Topical All-Trans Retinoic Acid Stimulates Collagen Synthesis In Vivo

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Histochemical and ultrastructural studies demonstrate that topical all-trans retinoic acid (RA) stimulates the deposition of a subepidermal band of collagen in photoaged hairless mice. The aim of this study was to examine the effect of RA treatment on collagen synthesis using biochemical and immunochemical techniques. Albino hairless mice were irradiated three times a week for 10 weeks with four minimal erythema doses of UVB from Westinghouse FS-40 bulbs. In the post-UV period, mice were either nontreated or treated with 0.05% RA or the ethanol-propylene glycol vehicle for up to 10 weeks. Antibodies against the aminopropeptide (AP) of type III procollagen were used in immunofluorescence microscopy and radioimmunoassay techniques. The AP of type III collagen is normally present throughout the dermis and in areas of active collagen synthesis (i.e., the dermal-epidermal junction). In this study, a similar distribution was seen in all untreated and vehicle-treated mice, and in mice treated with RA for 2, 4, and 6 weeks. However, increased staining, in a subepidermal band, was detected in the 8-week RA-treated skin. This region became intensely fluorescent to a depth of 100 μ in the 10-week RA-treated skins. As determined by radioimmunoassay, the content of the AP of type III procollagen increased twofold with 10-week RA treatment. Because the ratio of type I to type III collagens remained constant in treated and untreated skins, it is reasonable to assume that the content of type I collagen increased in proportion to type III collagen in RA-treated skins. *J Invest Dermatol 96:975–978, 1991*

Histochemical and electron microscopy studies have shown that topical application of all-trans retinoic acid (RA) for 10 weeks stimulates the deposition of a ~100 μ deep subepidermal band of collagen in photoaged albino hairless mice [1]. The collagen in this region appears histologically and ultrastructurally normal but it has not been characterized. The aims of this study were to quantify the newly synthesized collagen with radioimmunoassay techniques using antibodies against the aminopropeptide (AP) of type III procollagen and to localize this antigenic moiety in the subepidermal dermis with specific antibodies. It is the AP of type III procollagen that is retained when the procollagen is incorporated into mature collagen fibers [2,3], whereas the carboxyl propeptide is cleaved. Therefore, the amount of AP present reflects the synthesis of type III collagen. In addition, the ratios of type I to type III collagens were examined in total (pepsin soluble) collagen and in acid-soluble and CNBr-soluble (acid-insoluble) collagens.

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Abbreviations:
RA: retinoic acid
UV: ultraviolet

MATERIALS AND METHODS

Animals and Irradiation Schedules Eighty Skh-hairless-1 (albino) female mice, age 6–8 weeks (Skin & Cancer Hospital, Temple University Health Sciences Center, Philadelphia, PA), were housed individually with free access to food and water. Room lighting (12-h on/off cycle) was with General Electric F40 GO gold fluorescent bulbs, which emit no UV radiation. Mice were divided into four groups of 16–24 each to supply the various biopsy time points. Three groups were exposed to UVB radiation, whereas the fourth served as age-matched, unirradiated controls.

The UVB source was a bank of Westinghouse FS-40 sunlamps (280–400 nm: peak irradiance ~313 nm). Lamps were positioned 16 cm above the mice during the thrice-weekly exposures, which continued for 10 weeks. Each UV dose, reached gradually over the first 4 weeks by increments of ½ minimal erythemal doses (MED), was ~4.5 MEDs (0.07 J/cm^2 UVB). Irradiance was measured with an IL 700 Research Radiometer (International Light, Inc., Newburyport, MA) using a UVB sensor with peak sensitivity at ~290 nm.

Post-Irradiation Treatments Of the three irradiated groups of mice, one was not treated post UV. One group was treated on dorsal trunk skin with 100 μl of 0.05% all-trans RA in ethanol : propylene glycol (70 : 30 v/v) with 0.05% butylated hydroxytoluene whereas the third group was treated with the vehicle. Treatments were five times a week for 10 weeks.

Biopsies Mice were sacrificed by cervical dislocation, dorsal trunk skin was excised and frozen at −70°C until used. An adjacent strip of skin was prepared for light microscopy and stained with Luna's aldehyde fuchsin for assessment of new collagen deposition [1]. Samples for immunofluorescence microscopy were taken post UV on all irradiated groups at weeks 0, 2, 4, 6, 8, and 10 (two skins

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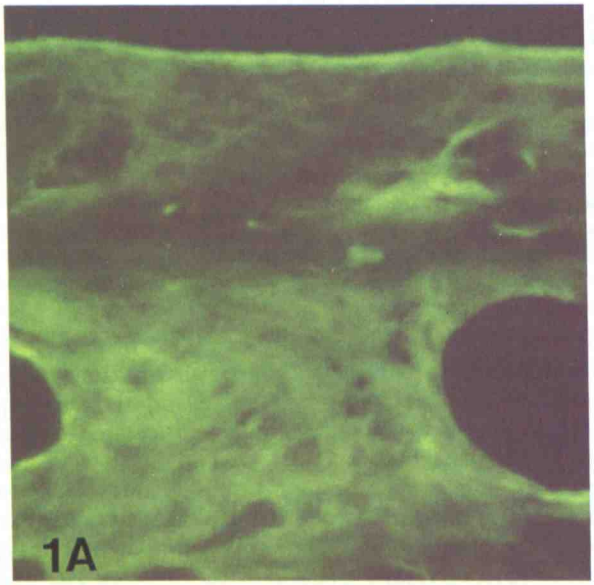

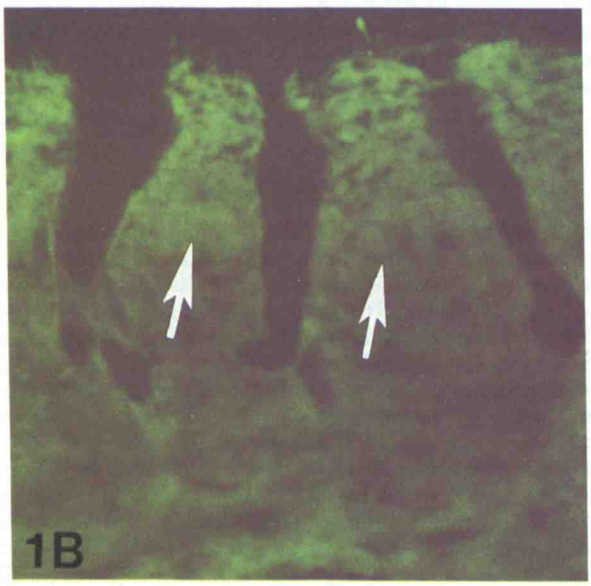

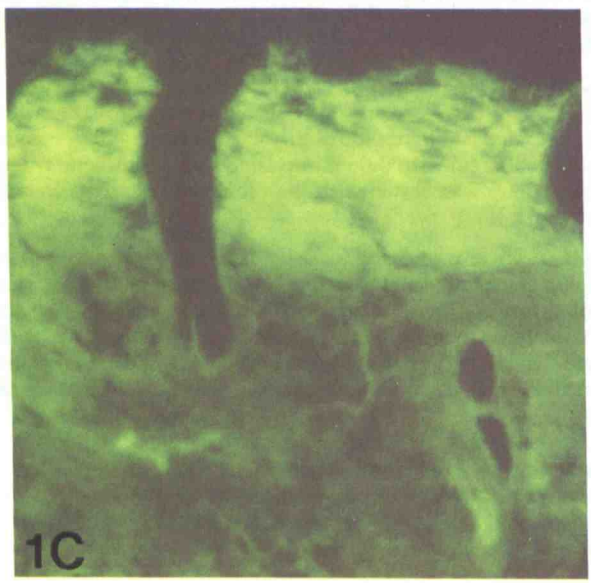

Figure 1. Indirect immunofluorescence of control and RA-treated mouse skin stained with antibodies directed against the AP of type III procollagen. *A*, age-matched untreated controls; fluorescence is diffuse throughout the dermis with a subepidermal brighter linear deposit; *B*, RA-treated for 8 weeks post UV: a slight increase in staining is detectable in a deep band in the subepidermal dermis. *Arrows*, lower limit of the band; *C*, RA-treated for 10 weeks post UV: the band of new collagen is deeper and is more intensely stained. The invaginations in the upper dermis in B and C are inactive hair follicles. *A–C*, magnification ×160.

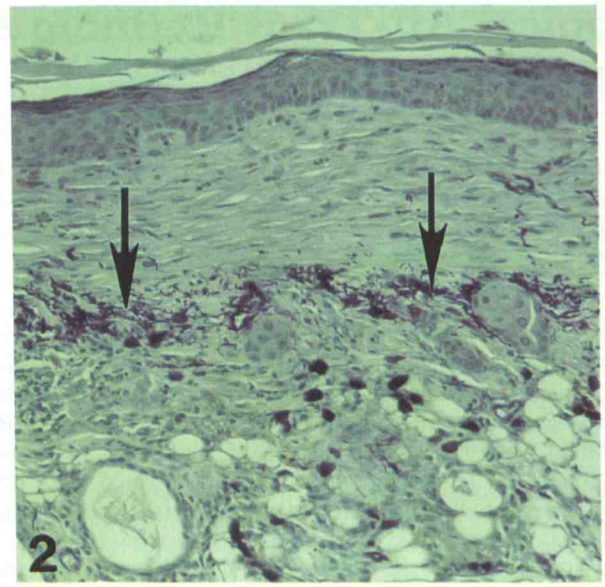

Figure 2. Luna's aldehyde fuchsin-stained paraffin section of skin treated with RA for 10 weeks post UV. The band of subepidermal collagen, delineated at its lower border by compressed elastic fibers (→), corresponds to the area of bright fluorescence in Fig 1*B,C*. Magnification ×115.

each time point). Biochemical and radioimmune analyses of collagen were performed on the UV-only group at 0 and 10 weeks post UV and on the topically treated groups at 10 weeks post UV (six skins each time point). Age-matched controls were sacrificed at similar time points.

Immunofluorescence Microscopy The immunofluorescence methodology was as previously described [4]. Briefly, frozen sections (8 μ) were treated with cold acetone for 5 min, rinsed with phosphate-buffered saline (PBS), and incubated for 30 min at 37°C with antibodies against the AP of type III procollagen. Sections were washed with PBS and treated for 30 min at 37°C with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (Organon Teknika-Cappel, West Chester, PA). Controls used IgG prepared from the sera of non-immunized rabbits. Purified antibody against the AP of type III procollagen was prepared in rabbits and purified as described by Nowack et al [5].

Radioimmunoassay (RIA) for pN Type III Collagen A RIA procollagen-III peptide kit (Behringwerke AG, Marburg, Federated Republic of Germany) for the radioimmunologic determination of procollagen III peptide was used to determine the content of AP type III procollagen as previously described [4].

Estimation of Types I and III Collagens (Intact Skins) Individual skins (100 mg wet weight) were sectioned in a cryostat (30 μ) in a plane parallel to the surface through the entire collagen-containing portion of the dermis to the beginning of the dermal cyst area. Pooled sections were pepsinized for 18 h at 4°C as previously described [4]. The pepsin-insoluble material was centrifuged, resuspended in fresh pepsin, and digested for an additional 18 h at 4°C. The supernatants from the two digestions were combined and types I and III collagens were resolved by interrupted gel electrophoresis

[6]. Gels were stained with Coomassie Brilliant Blue. The relative intensities of the α_1 (III), α_1 (I), and α_2 (I) bands were determined using a Beckman DU8 spectrophotometer (550 nm).

Estimation of Soluble Types I and III Collagens Defatted, dried skins were sequentially extracted with 1.0 M NaCl, 0.05 M Tris (pH 7.5), 2 mM phenylmethyl sulfonyl fluoride (PMSF), 0.02% sodium azide, and 10 μg/ml aprotinin (10 mg dry weight per ml buffer) and with 0.5N acetic acid, 2 mM PMSF, 0.02% sodium azide, and 10 μg/ml aprotinin overnight (0–4°C). Following centrifugation, the ratio of types I and III collagens in the acetic acid supernatant was measured by interrupted gel electrophoresis [6].

Estimation of Insoluble Types I and III Collagens The pellet obtained following the acetic acid extraction was treated with 9.5 M urea, 100 mM mercaptoethanol, 5 mM Tris HCl (pH 8), and proteolytic inhibitors overnight (0–4°C). The extraction mixture was then centrifuged and the pellets washed extensively with water and lyophilized.

The insoluble fraction was digested with cyanogen bromide as previously described [7]. The CNBr-soluble fraction contained peptides of types I and III collagens, which were resolved by 10–20% gradient SDS-PAGE. The Coomassie Blue stained gels were scanned with a Beckman DU8 Spectrophotometer. CNBr-digested standards for types I and III collagens (Sigma Chemical Co., St. Louis, MO) were included on each gel. The areas corresponding to α_1 (III) CB8 and α_1 (I) CB8 were used to determine the ratio of types I and III collagens [8].

Hydroxyproline Determination Lyophilized samples were hydrolyzed for 24 h in constant boiling HCl (Pierce, Rockford, IL) at 107°C. Amino acid residues (including hydroxyproline) were derivatized with 9 fluorenylmethyl chloroformate (FMOC) and then resolved by reverse-phase high-performance liquid chromatography (HPLC) using a 250 × 4.6-mm Spherisorb ODS-2 column [7,9].

RESULTS

Immunofluorescent Localization of the Aminopropeptide of Type III Procollagen In age-matched untreated controls a light, diffuse fluorescence was detected throughout the dermis and as a brighter linear deposit at the dermal-epidermal junction (Fig 1*A*). These findings are consistent with the reports that type III collagen is present in association with type I collagen [2,3]. No significant change in the staining pattern was detected at any time during the post-UV period (2, 4, 6, 8, or 10 weeks) in the untreated or vehicle-treated skins.

In RA-treated skins, no significant change was detected at 2, 4, or 6 weeks. After 8 weeks of treatment, a slight increase in staining was present in a deep band in the subepidermal dermis (Fig 1*B*). After 10 weeks, the band was significantly deeper and more intensely stained (Fig 1*C*). This band corresponds to what has been previously described histochemically as a repair zone (Fig 2).

Quantification of Collagen Synthesis The highly sensitive radioimmunoassay confirmed previous studies [4] showing that the content of AP type III procollagen is reduced in irradiated mouse skin (Table I). Treatment with RA in the post-UV period significantly raised the levels of the AP, returning it to close to that of normal controls. In contrast, the content of AP in vehicle-treated or non-treated samples showed little change (Table I).

Ratios of Types I and III Collagens The ratio of type I to type III collagen was determined for total (pepsin-solubilized) collagen, acid-soluble, and CNBr-soluble (acid-insoluble) collagen. Although ratios differed between the different extraction methods, they did not change significantly for treated and untreated skins within each extract fraction (Table II).

Table I. Quantification of the Aminopropeptide of Type III Procollagen by Radioimmunoassay

Groups	ng AP type III Procollagen/50 mg Dry Weight[a]
Untreated	17.2 ± 5.0
UV 10 weeks	1.7 ± 0.3
UV 10 weeks, untreated 10 weeks	7.0 ± 1.1
UV 10 weeks, vehicle 10 weeks	1.9 ± 0.5
UV 10 weeks, RA 10 weeks	13.8 ± 0.5[b]

[a] Mean ± SEM, n = 4.
[b] Significantly different ($p < 0.001$) from all other UV-treated groups.

Hydroxyproline Content Levels of hydroxyproline did not vary in intact skins or in the soluble and insoluble fractions of any of the treatment groups. Typical values ranged from 73 to 99 residues hydroxyproline per 1000 residues.

DISCUSSION

These studies confirm prior morphologic observations that topical all-trans RA stimulates collagen synthesis in the photoaged hairless mouse [1]. The increased levels of AP with RA treatment may reflect either increased synthesis or reduced degradation of type III procollagen. However, preliminary studies demonstrated increased mRNA levels for both types I and III collagen with RA treatment. Therefore, these results support the conclusion that RA stimulates collagen synthesis. This is in contrast to in vitro studies that report inhibition of collagen synthesis by retinoids. Nelson and Balian [10] showed, in fetal dermal fibroblasts, a non-specific RA-induced decrease in collagen that paralleled inhibition of non-collagenous protein synthesis and cell proliferation. Oikarinen et al [11], however, reported a specific inhibition of collagen synthesis by all-trans RA in human skin fibroblast cultures. This was accompanied by a decrease in mRNA levels for type I procollagen without a concommitant decrease in fibronectin and B-actin mRNA. There have been similar findings in human keloid fibroblast cultures [11,12].

The discrepancy between in vivo and in vitro findings extends to another retinoid. In vivo, 13-cis RA stimulated the production of a morphologically defined repair zone of new collagen in the same photoaged mouse model [13]. It also increased, by 2–4 times, mRNA levels for types I and III collagens [14]. In vitro, however, 13-cis RA has been reported to reduce collagen biosynthesis and mRNA levels in human skin fibroblast cultures [11,15]. In preliminary studies, we also found increased mRNA levels for both types I and III collagens with all-trans RA treatment of photoaged mice

Table II. Ratio of Types I/III Collagens in Various Extracts[a]

Groups	Pepsin-Soluble	Acid-Soluble	CNBr
Untreated	11.5	4.4	1.6
UV 10 weeks	8.0	4.7	1.5
UV 10 weeks, untreated 10 weeks	8.1	ND[b]	1.5
UV 10 weeks, vehicle 10 weeks	11.5	3.9	1.3
UV 10 weeks, RA 10 weeks	10.8	3.9	1.5

[a] Values represent the mean of triplicate determinations.
[b] ND, not determined.

(unpublished results).* The 10-week course of irradiation had little effect on α_1 (I) and α_1 (III) procollagen mRNA. When this was followed by 10 weeks of topical treatment with all-trans RA, there was a 2–3 times increase in the levels of both mRNA as corrected against B-actin. These results support our findings of unchanged ratios of types I and III collagens, suggesting a proportional stimulation of the synthesis of both types of collagen by all-trans RA. Because the conditions are so different, it is not unusual for in vivo and in vitro studies to be antithetic. In this in vivo study, there is agreement between the biochemical and morphologic findings.

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