNA, nanomoles × hour^{-1}/milligram of DNA.

For assay of prolyl hydroxylase activity, the fibroblasts were scraped and homogenized in 50 mM Tris-HCl, pH 7.5, containing 0.2 M NaCl and 0.01% Triton X-100. After a 60-min extraction at 4°C, the homogenates were centrifuged at 30,000 *g* for 30 min. Aliquots of the supernatant were assayed for prolyl hydroxylase activity by incubating with [^{3}H]protocollagen substrate, prepared from chick embryo tendons (19), for 60 min at 37°C in the presence of 2 mM ascorbic acid, 8 mM $FeSO_4$, and 0.5 mM α-ketoglutarate (20). After incubation, the samples were hydrolyzed in 6 M HCl and assayed for [^{3}H]hydroxyproline (6). The enzyme activity was expressed as disintegrations per minute of [^{3}H]hydroxyproline synthesized × hour^{-1} per milligram of protein.

Cell viability was studied by incubating cells in serum-free medium with all-*trans*-RA or ethanol for 6 h. The viability of the cells was then examined using the trypan blue exclusion test. For assay of DNA synthesis, the fibroblasts were preincubated in 96-well microtiter plates with all-*trans*-RA or ethanol for 60 min and then labeled with [^{3}H]thymidine (New England Nuclear, Boston, MA), 10 μCi per well. After 6 h of incubation, the cells were collected by a cell harvester (PHD, Cambridge Technology, Inc., Cambridge, MA), and the ^{3}H-radioactivity collected on glass fiber filters was determined by a liquid scintillation counter.

The mannosylation of procollagen was studied by labeling cells simultaneously with [2-^{3}H]mannose (300 μCi/flask) and [^{14}C]proline (30 μCi/flask) in serum-free medium containing ascorbic acid and all-*trans*-RA or ethanol, for 8 h. The medium was removed, protease inhibitors and fetal calf serum were added, and procollagen was precipitated by ammonium sulfate (176 mg/ml) for 2 h at 4°C. The precipitates were collected by centrifugation, dissolved in 3% SDS that contained protease inhibitors, boiled and dialyzed against 0.125 M Tris-HCl, pH 6.8, containing 2% SDS, 0.01% bromphenol blue, and 10% glycerol. The samples were electrophoresed after reduction with 2-mercaptoethanol on 6% polyacrylamide gel (21) and radioactive peptides were visualized by fluorography (22). Bands corresponding to proα-chains of procollagen were cut out from the gel and treated with protosol tissue solubilizer (New England Nuclear). The radioactivities of ^{3}H and ^{14}C were then counted by liquid scintillation counting using a two-channel double-label counting program.

DNA was assayed as described by Burton (23), and protein according to Lowry et al. (24).

For statistical analyses, Student's two-tailed *t* test was used.

Results

Inhibition of procollagen production by all-trans-RA. To examine the effects of retinoids on procollagen production by human skin fibroblasts, the cells were first cultured in a medium containing fetal calf serum. Preliminary experiments indicated that all-*trans*-RA had little, if any, effect on collagen production by the cells when added in a serum-containing medium. Because the lack of effect could probably be explained on the basis of retinoid binding to serum proteins (25), in subsequent experiments the retinoids were added to the culture in a serum-free medium for time periods short enough not to affect the viability of the cells. Thus, after the cells had reached early visual confluency, the medium was replaced with fresh medium containing the retinoid and ascorbic acid, but devoid of serum; after a 60-min preincubation, [^{3}H]proline was added. In the first set of experiments, varying concentrations of all-*trans*-RA were tested. The results indicated that the synthesis of [^{3}H]hydroxyproline in the nondialyzable fraction was significantly decreased with all-*trans*-RA in 1–5 × 10^{-5} M concentration, when compared with corresponding controls containing the same amount of solvent used to dissolve the retinoid (Fig. 1 and Table I). Lower concentrations of all-*trans*-RA did not have a significant effect on [^{3}H]hydroxyproline production in these experiments. In the experiment shown in Fig. 1, the total incorporation of ^{3}H-radioactivity into nondialyzable macromolecules in the presence of 10^{-5}, 2.5 × 10^{-5}, and 5 × 10^{-5} M all-*trans*-RA was 92.1, 86.0, and 79% of the control incubated with ethanol alone. However, the suppression of the production of [^{3}H]hydroxyproline-containing macromolecules by all-*trans*-RA was more pronounced in that the inhibition of [^{3}H]hydroxyproline production in each case was larger than the inhibition of the total incorporation of ^{3}H-radioactivity. Specifically, in the experiment shown in Fig. 1, the ratio of [^{3}H]hydroxyproline/total ^{3}H-radioactivity × 100 in the control cultures was 11.9, whereas in the cultures incubated with 10^{-5} M, 2.5 × 10^{-5} M, and 5 × 10^{-5} M all-*trans*-RA, the corresponding values were 9.9, 7.3, and 5.1%, respectively.

Several control experiments were performed to examine the mechanism of the observed reduction in the [^{3}H]hydroxyproline production. First, the specific activity of the intracellular prolyl-tRNA pool was assayed in the cultures treated with all-*trans*-RA. No significant differences were observed between the cultures treated with 10^{-5} M all-*trans*-RA and the corresponding control containing the equivalent concentration of ethanol; the values for the activity of [^{3}H]prolyl-tRNA in these two cultures were 69.7±22.9 × 10^3 and 79.4±9.2 × 10^3 cpm/mg of DNA, respectively.

Secondly, the level of posttranslational hydroxylation of prolyl residues in the collagenous domain of procollagen polypeptides was examined. For this purpose, the newly synthesized collagen polypeptides were isolated by limited pepsin proteolysis and differential salt precipitation. Amino acid anal-

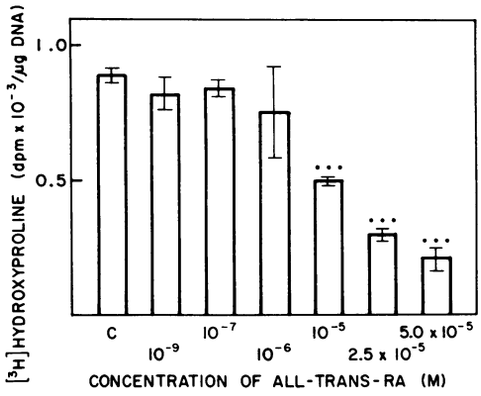

Figure 1. Dose-dependent inhibition of procollagen production by all-*trans*-RA in human skin fibroblast cultures. Cell cultures were preincubated for 60 min in the presence of the retinoid in concentrations indicated and then labeled with [^{3}H]proline for 6.5 h. The synthesis of [^{3}H]hydroxyproline in nondialyzable fraction was then assayed, as described in Methods and in Table I. The values are mean±standard deviation of three parallel flasks. *C*, Control cultures. *** $P < 0.001$.

yses indicated that the ratio of [^{3}H]hydroxyproline/[^{3}H]proline in collagen α-chains synthesized in the control cultures was 0.69; the corresponding value in collagen synthesized in the presence of all-*trans*-RA in 10^{-5} M concentration was 0.60. Thus, the reduction in [^{3}H]hydroxyproline production noted in the presence of all-*trans*-RA could not be explained either by changes in the specific activity of prolyl-tRNA pools or by a reduction in the level of posttranslational hydroxylation of prolyl residues in the polypeptides.

Examination of the newly synthesized procollagens indicated that the proα1(I)- and proα2(I)-chains were synthesized in the control cultures in an apparent 2.6:1 ratio. The corresponding ratio in the cultures incubated with the all-*trans*-RA was 3.1:1. Examination of the ratio of α1 to α2 chains in triple-helical collagen, isolated by limited pepsin proteolysis, indicated that the ratio in the control cultures was 2.6:1, whereas the corresponding value in all-*trans*-RA-treated cultures was 2.3:1. Since these values are slightly higher than the expected 2:1 ratio, it is conceivable that the band migrating in the position of proα1- or α1-chains contained polypeptides representing both type I and type III procollagens. It should be noted that essentially all collagenous polypeptides were recovered as intact proα-chains both in the control and all-*trans*-RA-treated cultures. Thus, no evidence of the conversion of proα-chains to α-chains or intermediate polypeptides, pC- and pN-chains was noted.

Further control experiments demonstrated that the viability of cells cultured in the presence of all-*trans*-RA (10^{-5} M) and the controls containing 0.2% ethanol were similar, when examined by the trypan blue exclusion test. Specifically, at the end of a 6-h incubation in the medium devoid of serum, 77% of the cells in both cultures were viable by this test. Furthermore, DNA synthesis, measured by uptake of [^{3}H]thymidine was not affected significantly by all-*trans*-RA. The values in cultures incubated with ethanol and 10^{-5} M all-*trans*-RA were 1,746±494 and 1,140±209 cpm/well, respectively, whereas the corresponding value for the control cell line incubated without ethanol was 1,375±217 cpm/well. Because 0.2% ethanol, which was used as a solvent for retinoids in most experiments, caused a small inhibition of [^{3}H]hydroxyproline synthesis, in further experiments, the effect of 10^{-5} M all-*trans*-RA was tested in media containing the final concentrations of 0.1% ethanol or 0.125% DMSO; these concentrations of the solvents alone did not have any effect on the production of [^{3}H]hydroxyproline (Table I). In the presence of 0.1% ethanol or 0.125% DMSO,

Table I. Effect of All-trans-RA on Collagen Production by Human Skin Fibroblasts

Culture	Total ^{3}H-incorporation		[^{3}H]Hydroxyproline		100 × [^{3}H]hydroxyproline/total ^{3}H
	dpm × 10^{-3}/µg DNA	*% of ethanol control**	*dpm × 10^{-3}/µg DNA*	*% of ethanol control**	*%*
Experiment 1					
Control	13.1±1.7	—	1.00±0.05	—	7.6
Ethanol (0.2%)	12.0±1.5	100.0	0.81±0.12	100.0	6.8
All-*trans*-RA (10^{-5} M)	10.6±0.5	88.3	0.49±0.03‡	60.5	4.6
Ethanol (0.02%)	12.9±1.7	100.0	0.98±0.08	100.0	7.6
All-*trans*-RA (10^{-6} M)	12.8±1.4	99.2	0.83±0.19	84.7	7.4
Experiment 2					
Control	16.1±3.0	—	0.92±0.12	—	5.7
Ethanol (0.2%)	15.4±1.2	100.0	0.91±0.08	100.0	5.9
All-*trans*-RA (10^{-5} M)	16.0±1.0	103.9	0.71±0.07§	78.0	4.4
Experiment 3					
Control	7.2±0.5	—	0.87±0.13	—	12.1
Ethanol (0.1%)	7.2±0.7	100.0	0.95±0.08	100.0	13.2
All-*trans*-RA (10^{-5} M)	5.1±0.2‡	71.7	0.57±0.07‡	60.2	11.2

Fibroblast cultures from normal human subjects were preincubated with various concentrations of all-*trans*-RA or ethanol for 60 min, and the cultures were labeled with [^{3}H]proline for 8 h. Total nondialyzable radioactivity and [^{3}H]hydroxyproline in cells plus medium were then assayed as described in Methods. The values are the mean±standard deviation of three parallel flasks each assayed in duplicate. A different cell line was used in each separate experiment. * The percentages are calculated from the parallel control flasks containing the same concentrations of ethanol as the all-*trans*-RA containing flasks. ‡ Statistically different from control, $P < 0.01$. § Statistically different from control, $P < 0.05$.

all-*trans*-RA in a 10^{-5} M concentration equally reduced the [^{3}H]hydroxyproline production (Table I). Thus, it appears that the effects of all-*trans*-RA on procollagen production are independent of the solvent effects.

Reduction of type I procollagen mRNA levels. To examine in further detail the mechanism by which all-*trans*-RA inhibited the procollagen production in fibroblast cultures, the levels of type I procollagen-specific mRNA were assayed. For quantitative estimation of the recovery of human poly(A)-RNA, rabbit globin mRNA was added as an internal standard during the isolation procedure. Dot blot hybridizations with globin-specific DNA indicated that the recoveries of poly(A)-RNA in control and all-*trans*-RA treated cultures were 50.6 and 53.4%, respectively. Northern blot analyses revealed no qualitative differences between the poly(A)-RNA preparations isolated from control or all-*trans*-RA treated cultures (Fig. 2 *A*). Specifically, a human proα2(I) type I collagen-specific cDNA (Hf 32) (10) hybridized to three major transcripts corresponding to the sizes of 5.2, 4.6, and 4.4 kb, respectively. This finding confirms the specificity of the hybridization conditions, further indicating that the mRNAs in retinoid-treated cultures were full length. Quantitative analyses by dot blot hybridizations with the same cDNA probe (Hf 32) indicated that the levels of procollagen mRNA in the presence of 10^{-5} M all-*trans*-RA were reduced in two different cell lines by 66.1±2.4 and 71.4±5.6% (mean±SD; $n = 3$), as compared to the corresponding controls containing ethanol (Fig. 2 *B*). The yield of poly(A)-RNA, isolated by oligo-dT-cellulose chromatography, was in the corresponding control and all-*trans*-RA-treated cultures 0.28 and 0.30 $\mu g/10^6$ cells, respectively. Thus, these observations suggest that the inhibition of procollagen production by all-*trans*-RA predominantly results from the reduction of the corresponding mRNA levels in the cell culture.

Evidence for selectivity in the inhibition of procollagen production. Three independent approaches were taken to examine the possible selectivity of the inhibition of procollagen production elicited by all-*trans*- and 13-*cis*-RA. First, the synthesis of noncollagenous proteins was measured by the incorporation of [^{3}H]leucine into bacterial collagenase-resistant polypeptides and compared with the synthesis of collagenase-digestible peptides in the same cultures. The incorporation of [^{3}H]leucine into noncollagenous proteins in control cultures incubated with ethanol alone was $5.2\pm1.0 \times 10^5$ cpm/mg protein (mean±SD), while the corresponding values in cultures incubated with 10^{-5} M all-*trans*-RA and 13-*cis*-RA were $4.9\pm1.6 \times 10^5$ and $5.1\pm0.9 \times 10^5$, respectively; the latter values are not significantly different from the controls. The incorporation of [^{3}H]leucine into the collagenase-digestible polypeptides in the control cultures was $8.3\pm1.4 \times 10^3$ cpm/mg protein, whereas the corresponding values in all-*trans*- and 13-*cis*-RA treated cultures were $4.8\pm0.9 \times 10^3$ and 6.1±0.4 ($P < 0.01$ and 0.05, respectively).

Secondly, total RNA was isolated from the fibroblast cultures incubated either with 10^{-5} M all-*trans*-RA or 13-*cis*-RA, or with the corresponding concentration of ethanol, and mRNA levels were determined by hybridizations with human proα2(I) collagen, fibronectin, and β-actin specific DNA probes. In accordance with the results obtained with hybridizations with poly(A)-RNA (see above), the levels of proα2(I) collagen mRNA were significantly reduced (Table II). In contrast, incubation of fibroblasts with 10^{-5} M all-*trans*-RA had no effect on the levels of fibronectin and β-actin mRNAs. In the presence of 10^{-5} M 13-*cis*-RA, the level of β-actin mRNA was unaffected while the level of fibronectin mRNA was significantly increased.

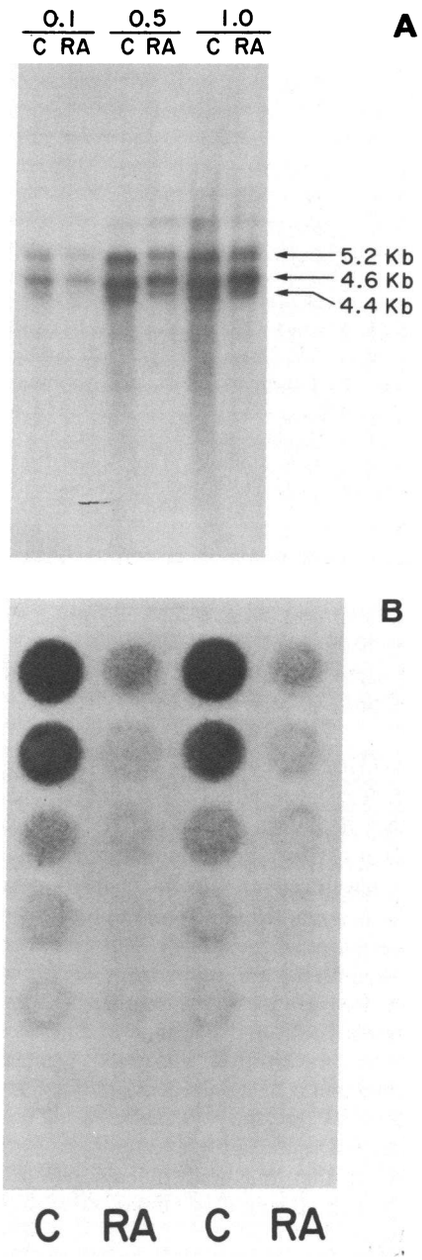

Figure 2. Northern blot and dot blot hybridizations of poly(A)-RNA isolated from human skin fibroblast cultures incubated with all-*trans*-RA. Two different human skin fibroblast cell lines were incubated either with 10^{-5} M all-*trans*-retinoic acid (*RA*) or with 0.2% ethanol (*C*) for 7 h. The cells were removed and poly(A)-RNA was isolated as described in Methods. (*A*) Poly(A)-RNA, 0.1, 0.5, or 1.0 μg, from control and RA-treated cells was electrophoresed on 1% agarose gel under denaturing conditions, transferred to nitrocellulose filters by Northern blotting, and hybridized with ^{32}P-labeled cDNA (Hf 32) corresponding to human proα2(I) of type I collagen sequences. Autoradiogram revealed three major mRNA species with estimated molecular weights of 5.2, 4.6, and 4.4 kb, respectively. (*B*) Poly(A)-RNA, in concentrations varying from 100 to 3.38 ng in 1:2 dilutions, was dot blotted on the nitrocellulose filters. The filters were hybridized as in *A* and subjected to autoradiography.

Table II. Levels of Proα2(I)-Collagen, Fibronectin, and β-Actin mRNAs in Fibroblast Cultures Incubated with All-trans-RA or 13-cis-RA

Culture	mRNA levels					
	Proα2(I)		Fibronectin		β-Actin	
	*U/mg**	%§	*U/mg**	%§	*U/mg**	%§
Ethanol (0.1%)	387.0±81.9	100.0	77.3±18.1	100.0	194.8±46.8	100.0
All-*trans*-RA	188.2±84.0‡	48.6	73.8±29.4	95.5	216.4±13.6	111.1
13-*cis*-RA	202.8±20.3‡	52.4	156.3±40.1‡	202.2	202.5±48.5	104.0

Fibroblast cultures were incubated with 10^{-5} M all-*trans*-RA, 13-*cis*-RA, or with the corresponding concentration of ethanol used as solvent, for 6 h in serum-free medium. Total RNA was isolated by a CsCl density-gradient centrifugation (see Methods). RNA, in amounts varying from 4.0 μg to 31.25 ng, was dotted on the nitrocellulose filters, and hybridized with ^{32}P-labeled DNA probes specific for human α2(I)-collagen, fibronectin, and β-actin sequences, as indicated in Methods. The filters were washed, exposed to x-ray films, and the autoradiograms scanned at 700 nm. * Expressed as absorbance units/mg of total RNA per sample, calculated from the linear range of the RNA dose curve. The values are mean±standard deviation of three parallel samples. Values indicated by ‡ statistically different from the control cultures incubated with 0.1% ethanol, $P < 0.01$. § Expressed as percent of the controls incubated with ethanol alone.

Thirdly, the activities of three fibroblast enzymes, collagenase, prolyl hydroxylase and an elastaselike neutral protease, were assayed in cultures incubated with 10^{-5} M all-*trans*-RA. As indicated below, the activity of collagenase was slightly reduced in the retinoid-treated cultures whereas the activities of an elastaselike neutral protease and prolyl hydroxylase were unaffected.

Modulation of procollagen production by other retinoids. In further studies, the effects of a variety of other retinoids were compared with that noted with all-*trans*-RA. For this purpose, 13-*cis*-RA, RO-10-9359, retinol, retinal, and retinyl acetate, all in 10^{-5} M concentration, were incubated under conditions which resulted in a marked inhibition of procollagen production by all-*trans*-RA (see above). The results indicated that 13-*cis*-RA, retinol, and retinal also significantly decreased the production of [^{3}H]hydroxyproline in the cultures, as compared to the ethanol controls (Fig. 3 *A*). No significant difference between retinyl acetate and the controls was noted, whereas RO-10-9359 slightly stimulated collagen production (Fig. 3 *A*). The incorporation of total ^{3}H into the nondialyzable fraction in the same cultures was not significantly affected (Fig. 3 *B*). The results suggest, therefore, that structurally different retinoids may modulate procollagen production by human skin fibroblasts in a different manner.

All-trans-RA suppresses the production of trypsin-activatable collagenase but not elastaselike neutral protease or prolyl hydroxylase activities. Previous studies have indicated that retinoids can suppress the production of trypsin-activatable collagenase in both skin and synovial fibroblast cultures (26, 27). We, therefore, tested whether such inhibition could be observed under culture conditions utilized here. The results indicated that the collagenase activity, measured after a brief trypsin activation, was slightly decreased in cultures treated with 10^{-5} M all-*trans*-RA, the collagenase activity being 77% of the corresponding ethanol control (Table III). For comparison, the activities of two other fibroblast enzymes, an elastaselike neutral protease and prolyl hydroxylase, were also assayed. The results indicated that the elastaselike neutral protease activity assayed by using a synthetic substrate, SAPNA, was not significantly reduced in the cultures treated with all-*trans*-RA. Also, the values for prolyl hydroxylase activity were similar both in the control and all-*trans*-RA-treated cultures (Table III), suggesting that the reduction in collagenase production does not reflect a generalized reduction in protein synthesis.

All-trans-RA does not affect the mannosylation of procollagen. In further studies, the degree of mannosylation of procollagen was examined by incubating cells simultaneously with [^{3}H]mannose and [^{14}C]proline. The proα-chains of procollagen were isolated by SDS polyacrylamide gel electrophoresis and the degree of mannosylation was estimated from the ^{3}H to ^{14}C ratio, as determined by liquid scintillation counting. The results indicated that the degree of mannosylation was unaltered in cultures treated with 10^{-5} M all-*trans*-RA, as compared to the corresponding ethanol controls; the ratio of ^{3}H to ^{14}C in control and all-*trans*-RA-treated cultures were 48.2 and 52.4, respectively.

Inhibition of collagen production in keloid fibroblast cultures by all-trans- and 13-cis-RA. Excessive accumulation of collagen is the hallmark of several fibrotic cutaneous conditions, as for example, keloids (28). In further studies, we examined the effects of two of the retinoids, all-*trans*-RA and 13-*cis*-RA on the procollagen production by three fibroblast lines established from keloids. Two of these cell lines exhibited markedly elevated procollagen synthesis rates as compared with cell lines established from age-matched controls (Fig. 4). Incubation of keloid fibroblast cultures with 10^{-5} M all-*trans*-RA markedly reduced procollagen production in all three keloid cell lines, and the largest relative decrease was noted in cultures which initially showed the highest rate of procollagen production (Fig. 4). Similar decrease was noted when the cell cultures were incubated with 10^{-5} M 13-*cis*-RA (not shown).

To examine the mechanism of the inhibition of collagen production, the levels of proα2(I)-collagen mRNA were also measured in two keloid cell lines incubated with 10^{-5} M all-*trans*-RA. The results indicated that in two keloid cell cultures, collagen production was reduced by 56 and 75%, whereas type I procollagen mRNA levels in the corresponding cultures were reduced by 59 and 81%, respectively. Thus, the inhibition of procollagen production largely parallels the reduction in the mRNA levels in a similar manner as was noted in control fibroblast cultures (see above).

Discussion

The results of this study indicate that several retinoids, including all-*trans*-RA, 13-*cis*-RA, retinal, and retinol, markedly reduced

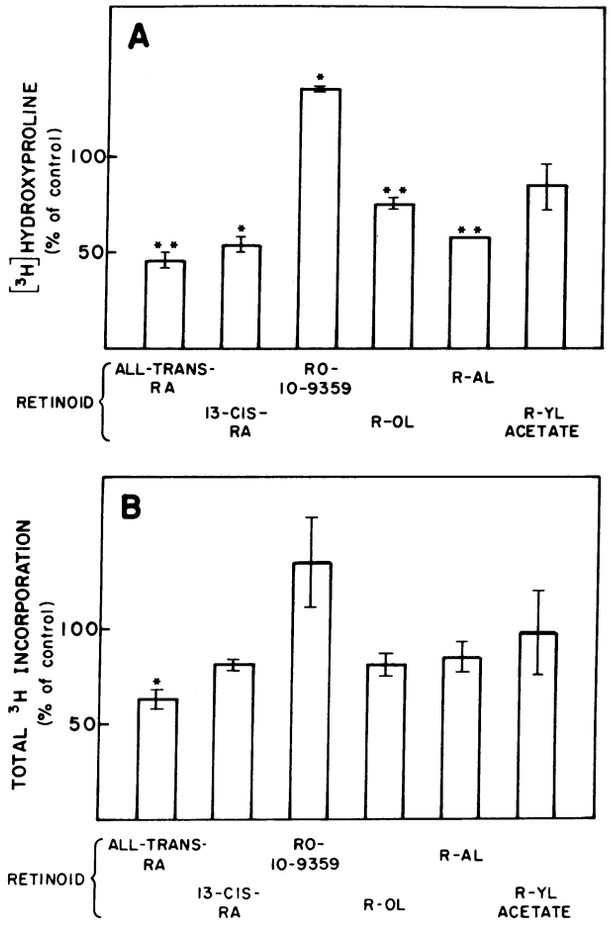

Figure 3. The effects of various retinoids on the synthesis of procollagen and the incorporation of total ^{3}H-radioactivity. Human skin fibroblast cultures were preincubated for 60 min in the presence of various retinoids in 10^{-5} M concentration. The cell cultures were then labeled with [^{3}H]proline for 6 h, and the synthesis of [^{3}H]hydroxyproline and the incorporation of total ^{3}H-radioactivity into nondialyzable fraction were assayed, as described in Methods. The results are expressed as the percentage of the corresponding control cultures incubated with 0.2% ethanol used as solvent for different retinoids. The values are mean±standard deviation of three parallel flasks. The retinoids used were all-*trans*-retinoic acid (*all-trans-RA*), 13-*cis*-retinoic acid (*13-cis-RA*), trimethylmethoxyphenyl retinoic acid ethylester (*RO-10-9359*), retinol (*R-OL*), retinal (*R-AL*), and retinyl acetate (*R-yl acetate*). *$P < 0.05$; **$P < 0.01$.

the production of procollagen by human skin fibroblasts in culture. The reduction of collagen synthesis appeared to be selective for collagen, in that the total incorporation of [^{3}H]proline into nondialyzable fraction was affected in the same fibroblast cultures to a lesser degree, and the ratio of [^{3}H]hydroxyproline to total ^{3}H was markedly reduced. Furthermore, the incorporation of [^{3}H]leucine into noncollagenous proteins was not affected by the retinoids in 10^{-5} M concentration. The reduction in procollagen production in RA-treated cultures was accompanied by a marked reduction in the proα2(I)-specific mRNA levels. In contrast, the abundance of mRNAs coding for fibronectin and β-actin was not altered in the same fibroblast cultures, further attesting to the selectivity of the inhibition of procollagen gene expression. The reduction in the mRNA levels was even more pronounced than the inhibition of the rate of procollagen production. This observation suggests that the reduction in mRNA levels by all-*trans*-RA may be accompanied by additional changes at different levels of procollagen gene expression, including the efficiency of message utilization, compartmentalization of the precursor amino acid pools, and intracellular degradation of the newly synthesized polypeptides (29–31). Northern blot analyses showed that qualitatively there were no changes in mRNA pattern, and both in control and RA-treated cultures three major transcripts corresponding to proα2(I) sequences were present. As discussed elsewhere (32), these three mRNA species code for the same polypeptide, and the molecular weight differences reflect differential utilization of polyA-addition signals. The results suggest that the rate of transcription of genomic DNA corresponding to procollagen may be reduced in retinoid-treated cultures. Such altered genomic expression of procollagen DNA could result from an interaction of the retinoid with the genome in the cell nucleus mediated by the specific cellular binding proteins for the retinoids (33, 34). Alternatively, the degradation and turnover of mRNA may be increased in these cultures, resulting in the reduced levels of mRNA. Control experiments indicated that the degree of prolyl hydroxylation, the specific activity of the intracellular prolyl-tRNA pools, and the viability of the cells were not affected by all-*trans*-RA in the concentration which effectively reduced the procollagen production. It should be noted that the effects of all-*trans*-RA on procollagen production appeared to be independent of the solvent required for solubilization of the retinoids.

Previous studies have indicated that the production of

Table III. Effect of All-trans-RA on the Production of Trypsin-activatable Collagenase and on the Activities of an Elastaselike Neutral Protease and Prolyl Hydroxylase in Human Skin Fibroblast Cultures

Culture	Collagenase activity*		Elastase activity‡		Prolyl hydroxylase activity§	
	cpm × 10^{-6}/h/mg DNA	%	*nmol × 10^{-2}/h/mg DNA*	%	*dpm × 10^{-3}/h/mg DNA*	%
Ethanol (0.2%)	7.09±0.51	100.0	6.80±1.5	100.0	1.61±0.30	100.0
All-*trans*-RA (10^{-5} M)	5.46±0.12‖	77.0	6.40±0.8	94.1	1.74±0.18	108.1

Fibroblast culltures were incubated with all-*trans*-retinoic acid or ethanol for 6 h. Aliquots of serum-free medium were assayed for collagenase activity, after a brief proteolytic activation of latent collagenase, and for elastase activity. The cell fraction was assayed for the activity of prolyl hydroxylase, as described in Methods. The values are mean±standard deviation of three parallel flasks, each assayed in triplicate. * Expressed as degradation of [^{3}H]proline-labeled type I collagen, cpm × 10^{-6}/h/mg of DNA. ‡ Expressed as hydrolysis of SAPNA, nmol × 10^{-2}/h/mg of DNA, as monitored at 410 nm. § Expressed as [^{3}H]hydroxyproline, dpm × 10^{-3}/h/mg of DNA, synthesized during a 60-min incubation with 5 × 10^4 cpm of [^{3}H]proline-labeled protocollagen substrate. ‖ Statistically different from control, $P < 0.05$.

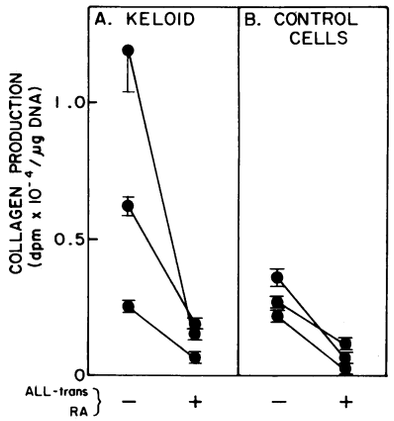

Figure 4. Inhibition of procollagen production by all-*trans*-RA in three keloid fibroblast cultures and in three control cultures obtained from age-matched, healthy subjects. The cultures were preincubated either with 10^{-5} all-*trans*-RA or 0.2% ethanol for 60 min, and then labeled with [^{3}H]proline for 6 h. The collagen production was assayed as synthesis of nondialyzable [^{3}H]hydroxyproline, expressed as disintegrations per minute per microgram of DNA in the corresponding culture. (−) cultures incubated without the retinoid; (+) cultures incubated with all-*trans*-RA. The values are mean±standard deviation of three parallel determinations.

activatable collagenase is reduced by retinoids in human skin fibroblast and synovial cell cultures (26, 27). Similar observations were made in the present study employing tissue culture conditions under which the procollagen production was reduced even to a larger extent. For comparison, we assayed the activity of an elastaselike neutral metalloprotease produced by the same fibroblast cultures. Furthermore, we assayed the activity of prolyl hydroxylase, an intracellular enzyme participating in the biosynthesis of procollagen (35). The results indicated that the activities of the latter enzymes were not affected by all-*trans*-RA, suggesting that the effects on the connective tissue metabolism may be selective. It should be noted that the selective suppression of procollagen production in this study was demonstrated in relatively short-term cultures. It is possible that in long-term cultures, inhibition of cell proliferation and a less-specific inhibition of protein synthesis could take place.

One of the major hypotheses concerning the molecular mode of retinoid action suggests that the retinoids might serve as a coenzyme in the membrane-mediated synthesis of glycoproteins, and specifically serve as a carrier of mannose into glycoproteins (36, 37). It was, therefore, of interest to study the degree of mannosylation of procollagen synthesized in the presence of a retinoid. The results indicated that mannosylation of procollagen, as determined by the ratio of [^{3}H]mannose to [^{14}C]proline, was unaffected by all-*trans*-RA. Thus, our results do not confirm earlier suggestions (38) that the mannosylation of procollagen might be increased in the presence of retinoids. It is possible that in our experimental situation the mannosylation of procollagen is already maximal and no sites are available for further mannosylation.

Excessive accumulation of collagen is the pathologic hallmark of a variety of fibrotic processes, as for example, pulmonary fibrosis or liver cirrhosis. Also, several dermal fibrotic conditions, including progressive systemic sclerosis, morphea, keloids, hypertrophic scars, and familial cutaneous collagenoma, result from excessive collagen accumulation in tissues (28). Although several pharmacologic approaches have been attempted in these conditions, there is currently no effective treatment for limiting collagen deposition in tissues (39). In this study, we demonstrated that procollagen production by keloid fibroblast lines, which initially showed significantly increased rates of collagen production, could be suppressed by all-*trans*-RA and 13-*cis*-RA. Consequently, further development of retinoids with appropriate biological activities, including selective collagen suppression, minimized toxicity, and efficient tissue targeting, might be useful for treatment of fibrotic processes.

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