Topical Tretinoin Increases the Tropoelastin and Fibronectin Content of Photoaged Hairless Mouse Skin

Elaine Schwartz and Lorraine H. Kligman

Departments of Dermatology, Mt. Sinai School of Medicine, New York, New York; and University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, U.S.A.

Topical tretinoin treatment of photoaged hairless mice has been shown in previous studies to stimulate formation of a subepidermal zone of new connective tissue characterized by enhanced collage synthesis. The aims of this study were to localize and/or quantify elastin, fibronectin, and glycosaminoglycans in the same model. Hairless mice (Skh-1) were irradiated thrice weekly for 10 weeks with gradually increasing doses of ultraviolet (up to 4.5 minimal erythema doses per exposure) from Westinghouse FS-40 bulbs. Mice were then treated five times a week with either 0.05% tretinoin, the ethanol:propylene glycol vehicle, or nothing for another 10 weeks. Controls included mice sacrificed after 10 weeks of ultraviolet treatment and age-matched untreated animals. The distribution of elastin and fibronectin was examined by immunofluorescence microscopy, which revealed fine fibrils in the subepidermal zone in tretinoin-treated skin. A quantitative slot-blot immunobinding assay showed that tretinoin induced a threefold higher amount of tropoelastin compared with controls. Insoluble elastin content (desmosine levels) was similar in all groups. Although fibronectin content was increased by ultraviolet radiation, tretinoin treatment reduced the largest increase. In contrast, the amount of glycosaminoglycans, although increased by UVB radiation, was reduced by tretinoin treatment. Key words: UV radiation/glycosaminoglycans/elastin/desmosine. J Invest Dermatol 104:518–522, 1995

During the development of the hairless mouse model for human photoaging, it was discovered that within a 10–15-week period after ultraviolet radiation (UVR), the damaged dermal matrix underwent partial and spontaneous restoration [1,2]. Histologic and ultrastructural studies demonstrated that topical treatment with tretinoin (all-trans-retinoic acid) in the post-UVR period accelerated this process by enhancing the production of a subepidermal repair zone of collagen [3,4]. A similar repair zone was reported by Bryce et al [5] using either tretinoin or isotretinoin in a different strain (HR/S) of photoaged mice.

More recently, using immunofluorescence microscopy and immunochemical and biochemical techniques, we reported that tretinoin enhanced collagen synthesis in the photoaged hairless mouse. The newly synthesized collagen was localized in the histochemically defined repair zone [6]. Using a UVR schedule similar to that of Kligman et al [3], other workers reported increased collagen synthesis after 6 weeks of treatment with tretinoin [7]. In addition, a two- to fourfold increase in steady-state mRNA levels for types I and III collagen was induced in the photoaged mouse by tretinoin [6,8] and isotretinoin treatments [8].

UVR-induced changes in the noncollagenous dermal matrix have been documented extensively. Increased amounts of elastic fibers, fibronectin, and glycosaminoglycans have been reported in the photoaged hairless mouse and in human skin by histochemical [1,9–13], immunohistochemical [14,15], and biochemical means [14,16,17]. However, there is little information concerning the effect of tretinoin on the noncollagenous dermal matrix in photoaged skin. One study of the hairless mouse reported that tretinoin treatment did not affect glycosaminoglycan synthesis [18]. A second study failed to find an effect of tretinoin on the UVR-induced desmosine content [5]. Recent studies on human photoaged skin have focused mainly on the clinical effects of tretinoin [19,20] or on collagen deposition [21,22]. The only reports of tretinoin-induced changes in noncollagenous proteins involved the epidermis or cell culture results. Lunden et al [23] reported that 6 months of tretinoin treatment did not alter hyaluronic acid content in the epidermis or in blister fluid. Increased amounts of fibronectin were reported by Varani et al [24–26] in fibroblast cultures, whereas in keratinocyte cultures, fibronectin synthesis was decreased after tretinoin treatment.

The aim of this study was to determine the effects of topical tretinoin on the elastin, fibronectin, and glycosaminoglycan contents of the photoaged hairless mouse dermis.

MATERIALS AND METHODS

Animals and Irradiation Schedules One hundred Skh-hairless-1 (albino) female mice, ages 6–8 weeks (Charles River Laboratories, Wilmington, MA) 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 UVR. Four groups of 20 mice each were
exposed to UVB radiation, whereas the fifth group served as age-matched, unirradiated controls.

The UVR source was a bank of eight Westinghouse FS-40 “sunlamps” (280–400 nm; peak irradiance at 313 nm). Lamps were positioned 16 cm above the mice for the thrice-weekly exposures, which continued for 10 weeks. Each UVR dose, reached gradually over the first 4 weeks by increments of 0.5 minimal erythemal doses (MED), was 4.5 MED (0.07 J/cm² UVB). Irradiance was measured with an IL 700 Research Radiometer (International Light, Inc., Newburyport, MA) using a UV sensor with peak sensitivity at 290 nm.

Post-Irradiation Treatments At the end of the 10-week irradiation period, one irradiated group was sacrificed to provide tissue for the UVR-only baseline values. The remaining three groups were treated as follows: 1) no further treatment, 2) 0.05% tretinoin in an ethanol:propylene glycol vehicle (70:30 v/v) containing 0.05% butyraldehyde, and 3) vehicle. Dorsal applications (100 µl each) were made five times a week for 10 weeks.

Biopsies and Histologic Stains The mice were sacrificed by cervical dislocation, and 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 [27] to monitor depth of the repair zone. Movry’s colloidal iron stain was used to assess glycosaminoglycan content.

Immunofluorescence Microscopy Frozen sections (8 µm) were treated with cold acetone for 5 min and rinsed with phosphate-buffered saline. The specimens were then incubated for 30 min at 37°C with either rabbit anti-bovine elastin or rabbit anti-mouse fibronectin antibodies, washed with phosphate-buffered saline, 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 nonimmunized rabbits.

Tropoelastin and Insoluble Elastin Analyses Equal wet weights of skin were homogenized and defatted with butanol:disopropyl ether (40:60 v/v). Insoluble experiments have determined that tropoelastin is not extracted by this procedure. Extraction was onward at room temperature, with stirring, in 0.05 M Tris HCl (pH 7.5), 1% sodium dodecyl sulfate, and 0.33 M mercaptoethanol containing proteolytic inhibitors. This was followed by centrifugation at 10,000 rpm for 30 min. The supernatants were electrophoretically concentrated fivefold at 2.5 mA/sample for 90 min (Little Blue Tank, Isco, Lincoln, NE) and analyzed for tropoelastin by a quantitative immunobinding assay [28]. Dilutions of extracts and standards were immunobilized on a nitrocellulose sheet. The sheet was then blocked with bovine serum albumin, washed, and incubated with antibodies directed against bovine alpha elastin. The sheets were again washed and treated with 125I-labeled protein A. After a third washing, the nitrocellulose was dried and exposed to film with intensifying screens. The films were scanned using a Beckman DU8 Spectrophotometer (550 nm).

The insoluble residues were washed extensively, lyophilized, and hydrolyzed in 6 N HCl for 72 h at 107°C to release the desmosines. Hydrolysates were applied to mini-cellulose columns and the desmosines were eluted, dried, and analyzed by high performance liquid chromatography [29].

Fibronectin Analysis Fibronectin was quantified as described previously [30]. Five skins from each group were individually trimmed to equal surface area, homogenized, defatted, and extracted for 48 h with 5 ml/100 mg dry weight of 0.05 M Tris HCl (pH 7.5), 1.0% sodium dodecyl sulfate, 0.33 M mercaptoethanol, and proteolytic inhibitors (1 µg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 1 mM N-ethyl maleimide). The latter are serine protease inhibitors, and sodium dodecyl sulfate is an effective general protease inhibitor. Many of the plasma enzymes are serine proteases, including elastase. The suspension was centrifuged at 10,000 rpm for 30 min. Supernatants were electrophoretically concentrated fivefold at 2.5 mA/sample for 90 min (Isco Inc.).

Fibronectin content was analyzed by a specific enzyme-linked immunosorbent assay (ELISA) using rabbit anti-mouse fibronectin and mouse fibronectin (Chemicon, Temecula, CA), as follows. Mouse fibronectin (400 ng) was added to wells in a flat-bottom microtiter plate and allowed to incubate overnight at room temperature. To wells in a round-bottom microtiter plate, 110 µl of rabbit anti-mouse fibronectin antigen (diluted 1:15,000) and 110 µl of dilutions of either standard mouse fibronectin (1.25 µg/ml diluted to 1 ng/ml) or samples were mixed for 1 h at room temperature. The plate was incubated overnight at 4°C, and then kept refrigerated at 0–4°C. Excess antigen was removed from the flat wells, which were then washed, and 0.35 ml of 1% bovine serum albumin was added. After 1 h, the bovine serum albumin was removed, the wells were washed, and 200 µl from the round wells was added. The plate was incubated at 37°C for 45 min and washed, and anti-rabbit IgG-linked alkaline phosphatase (1:1000 dilution) was added to the wells. After incubation at 37°C for 1 h and washing, the wells were rinsed rapidly with substrate buffer (10% diethanolamine, pH 9.8), and 0.2 ml of a solution of substrate p-nitrophenyl phosphate (1 mg/ml) in substrate buffer was added. The reaction was allowed to proceed at 37°C for 15–30 min and was terminated by the addition of 0.1 ml of 1 N NaOH. Products were quantified by reading of the plate at 405 nm. Appropriate controls lacking antigen, antibody, enzyme, or substrate were run simultaneously.

Glycosaminoglycan Analysis Glycosaminoglycans were quantified as described previously [30]. Weighed skins were minced, defatted in acetone:ether (1:1), dried, and suspended in 10 ml of 0.1 M phosphate buffer (pH 6.5) with 0.005 M ethylenediamine tetraacetic acid and 0.005 M cysteine HCl. Digestion was with papain (50 ml containing 24 IU/ml; Sigma Chemical Co., St. Louis, MO) for 24 h at 65°C. The digest was diluted to 40 ml with water and centrifuged for 2 h at 12,000 rpm (0–4°C). A 1% suspension of cetylpyridinium chloride was added dropwise to the supernatant [31]. After storage overnight at 4°C, the suspension was centrifuged at 12,000 rpm for 1 h at 0–4°C. The precipitate was dissolved in 300 µl of n-propanol and 75 µl of 1% cetylpyridinium chloride. The glycosaminoglycans were further precipitated with 5 ml of absolute ethanol and a few drops of sodium acetate. The precipitate was washed with ethanol and diethyl ether, air dried, redissolved in 1 ml of water, transferred to preweighed tubes, and dried, after which the glycosaminoglycan weight was calculated. Glycosaminoglycans were redissolved in 1 ml of water, and the uronic acid content was determined by the carbazole assay [32].

RESULTS

Histochemical Findings Confirm Tretinoin Enhancement of Dermal Matrix Deposition Luna’s stain revealed the subepidermal zone of new collagen in all specimens at 10 weeks after UVR, as reported in previous studies [3,4]. In tretinoin-treated skin, the zone was considerably deeper, more extensive across the specimens, and better delineated, with compressed elastosis at the lower border (data not shown). Movry’s stain showed increased glycosaminoglycans in all specimens. Histologically, the increases induced by UVR alone appeared to regress too slowly in the post-UVR period to make histologic quantification of the effect of tretinoin difficult (data not shown).

Immunofluorescence and Immunobinding Studies Locate and Quantify Tretinoin-Enhanced Tropoelastin Accumulation

Immunofluorescence: Sections from normal, 10-weeks UV-irradiated, post-UVR untreated, and vehicle-treated skins showed similar patchy fluorescence throughout the dermis (Fig 1a). After 10
weeks of UVR treatment with tretinoin, fluorescent strands of new elastic tissue were present in the mainly unstained subepidermal dermis (Fig 1b), which corresponds to the morphologically defined repair zone [3,4]. A concentration of patchy fluorescence was present at the lower border of the zone, corresponding to the compressed elastosis seen histologically. Fluorescence was not visible in controls treated with normal rabbit serum.

**Immunobinding Assay:** The autoradiogram of the slot-blot immunobinding assay using antibodies against bovine alpha elastin and 125I-labeled protein A clearly showed an increase in tropoelastin in tretinoin-treated skin (Fig 2). Densitometric scanning of the autoradiogram films showed that the increase was approximately threefold higher than in any of the other treatment groups (Table I).

**High Performance Liquid Chromatography for Insoluble Elastin:** Determination of desmosine content indicated no significant increase in insoluble elastin in any of the groups (Table I). The values ranged from 10.1 to 11.9 ng desmosine/mg wet weight.

**Tretinoin-Enhanced Fibronectin Content is Demonstrated by Immunofluorescence and Enzyme-Linked Immunosorbent Assay**

**Immunofluorescence:** Fluorescent staining was more abundant but otherwise similar to that seen for elastin in normal, 10-week UV-irradiated, post-UVR untreated, and vehicle-treated skins (Fig 3a). After 10 weeks of UVR treatment with 0.05% tretinoin, the subepidermal repair zone was filled with intensely stained fine fibrillary fibronectin (Fig 3b). Fluorescence was not visible in controls treated with normal rabbit serum.

**Quantification by Enzyme-Linked Immunosorbent Assay:** Whether calculated by area, wet weight, or dry weight, UVR significantly increased fibronectin levels compared with normal controls (Table II). These levels decreased during the 10-week post-UVR period with vehicle or no topical treatment, but remained significantly higher than in normal controls. The greatest increase was induced by tretinoin (more than twofold).

**Tretinoin Diminishes the UVR-Induced Increase in Glycosaminoglycans** Quantification by the carbazole method showed that glycosaminoglycan levels were significantly increased after 10 weeks of UVR. The levels normalized in the post-UVR period with vehicle or no topical treatment. However, tretinoin treatment resulted in a significant lowering of glycosaminoglycan levels when compared with normal or 10-week UVR controls (Table III).

**Table I. Effect of UVR and Tretinoin on Elastin Accumulation**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Immunobinding Assay (ng/elastin/mg wet weight)</th>
<th>Desmosine (ng/mg wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Untreated</td>
<td>150 ± 16</td>
<td>11.4 ± 1.2</td>
</tr>
<tr>
<td>II. UVR 10 weeks</td>
<td>175 ± 39</td>
<td>10.9 ± 3.8</td>
</tr>
<tr>
<td>III. UVR 10 weeks; untreated 10 weeks</td>
<td>198 ± 29</td>
<td>10.1 ± 2.3</td>
</tr>
<tr>
<td>IV. UVR 10 weeks; vehicle 10 weeks</td>
<td>184 ± 29</td>
<td>10.4 ± 0.6</td>
</tr>
<tr>
<td>V. UVR 10 weeks; tretinoin 10 weeks</td>
<td>479 ± 62</td>
<td>11.9 ± 1.1</td>
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</tbody>
</table>

*a Hairless mice were irradiated with UVR for 10 weeks and then treated with either 0.05% tretinoin, the vehicle, or nothing. Skins were homogenized, defatted, extracted, and analyzed by a quantitative immunobinding assay [28]. Insoluble residues were hydrolyzed in 6 N HCl, and desmosines were eluted on mini-cellulose columns, dried, and analyzed by high-performance liquid chromatography [29].

**DISCUSSION**

Previous histologic [3-5], immunocohemical, and biochemical studies [6,7] have shown that topical tretinoin treatment of photoaged hairless mouse skin stimulates the deposition of a subepidermal repair zone of newly synthesized collagen. This study expands the in vivo findings, demonstrating a tretinoin-induced threefold increase in soluble elastin and a greater than twofold increase in fibronectin. In agreement with Bryce et al [5], we failed to find a

**Figure 3. Detection of fibronectin in the subepidermal dermis by immunofluorescence with antibodies directed against mouse fibronectin.** A UV for 10 weeks. Fluorescence highlights a diffuse network of fibronectin throughout the dermis. Arrowheads, dermoepidermal junction. Bar, 10 μm. B UV for 10 weeks followed by tretinoin for 10 weeks. Dense parallel fluorescent strands of fibronectin fill the subepidermal (SE) band of new connective tissue. Note that fibronectin in the lower dermis retains the network appearance. Arrowheads, dermoepidermal junction. Bar, 10 μm.
tretinoin-induced change in insoluble elastin as measured by desmosine content in the 10-week post-UVR period. In contrast to our findings, Bryce et al. [5] reported that insoluble elastin was increased as a result of the UVR exposures. The most likely reason for this divergence lies in the different protocols. The 5–6 months of UVR used by Bryce et al. to produce photoaging in the HRS/J mice, compared with our 10-week schedule, allowed more time for tropoelastin to accumulate and become crosslinked into mature fibers.

The small but significant increase in glycosaminoglycans after 10 weeks of UVR was expected on the basis of histochemical evaluation. However, the considerable decrease with tretinoin treatment was not anticipated. This finding supports the premise of Chen et al. [18] that glycosaminoglycans may not be responsible for the wrinkle effacement reported in these mice with tretinoin [5]. These authors found no increase in sulfated glycosaminoglycan or hyaluronic acid synthesis in explants of UV-irradiated, tretinoin-treated hairless mouse skin as compared with irradiated, vehicle-treated controls. Unfortunately, the effect of 10 weeks of irradiation alone could not be assessed because Chen et al. [18] did not include these or normal controls in their study.

Other studies with hairless mice examined either UVR alone or tretinoin alone. In agreement with our findings on the effect of UVR alone, Margelin et al. [33] found increased glycosaminoglycan synthesis in explants from UV-irradiated hairless mice. However, the increase was seen only in the proteoglycans heparin and heparan sulfate. Synthesis of dermatan sulfate and hyaluronic acid was not affected. An in vitro study [34] that examined lifetime application of tretinoin to unirradiated hairless mice demonstrated histochemically a large increase in dermal and epidermal glycosaminoglycans. Taken together with our current findings, the evidence suggests that UVR may increase some classes of glycosaminoglycans, whereas tretinoin acts to decrease UVR-induced levels. However, in unirradiated skin, tretinoin may increase this dermal matrix component [34].

The difficulty of obtaining sufficient human biopsy material has limited extensive biochemical studies of tretinoin effects on non-collagenous dermal matrix. The few in vitro studies have examined the effects of UVR or tretinoin, but not the combination of the two. For example, Schwartz and Cruickshank† irradiated normal human

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### Table II. Effect of UVR and Tretinoin on Fibronectin Content

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>ng/cm²</th>
<th>ng/mg Wet Weight</th>
<th>ng/mg Dry Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Untreated</td>
<td>64.4 ± 2.3</td>
<td>0.80 ± 0.06</td>
<td>3.64 ± 0.28</td>
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<tr>
<td>II. UVR 10 weeks</td>
<td>119.5 ± 19.6</td>
<td>1.39 ± 0.22</td>
<td>7.32 ± 1.1</td>
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<tr>
<td>III. UVR 10 weeks; untreated 10 weeks</td>
<td>73.9 ± 3.3</td>
<td>1.20 ± 0.03</td>
<td>4.99 ± 0.21</td>
</tr>
<tr>
<td>IV. UVR 10 weeks; vehicle 10 weeks</td>
<td>88.5 ± 4.5</td>
<td>1.39 ± 0.08</td>
<td>6.72 ± 0.52</td>
</tr>
<tr>
<td>V. UVR 10 weeks; tretinoin 10 weeks</td>
<td>160.9 ± 23.9</td>
<td>2.08 ± 0.24</td>
<td>10.38 ± 1.16</td>
</tr>
</tbody>
</table>

### Table III. Effect of UVR and Tretinoin on Glycosaminoglycan Content

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Carbazole Method (µg/cm²)</th>
<th>µg/mg Wet Weight</th>
<th>µg/mg Dry Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Untreated</td>
<td>21.77 ± 0.72</td>
<td>0.286 ± 0.018</td>
<td>1.54 ± 0.10</td>
</tr>
<tr>
<td>II. UVR 10 weeks</td>
<td>29.6 ± 2.9</td>
<td>0.329 ± 0.024</td>
<td>1.87 ± 0.13</td>
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<tr>
<td>III. UVR 10 weeks; untreated 10 weeks</td>
<td>21.68 ± 1.63</td>
<td>0.314 ± 0.009</td>
<td>1.73 ± 0.043</td>
</tr>
<tr>
<td>IV. UVR 10 weeks; vehicle 10 weeks</td>
<td>21.88 ± 3.39</td>
<td>0.303 ± 0.039</td>
<td>1.71 ± 0.233</td>
</tr>
<tr>
<td>V. UVR 10 weeks; tretinoin 10 weeks</td>
<td>14.76 ± 2.52</td>
<td>0.196 ± 0.038</td>
<td>1.09 ± 0.207</td>
</tr>
</tbody>
</table>

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dermal fibroblasts in vitro in the absence of tretinoin and found increased synthesis of both fibronectin and tropoelastin, a finding confirmed in the present in vivo study. As a second example, an in vivo study examined the effect of tretinoin on sun-protected buttock skin. Fisher et al [35] reported a tretinoin-induced increase in the transforming growth factor-β-induced “mucin” or glycosaminoglycans and proteoglycans. Thus, there is sparse biochemical information on human studies with which to compare the effect of tretinoin on noncollagenous dermal matrix in the photoaged hairless mouse.

It has been established that many of the UVR-induced changes in the hairless mouse correlate well with those occurring in human photoaged skin [36]. As trials of tretinoin-treated human skin approach and exceed 2 years, it is becoming apparent that the hairless mouse model is also a predictive one for the repair of human photoaging. Recent reports have demonstrated a tretinoin-induced subepidermal repair zone of new collagen [19,37], along with reduction in the abundant glycosaminoglycans present in photoaged skin [37]. The ultrastructural reappearance of type VII collagen anchoring fibrils has been reported after 2 years of treatment [21]. Most recently, Griffiths et al [22] demonstrated increased intracellular and extracellular type I collagen synthesis in the papillary dermis after 12 months of tretinoin treatment. In accordance with its short life span, metabolic processes occur more rapidly in mice than in humans. It is therefore likely that with time, much of what has been discovered in tretinoin-treated photoaged mouse skin will be confirmed in human skin.

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