All-*trans* Retinoic Acid (RA) Stimulates Events in Organ-cultured Human Skin That Underlie Repair

Adult Skin from Sun-protected and Sun-exposed Sites Responds in an Identical Manner to RA while Neonatal Foreskin Responds Differently

James Varani, Patricia Perone, Christopher E. M. Griffiths, Dennis R. Inman, Suzanne E. G. Fligiel, and John J. Voorhees
Departments of Pathology and Dermatology, The University of Michigan Medical School, Ann Arbor, Michigan 48109; and The Department of Pathology, Wayne State University, Veterans Affairs Medical Center, Allen Park, Michigan 48101

Abstract

Adult human skin from a sun-protected site (hip) and from a sun-exposed site (forearm) was maintained in organ culture for 12 d in the presence of a serum-free, growth factor–free basal medium. Cultures were incubated under conditions optimized for keratinocyte growth (i.e., in 0.15 mM extracellular Ca^{2+}) or for fibroblast growth (i.e., in 1.4 mM extracellular Ca^{2+}). Treatment with all-*trans* retinoic acid (RA) induced histological changes in the organ-cultured skin under both conditions which were similar to the changes seen in intact skin after topical application. These included expansion of the viable portion of the epidermis and activation of cells in the dermis. In sun-damaged skin samples, which were characterized by destruction of normal connective tissue elements and presence of thick, dark-staining elastotic fibers, a zone of healthy connective tissue could be seen immediately below the dermo–epidermal junction. This zone was more prominent in RA-treated organ cultures than in matched controls. Associated with these histological changes was an increase in overall protein and extracellular matrix synthesis. In concomitant studies, it was found that RA treatment enhanced survival and proliferation of adult keratinocytes and adult dermal fibroblasts under both low- and high-Ca^{2+} conditions. In all of these assays, responses of sun-protected and sun-exposed skin were identical. In contrast, responses of neonatal foreskin to RA were similar to those of adult skin in the presence of low-Ca^{2+} culture medium, but under conditions of high extracellular Ca^{2+} RA provided little or no additional stimulus. Together these studies suggest that the ability of RA to enhance repair of sun-damaged skin (documented in previous studies) may reflect its ability to influence the behavior of skin in a manner that is age dependent but independent of sun-exposure status. (*J. Clin. Invest.* 1994. 94:1747–1756.) Key words: all-*trans* retinoic acid • keratinocytes • fibroblasts • skin • repair

Introduction

Past studies have shown that proliferative responses of human keratinocytes and fibroblasts decrease as a function of age (1–12). This can be seen either when embryonic or neonatal cells age in vitro or when cells taken from individuals of varying age are examined in vitro. Decreased proliferative capacity with age does not appear to be growth factor specific as similar results have been obtained with numerous different factors. With fibroblasts, there is a parallel decrease in growth response to extracellular Ca^{2+} (13–16). Changes in fibroblast and keratinocyte proliferative capacity in monolayer culture are correlated with age-related alterations in the appearance of the skin (17–21). Characteristically, the skin of older individuals is looser, dryer, paler, and more finely wrinkled than the skin of younger people. The epidermis tends to be thinner in aged skin although the normal features of differentiation can be seen. The dermis is also thinner. The average number of dermal fibroblasts present is decreased as is the total amount of dermal collagen. Likewise, dermal vascularity of aged individuals is decreased as compared with that of younger people.

Superimposed on the structural and functional changes in the skin that result from intrinsic aging are effects of chronic sun exposure. Past studies have documented the deleterious effects of ultraviolet irradiation on both dermal and epidermal cellular populations in skin. Studies by Gilchrest (22, 23) have shown that keratinocytes and fibroblasts taken from sun-exposed skin have a reduced lifespan in vitro as compared with the same cells taken from adjacent (sun-protected) skin. Histological differences between sun-exposed and sun-protected skin can also be seen (24–31). Epidermis of sun-exposed skin is compacted and thinned. Polarity of basal cells is lost, and nuclear atypia can be seen. In the dermis one sees accumulation of thickened, degraded elastic fibers and a decrease in intact collagen fibers. This is accompanied by a reduction in the interstitial cell population (i.e., mostly fibroblasts) and a deterioration of the capillary beds of the papillary dermis. Although it was at one time thought that sun damage to skin was irreversible, more recent studies in humans and in experimental animals have documented that repair can occur upon cessation of exposure (32, 33). Further, it has been shown that treatment of sun-damaged skin with agents such as all-*trans* retinoic acid (RA)[1] can hasten the repair process (34, 35). How RA facilitates repair of sun-damaged skin is not fully understood. It may act by inhibiting or reversing processes that are triggered directly by exposure to ultraviolet light (36–39). Alternatively, RA is a multifunctional molecule that is known to regulate directly or indirectly many processes in a variety of cell types (40). The

Address correspondence to James Varani, Ph.D., Department of Pathology, The University of Michigan Medical School, 1301 Catherine Road, Box 0602, Ann Arbor, MI 48109.

Received for publication 1 December 1993 and in revised form 30 June 1994.

1. *Abbreviations used in this paper:* KBM, keratinocyte basal medium; KGM, keratinocyte growth medium; RA, all-*trans* retinoic acid; SLS, sodium lauryl sulfate; vitamin D, 1,25 dihydroxyvitamin D_3.

ability of RA to enhance repair of sun-damaged skin may reflect this pleotropic activity.

We demonstrated recently that human skin could be maintained in organ culture for up to 12 d under serum-free, growth factor–free conditions (41–43). It was shown in these studies that treatment of organ-cultured skin with RA induced changes that were analogous to those seen in intact skin after topical RA treatment. These changes include expansion of the viable portion of the epidermis (41, 42) and induction of new extracellular matrix production (43). In our original studies, only skin from a sun-protected site was used. In this study we have used the same organ culture approach to compare skin from three sources, i.e., adult sun-protected skin, adult sun-exposed skin, and neonatal foreskin, in their responses to RA. We report here that, by a variety of criteria, adult skin from sun-protected and sun-exposed sites responds in a qualitatively and quantitatively similar manner to RA while there are significant differences between adult skin from either site and neonatal foreskin in these same responses.

Methods

Culture media and reagents. Keratinocyte basal medium (KBM) was used as culture medium for these experiments. KBM is a serum-free, growth factor–free modification of MCDB-153 (Clonetics Corp., San Diego, CA). The Ca^{2+} concentration of KBM is 0.15 mM. It was used without further modification or supplemented with calcium chloride to bring the final Ca^{2+} concentration to 1.4 mM. In certain experiments, keratinocyte growth medium (KGM) (Clonetics Corp.) was used in place of KBM. KGM consists of the same basal medium as KBM but is supplemented with a number of growth factors including epidermal growth factor, insulin, hydrocortisone, ethanolamine, phosphoethanolamine, and pituitary extract.

RA was obtained from the R. W. Johnson Pharmaceutical Research Institute (Raritan, NJ) and prepared as a 20 mg/ml solution in dimethylsulfoxide (DMSO). The stock solution was stored at −20°C in the dark until use. At the time of use, it was diluted directly in KBM and added to the cultures. RA-treated cultures (as well as controls) were kept in the dark throughout the incubation period. In cultures treated with 3 μM RA (highest concentration used), the final concentration of DMSO in the culture medium was 0.005%. This amount of DMSO had no detectable effect by itself. Retinol (Sigma Chemical Co., St. Louis, MO) was used in place of RA in certain experiments. It was prepared as a 20 mg/ml solution in DMSO and handled exactly as RA. In other experiments, 1,25 dihydroxyvitamin D_3 (vitamin D) and the detergent sodium lauryl sulfate (SLS) were used. Vitamin D was obtained from Hoffmann-La Roche (Nutley, NJ). It was prepared in DMSO and handled as RA. High-purity, electrophoresis-grade reagent (Bio Rad Laboratories, Melville, NY) was the source of SLS. It was prepared as a stock solution in KBM and kept at 4°C. At the time of use, it was warmed and diluted directly into culture medium.

Organ cultures. Organ cultures were established from healthy adult volunteers and maintained as described (41–43). Briefly, 2-mm full-thickness punch biopsies were obtained from the hip (sun-protected site) or forearm (sun-exposed site) of healthy adult volunteers (normally four biopsies per site) and were immediately placed into wells of a 24-well dish containing 0.5 ml of KBM. Foreskin tissue was obtained after circumcision and cut into 2-mm full-thickness pieces and placed into wells containing 0.5 ml of KBM. One tissue from each specimen was incubated in KBM alone containing either 0.15 or 1.4 mM extracellular Ca^{2+}, while RA (3 μM) was added to the KBM in other wells. This concentration of RA has been shown to be optimal for preservation of histological features in adult sun-protected skin (41). Furthermore, recent studies have shown that topical RA treatment protocols that improve the clinical appearance of intact skin result in a 2–3 μM concentration of RA in the viable portion of the epidermis (44). The organ cultures were then incubated at 37°C and 5% CO_2 with fresh culture medium and treatments provided at 2-d intervals. After incubation for the desired period of time (normally 12 d), the organ-cultured tissue was examined in one of several ways as indicated below.

Histological evaluation. After incubation, the organ-cultured tissue was fixed in 10% buffered formalin and embedded in paraffin. 5-μm thick sections were cut, and representative sections of each biopsy were selected for histological studies. The epidermis was examined for overall viability. Nonpyknotic cells in the basal epithelial layer were counted using a microscope with a calibrated grid in the eyepiece (at a magnification of 200) to provide an estimate of basal cell density. The height of the viable portion of the epidermis (from the basement membrane to the top of the stratum granulosum) was measured to provide an estimate of the width of the viable portion of the epidermis. Evaluation of the dermis consisted of counting numbers of nonpyknotic interstitial cell nuclei (i.e., those not associated with epithelial structures or capillaries) over each entire section. Additional histological characteristics were assessed in skin samples from the sun-exposed site. Specifically, each specimen was examined for destruction of normal connective tissue elements and for presence of elastotic fibers. The degree of sun damage was assessed on a 0 to 2 ordinal scale (0 indicating an appearance virtually indistinguishable from that of sun-protected skin and 2 indicating clear histological evidence of damage, including degradation of collagenous fibers and/or presence of elastotic fibers). Of the 36 skin samples from sun-exposed sites that were examined, 21 received a score of 2 and were considered to be sun damaged. In some of the sun-damaged skin samples, abnormal histological features could be seen extending almost to the dermo–epidermal junction. In others, a thin zone of healthy appearing connective tissue could be seen immediately beneath the dermo–epidermal junction. The width of this zone was measured.

Protein synthesis. Overall protein synthesis was assessed in organ-cultured skin as described previously (41). Briefly, 1 μCi of [^{3}H]leucine was added per well, and the cultures were incubated for an additional 72-h period. After this, the tissue and culture fluid were collected together in a phosphate-buffered saline solution containing three detergents (1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecylsulfate; all obtained from Sigma Chemical Co.) and protease inhibitors including 20 mM EDTA, 5 mM *N*-ethylmaleimide, and 2 mM phenylmethyl sulfonyl fluoride. The lysed tissue was frozen at −80°C, thawed, and clarified by centrifugation (37,000 *g* for 60 min). Samples of each extracted tissue were precipitated with 10% (final concentration) trichloroacetic acid (TCA) and washed two times in 10% TCA. After the final wash, the samples were counted directly in a β scintillation counter to obtain an estimate of total radioactivity incorporated into TCA-precipitable material as an indicator of protein synthesis.

Enzyme-linked immunosorbent assay (ELISA) for fibronectin. An ELISA was used to assess fibronectin concentrations in the culture fluids of control and RA-treated organ cultures. Organ culture medium collected from the wells at 2-d intervals was analyzed. Commercially available rabbit polyclonal serum prepared against human plasma fibronectin (Telios Pharmaceuticals, Inc., San Diego, CA) was used as the primary antibody along with alkaline phosphatase–labeled goat anti–rabbit IgG (Sigma Chemical Co.) as the secondary antibody. Human plasma fibronectin was used as a standard. Briefly, 0.1 ml of culture fluid was added to wells of a 96-well dish (Falcon Plastics, Oxnard, CA) from lots that had been prescreened for acceptibility in ELISA. Low-Ca^{2+} and high-Ca^{2+} KBM alone served as negative controls, and human plasma fibronectin diluted in the same culture media was used to generate standard curves. The ELISA was run as described in previous studies (45), and the values obtained with the culture fluids were compared directly with the values obtained from the negative control wells and standard curves.

Cellular outgrowth from organ-cultured tissue. A number of specimens were used to assess keratinocyte and fibroblast viability after organ-culture incubation. For this, tissue specimens that had been incubated in either KBM alone (0.15 or 1.4 mM Ca^{2+}) or in the same basal medium supplemented with RA were harvested and minced with scissors

and forceps. The minced tissue pieces were transferred to 25-cm^2 flasks and allowed to attach firmly to the tissue culture plastic. After allowing the tissue pieces to attach, minimal essential medium of Eagle with Earle's salts, nonessential amino acids, and 10% fetal bovine serum (MEM-FBS) was added. Cultures were provided fresh medium as needed and examined for outgrowth of fibroblasts and keratinocytes. If no outgrowth was seen by 2 wk, the flasks were discarded. Although this assay is insensitive, it is definitive in that recovery of keratinocytes and/or fibroblasts provides proof that these cells were viable at the end of the organ-culture period.

Monolayer culture assays. Keratinocytes were obtained from adult sun-protected skin and neonatal skin by the method of Liu and Karasek (46). Culture medium consisted of KGM. Fibroblasts were obtained from minced tissue (adult sun-protected, adult sun-exposed, and neonatal) and cultured in MEM-FBS as described previously (47). Both keratinocytes and fibroblasts were grown at 37°C in an atmosphere of 5% CO_2 and subcultured as required. Cells were routinely used at passages 2–4. For use in proliferation assays, the cells were plated at 5×10^4 cells/well in a 24-well culture dish. KGM was used as plating medium for keratinocytes, and MEM-FBS was used as plating medium for fibroblasts. After allowing the cells to attach and spread, cells were washed twice in KBM (either 0.15 or 1.4 mM Ca^{2+}) and incubated for an additional 2 d in this medium alone or in the same medium supplemented with RA (3 μM). At the end of the incubation period, cells were harvested and counted. An electronic particle counter was used to assess cell numbers after first verifying that the cells were in single-cell suspension. Additional studies were conducted in the exact same manner except that (growth factor–supplemented) KGM rather than KBM was used as incubation medium.

Results

Effects of RA on epidermal features of organ-cultured skin. Adult sun-protected and sun-exposed skin and neonatal foreskin were incubated for 12 d in organ culture under conditions optimized for keratinocyte growth (i.e., in KBM containing 0.15 mM Ca^{2+}) (46, 48–50) or for fibroblast growth (i.e., in KBM containing 1.4 mM Ca^{2+}) (47, 51), based on monolayer culture studies. Cultures were left as control or concomitantly treated with 3 μM RA. At the end of the incubation period, tissues were fixed for histology, stained with hematoxylin and eosin, and examined microscopically. In the absence of RA treatment, tissues from all three sites had a similar appearance. Specifically, they were completely necrotic after incubation in low-Ca^{2+} culture medium for 12 d, but remained histologically intact when incubated for the same period of time in high-Ca^{2+} KBM. Adult sun-protected skin and neonatal foreskin after incubation in low-Ca^{2+} KBM are shown in Fig. 1, *A* and *E,* respectively, and skin from the same sites after incubation in high-Ca^{2+} KBM are shown in Fig. 1, *C* and *G.* Adult sun-exposed skin (not shown) had an appearance identical to that of sun-protected skin.

Treatment of adult sun-protected or sun-exposed skin with RA under either low-Ca^{2+} or high-Ca^{2+} conditions resulted in dramatic changes in the histological appearance of the epidermis. Under low-Ca^{2+} conditions, RA prevented epidermal necrosis. The basal epithelial layer remained intact, and there were several layers of viable epithelial cells above. However, normal epithelial differentiation did not occur, and the upper layers of the epidermis separated from the viable portion beneath (Fig. 1 *B*). Under high-Ca^{2+} conditions, concomitant treatment with RA resulted in a significant expansion of the viable portion of the epidermis (Fig. 1 *D*). When neonatal foreskin was treated with RA in organ culture, different results were obtained. Specifically, neonatal foreskin incubated in low-Ca^{2+} KBM responded to RA in much the same manner as adult skin (Fig. 1, *F*). However, there was essentially no response to treatment with RA under high-Ca^{2+} conditions. Control and RA-treated skin were virtually identical in histological appearance (Fig. 1, *G* and *H*). Quantifiable information from these tissues is presented in Table I.

Although RA was used with most of the skin specimens, retinol, vitamin D, and SLS were also examined with a number of adult sun-protected specimens. To summarize findings with these agents, the same changes in epidermal structure were seen after retinol treatment as after RA treatment. However, retinol was less effective than RA. It required a higher concentration to elicit a maximal response (7.5 vs 3 μM, respectively), and the response of the tissue to an optimal concentration was less (on average) with retinol than with RA (Table I). In contrast, vitamin D and SLS were completely without effect in organ culture when examined over a wide range of concentrations (0.1–10 μM with vitamin D and 0.1–50 μM with SLS). There was no preservation of the basal epithelial cell layer under low-Ca^{2+} conditions with either agent and no expansion of the viable portion of the epidermis under high-Ca^{2+} conditions (Table I).

Effects of RA on dermal features of organ-cultured human skin. Dermal histological features were also assessed in the same specimens. With skin from all three sites, incubation in low-Ca^{2+}–containing medium resulted in extensive pyknosis in the interstitial cell population (mainly fibroblasts) and in degeneration of both connective tissue structures and capillaries. These degenerative changes were much less evident in tissues that had been incubated either in low-Ca^{2+} KBM supplemented with RA or in high-Ca^{2+} KBM without RA. The combination of high Ca^{2+} and RA provided the best response. Although these dermal features are difficult to quantify, numbers of nonpyknotic interstitial cells (Table I) indicate the differences between treatment groups. As with the epidermal features, there was no difference between adult and neonatal tissues in their response to RA under low-Ca^{2+} conditions, while only the adult tissue responded to RA under high-Ca^{2+} conditions (Table I).

In addition to these findings, which appeared to be independent of sun-exposure status or degree of sun damage, additional dermal features were evident in sun-damaged skin specimens. The dermis of these specimens was characterized by disruption of normal connective tissue structures and by the presence of dark-staining, thickened, elastotic fibers throughout the tissue sections. The appearance of one such specimen after 12 d in culture in KBM containing 1.4 mM Ca^{2+} with or without RA is shown in Fig. 2. Incubation in organ culture for this short period of time (either with or without RA) did not reverse the solar damage (i.e., dermal elastosis) that had already accumulated. However, consistent with findings from in vivo studies (32–34, 36), a zone of healthy connective tissue could be seen immediately below the dermo–epidermal junction in both specimens. The major difference between the control and RA-treated tissue was in the width of this zone. It was wider (on average) in the RA-treated sample (Fig. 2). Table II summarizes data from 21 specimens with detectable sun damage. The quantitative data presented in Table II substantiate the histological findings presented in Fig. 2; i.e., that the subepidermal zone of healthy connective tissue is wider in RA-treated specimens than in control.

Protein synthesis and extracellular matrix synthesis in RA-treated skin. In the next series of experiments, overall protein

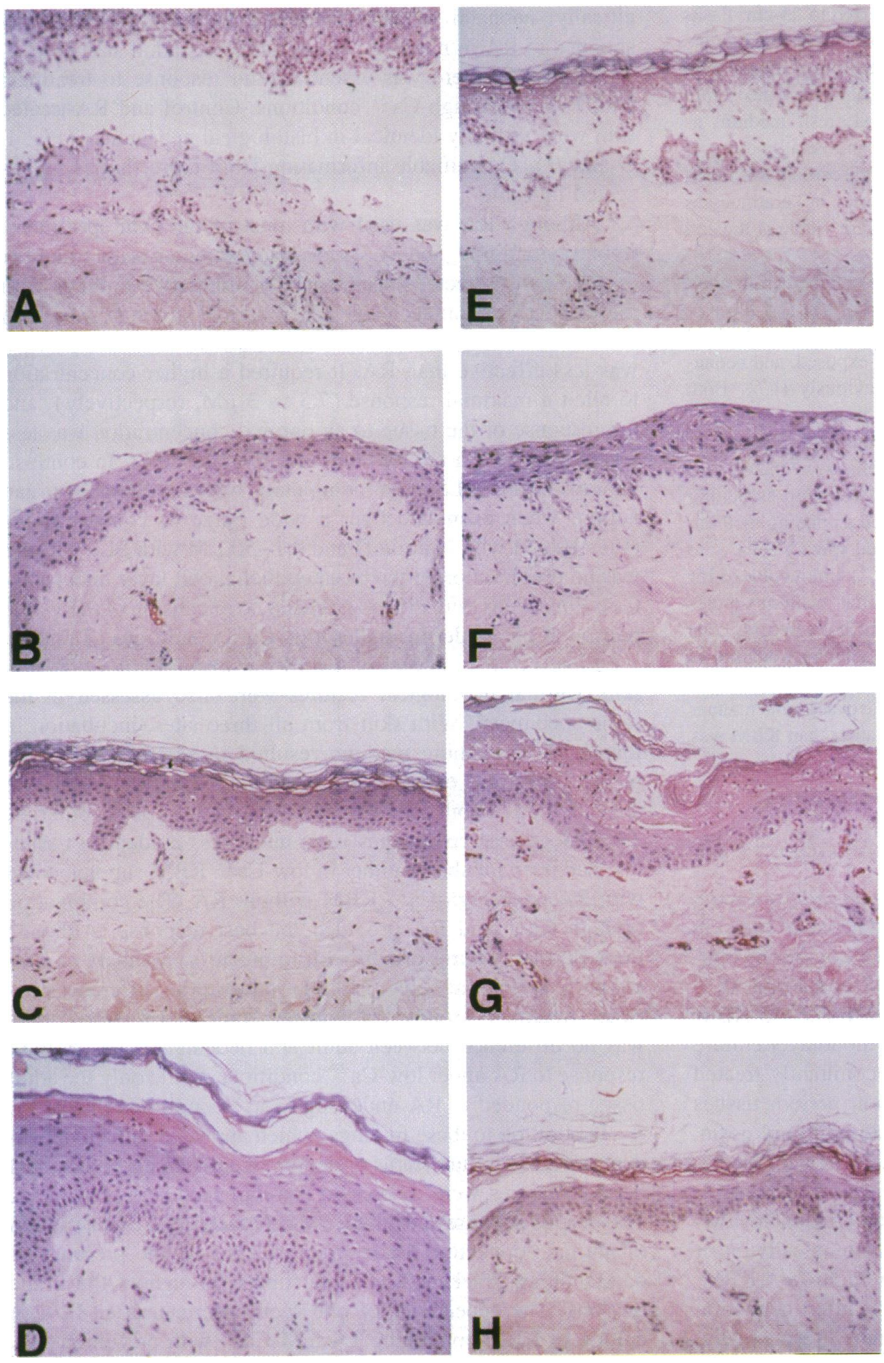

Figure 1. Epidermal features of skin after incubation in organ culture. Features of adult sun-protected skin are shown in *A–D*. Features of neonatal foreskin are shown in *E–H*. KBM alone, *A* and *E;* KBM + 3 μM RA, *B* and *F;* KBM containing 1.4 mM Ca^{2+}, *C* and *G;* KBM containing 1.4 mM Ca^{2+} + 3 μM RA, *D* and *H*. All photographs are hematoxylin and eosin stained. ×66.

synthesis and extracellular matrix (fibronectin) synthesis were examined in control and RA-treated skin organ cultures. Consistent with histological features, there was little protein synthesis and little extracellular matrix synthesis in the low-Ca^{2+} control tissues from either adult site and both were stimulated by RA (Figs. 3 and 4). Higher levels of both total protein and fibronectin synthesis were seen under high-Ca^{2+} conditions and both were further stimulated by RA. The relative increase in fibronectin synthesis was much greater than the increase in total protein synthesis (Figs. 3 and 4). As was observed histologically, neonatal tissue responded differently from adult tissue in regard to protein synthesis and extracellular matrix production. Specifically, protein synthesis was increased in the neonatal skin by RA under both conditions but the increase was much smaller than that seen with adult tissue from either site (Fig. 3). With regard to fibronectin synthesis, there was essentially no stimulation with RA under either condition (Fig. 4). It should be noted that tissue samples from adult sun-protected and sun-exposed sites were obtained from the same individuals and assayed in parallel. We, therefore, examined the strength of the linear relationship between the response to RA in individual arm and hip samples. Although there was a clear response to RA in both groups, the variability within each group prevented us from seeing a clear relationship between responsiveness in individual arm and hip samples (Figs. 3 and 4; *bottom panels*). Neonatal skin samples (in addition to having

Table I. Quantification of Histological Features of Human Skin in Organ Culture

Treatment group	Epithelial height	Basal epithelial cell density (cell per 2 mm of basement membrane; ×200)	Interstitial cell density (cells/ tissue section)
	μm		
Adult sun-protected (n = 36)			
Low-Ca^{2+} control	< 5	1±1	15±2
Low-Ca^{2+} RA-treated	21±2*	35±2*	25±16‡
High-Ca^{2+} control	45±1	46±3	24±2
High-Ca^{2+} RA-treated	72±2‡	53±3	32±3
Adult sun-exposed (n = 36)			
Low-Ca^{2+} control	< 5	1±1	11±1
Low-Ca^{2+} RA-treated	19±1*	34±1*	26±2‡
High-Ca^{2+} control	44±2	46±3	20±2
High-Ca^{2+} RA-treated	64±3‡	49±1	32±2‡
Neonatal foreskin (n = 6)			
Low-Ca^{2+} control	< 5	< 1	69±9
Low-Ca^{2+} RA-treated	23±3*	38±3*	91±16
High-Ca^{2+} control	28±3	41±3	94±16
High-Ca^{2+} RA-treated	18±1	42±1	88±12
Adult sun-protected (n = 8)			
Low-Ca^{2+} control	< 5	2±1	Not done
Low-Ca^{2+} retinol-treated	15±5*	30±5*	Not done
Low-Ca^{2+} vitamin D_3–treated	< 5	< 1	Not done
Low-Ca^{2+} SLS-treated	< 5	< 1	Not done
High-Ca^{2+} control	55±10	57±5	Not done
High-Ca^{2+} retinol-treated	65±5	50±6	Not done
High-Ca^{2+} vitamin D_3–treated	50±3	48±7*	Not done
High-Ca^{2+} SLS-treated	35±8	38±5	Not done

Epidermal height, basal epithelial cell density, and interstitial cell density were quantified as indicated in Methods. RA, 3 μM; retinol, 7.5 μM; vitamin D, 3 μM; SLS, 10 μM. Values shown represent means and standard errors of means. Statistical significance levels were determined by a series of t tests, comparing the treatment groups with their respective controls. ‡ Statistical significance at the $P < 0.05$ level; * Statistical significance at the $P < 0.01$ level.

no genetic relationship to the adult samples) were assayed independently. Therefore, it is difficult to draw quantitative comparisons between the adult and neonatal tissues. The more important comparison is the relationship between control and RA-treated samples from the same tissues.

Keratinocyte and fibroblast recovery from organ-cultured skin. Although these findings strongly indicate that human skin can be maintained in a histologically intact and biochemically active condition for several days in organ culture, it is difficult to directly assess cell viability in these cultures. A keratinocyte- and fibroblast-recovery assay was used, therefore, as a way to conclusively demonstrate cell viability after organ culture incubation under various conditions. The results of this assay are shown in Table III. It can be seen that when the organ-cultured adult skin from either site was incubated in low-Ca^{2+} KBM for 12 d there was a complete loss of keratinocyte viability as indicated by the inability to subsequently recover cells from minced tissue pieces. Likewise, fibroblasts were recovered from only a small percentage of these same tissues. Ability to recover both cell types was improved when RA was present throughout the organ culture incubation period. It can also be seen from Table III that keratinocyte and fibroblast viability was greater in high-Ca^{2+} KBM than in low-Ca^{2+} KBM. However, even in high-Ca^{2+} culture medium, RA promoted cell survival, and the highest recovery of both cell types was from tissues that had been incubated in high-Ca^{2+} KBM supplemented with RA.

Table III also shows results from studies with neonatal foreskin tissue. In contrast to what was observed with adult tissue, both keratinocytes and fibroblasts survived incubation in organ culture for 12 d in either low-Ca^{2+} or high-Ca^{2+} KBM. As a result, we were able to isolate both cell types from virtually all tissues in the presence or absence of RA. Additional studies were conducted in which neonatal skin was maintained in organ culture until day 20 and then examined for the presence of viable keratinocytes and fibroblasts. After 20 d, recovery of both cell types from tissues incubated in low-Ca^{2+} KBM alone was reduced as compared with day 12 recovery rates (Table III). Under conditions of low-Ca^{2+} culture medium, a protective effect of RA on both cell types was seen (Table III). Better recovery of both cell types occurred from cultures that had been incubated in high-Ca^{2+} KBM. The addition of RA to high-Ca^{2+} medium did not increase the recovery percentage further (Table III).

Although overall recovery percentage was the main criterion used in this study, we also noted a correlation between the rapidity with which cells could be detected in the outgrowth assay and the overall recovery percentage. Likewise, the total number of viable cells that could be seen microscopically in the culture flasks at any given time point during the 2-wk observation period appeared to correlate with overall recovery percentage. These parameters were difficult to quantify, however, because of culture to culture variability and the presence of both keratinocytes and fibroblasts in many of the same culture flasks.

Proliferation of keratinocytes and fibroblasts in monolayer culture. In a final set of experiments, keratinocytes and fibroblasts were isolated from adult and neonatal skin and established in monolayer culture. These cells were examined for ability to survive and proliferate in low-Ca^{2+} and high-Ca^{2+} KBM in the presence or absence of RA (Fig. 5). Neonatal keratinocytes underwent a proliferative response in either low-Ca^{2+} or high-Ca^{2+} culture medium while there was no net growth with the adult keratinocytes under either condition (Fig. 5, *bottom*). When RA was included in the culture medium, both neonatal and adult cells proliferated. The neonatal keratinocytes responded much more vigorously than the adult cells. In additional experiments, neonatal keratinocytes and adult keratinocytes (sun-protected site only) were examined for responsiveness to RA in growth factor–supplemented medium (KGM). Significant proliferation of both adult and neonatal cells occurred in the growth factor–supplemented medium, but, in contrast to the results obtained in basal medium, there was no response to RA in the already rapidly proliferating cells (not shown).

The fibroblast response to RA (Fig. 5, *top*) was significantly different from that of keratinocytes. When low-Ca^{2+} KBM was

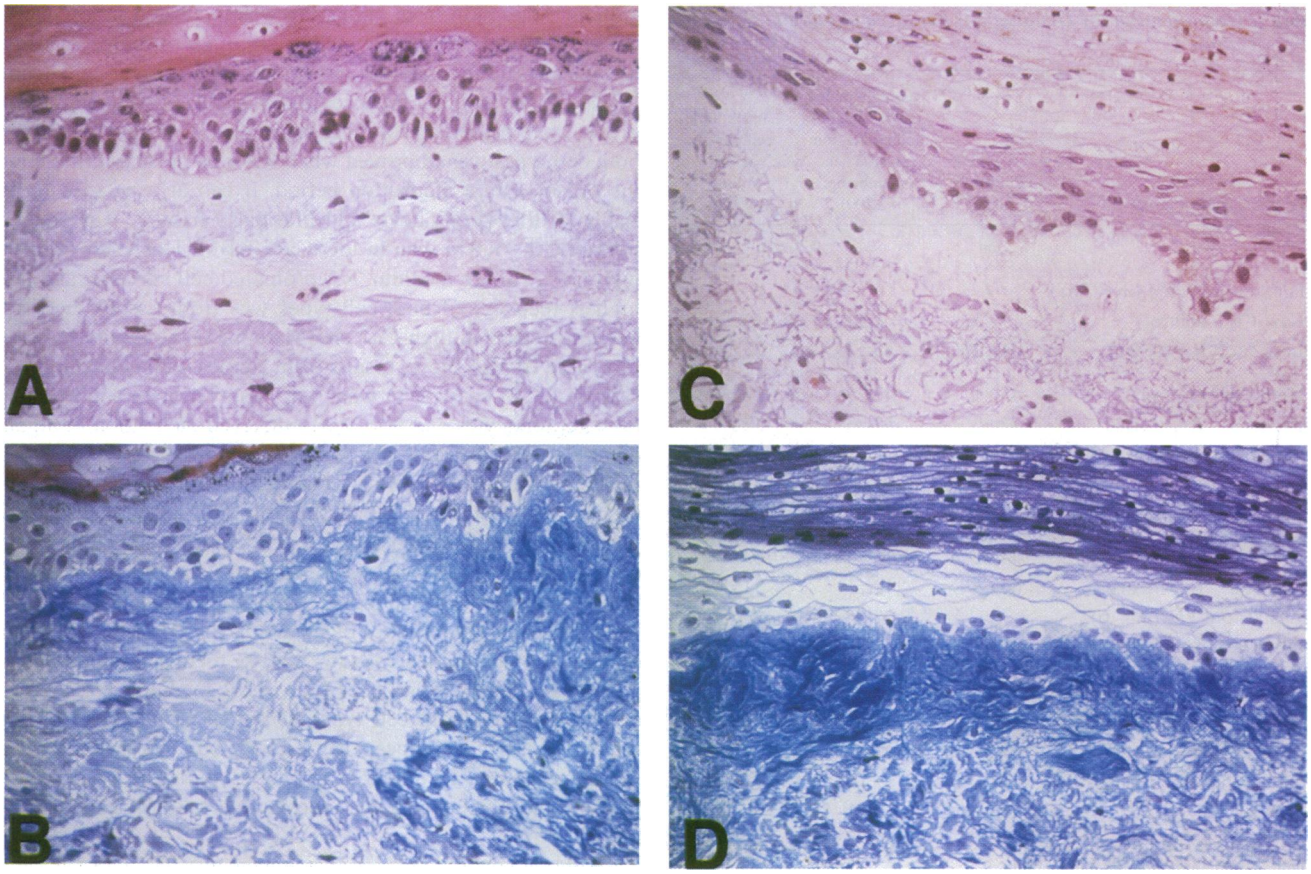

Figure 2. Dermal features of sun-exposed skin after incubation in organ culture. Features of adult sun-exposed (sun-damaged) skin after incubation for 12 d under control conditions (i.e., in high-Ca^{2+} KBM without RA) are shown in *A* and *B,* and after incubation in high-Ca^{2+} medium containing 3 μM RA are shown in *C* and *D.* Hematoxylin and eosin, *A* and *C;* Masson's trichrome stain, *B* and *D.* ×120.

used as culture medium, not only did the adult fibroblasts fail to proliferate, but a high percentage of these cells died. Although adult dermal fibroblasts did not survive incubation in low-Ca^{2+} KBM alone, addition of RA (3 μM) or supplementation with Ca^{2+} (1.4 mM, final concentration) prevented fibroblast death. This was seen equally with fibroblasts isolated from either sun-protected or sun-exposed tissue. Although incubation in high-Ca^{2+} KBM or in low-Ca^{2+} KBM supplemented with RA prevented cell death, neither treatment alone provided an optimal environment for proliferation. Significant growth was only observed in the presence of combined treatment with high Ca^{2+} and RA. Neonatal fibroblasts were also examined for survival and growth under the same culture conditions. Similar to what was observed with adult cells, the neonatal fibroblasts failed to proliferate in low-Ca^{2+} KBM but responded well to RA in this culture medium (Fig. 5). In contrast to the adult cells, however, neonatal fibroblasts proliferated maximally in high-Ca^{2+} culture medium without RA supplementation and RA did not enhance growth further. Indeed, the addition of RA reduced cell growth significantly (Fig. 5). Lastly, neonatal fibroblasts and adult fibroblasts from both sun-protected and sun-exposed sites were examined for responsiveness to RA in growth factor–supplemented medium (KGM). The results with all three populations were very similar to those shown in Fig. 5. That is, all three populations responded to RA in low-Ca^{2+} medium but only the adult cells responded in high-Ca^{2+} medium (not shown).

Table II. Measurement of the Subepithelial Zone of Repair in Control and RA-treated Sun-damaged Skin

Treatment group	Subepithelial zone width
	μm
Adult sun-exposed (n = 21)	
Low-Ca^{2+} control	3.6±3.8
Low-Ca^{2+} RA-treated	10.0±5.8*
High-Ca^{2+} control	5.8±2.8
High Ca^{2+} RA-treated	12.0±4.8*

Width of the subepithelial zone of repair was quantified as indicated in Methods. Values shown represent means and standard deviations. Statistical significance levels were determined using the t test, comparing the treatment groups with their respective controls. * Statistical significance at the $P < 0.05$ level.

Discussion

Topical RA treatment has been shown to improve the clinical appearance of sun-damaged skin (34–36), but how this occurs is poorly understood. Previous studies, based largely on murine models, have suggested that RA works by reversing events

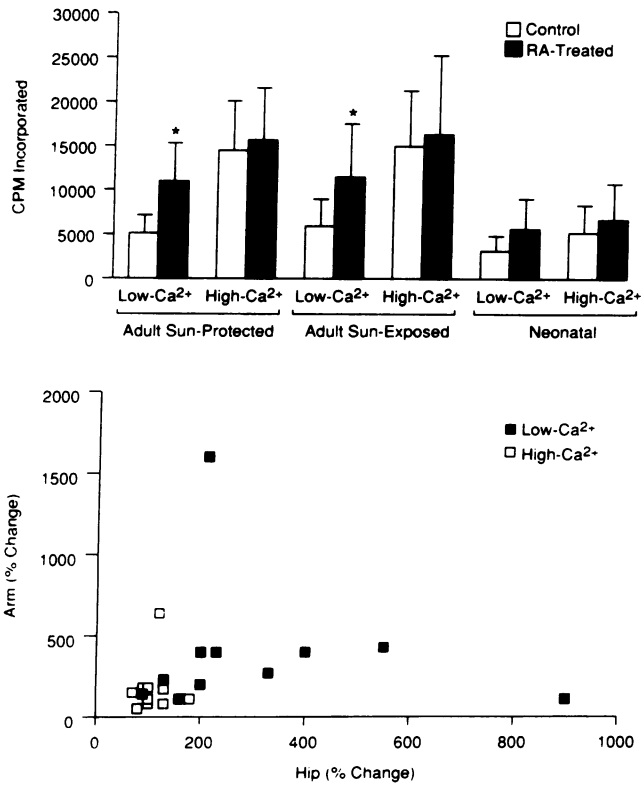

Figure 3. Protein synthesis by organ-cultured adult sun-protected, adult sun-exposed, and neonatal skin. (*Top*) Values shown represent total counts per minute of [^{3}H]leucine incorporated per tissue±standard deviations. Values are based on matched specimens from 11 different volunteers for adult sun-protected and adult sun-exposed skin and from specimens obtained from 9 different foreskins. Statistical significance levels were assessed using a series of *t* tests, comparing each treatment group with its respective control. *Statistical significance at the $P < 0.05$ level. (*Bottom*) Scattergram showing relationship between response to RA in tissue from arm and hip of individual volunteers. The strength of the linear relationship was assessed using Pearson's correlation analysis. The linear relationship was not significant at the $P < 0.05$ level.

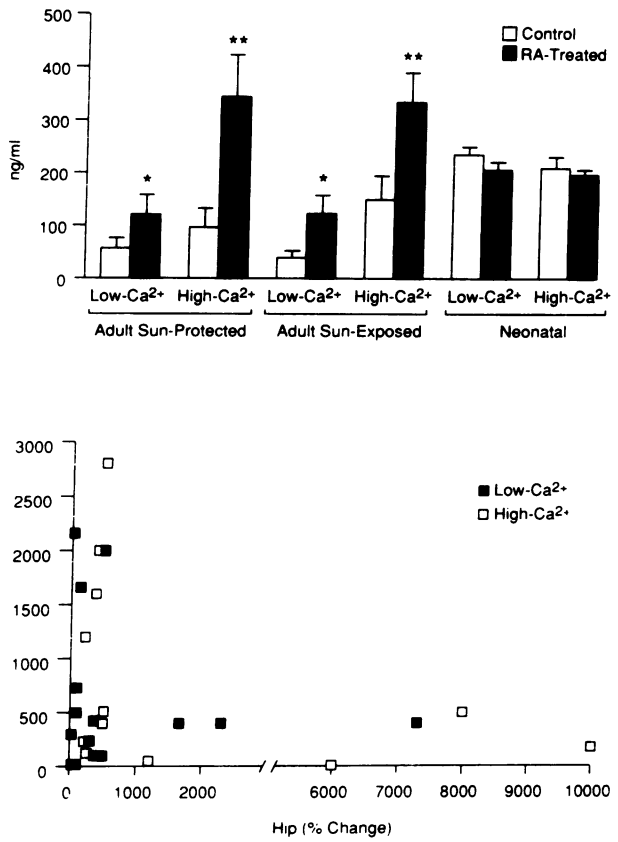

Figure 4. Measurement of immunoreactive fibronectin in organ culture fluid by ELISA. (*Top*) Values shown represent nanograms of immunoreactive fibronectin per 100 μl of culture fluid±standard errors. Values are based on matched specimens from 15 different volunteers for adult sun-protected and adult sun-exposed skin and from specimens obtained from 7 different foreskins. Statistical significance levels were assessed using a series of *t* tests, comparing each treatment group with its respective control. *Statistical significance at the $P < 0.05$ level; **Statistical significance at the $P < 0.01$ level. (*Bottom*) Scattergram showing relationship between response to RA in tissue from arm and hip of individual volunteers. The strength of the linear relationship was assessed using Pearson's correlation analysis. The linear relationship was not significant at the $P < 0.05$ level.

"triggered" by exposure to ultraviolet light (36–39). Specifically, the histological changes seen in the dermis of sun-damaged skin reflect degradation of dermal collagen. This could result either from reduced synthesis of new collagen or from increased breakdown of existing collagen. Since RA has the capacity to both stimulate new collagen synthesis (51–54) and decrease expression of collagen-degrading enzymes (55, 56), one or both of these effects could alter the balance between collagen production and breakdown and could result in dermal repair. Alternatively, the recent observation of Kligman et al. (21) that RA improves clinical and histological appearance of sun-protected as well as sun-exposed skin in elderly people suggests that RA may not act specifically to reverse events caused by excessive sun exposure but, rather, may have a more general effect on the physiology of skin.

The studies described here provide insight into this issue. Based on a variety of experimental approaches using organ-cultured human skin as well as keratinocytes and fibroblasts in monolayer culture, we observed identical responses to RA in adult skin taken from either a sun-exposed or sun-protected site. In contrast, clear differences were evident when adult skin from either site was compared with neonatal foreskin. Specifically, neonatal foreskin responded to RA treatment under low-Ca^{2+} conditions in a similar fashion to adult tissue, but under conditions of high extracellular Ca^{2+} was virtually nonresponsive. Adult skin was responsive under both low-Ca^{2+} conditions and in the presence of a physiological level of extracellular Ca^{2+}. The implication of this is that RA-induced repair of sun-damaged skin (34–36) may reflect the capacity of retinoids to modulate the behavior of fibroblasts and keratinocytes in a manner that is age related but independent of their sun-exposure status. Alternatively, the possibility must be considered that foreskin differs from adult skin as a result of site-specific rather than age-specific factors. We don't feel this is likely, however, for as pointed out by Plisko and Gilchrest (3) adult cells show decreased responsiveness to a number of different growth factors that do not have selective effects for genital tissue. Regardless of underlying mechanism, our data do not address the issue of intrinsic aging per se, in any event. Nonetheless, they do support the suggestion by Kligman et al. (21) that retinoids

Table III. Effects of RA on Keratinocyte and Fibroblast Recovery from Organ-cultured Skin

Treatment group	Recovery (%) Fibroblasts	Keratinocytes
Adult, sun-protected (day 12)		
Low-Ca^{2+} control	3/20 (15)	0/20 (0)
Low-Ca^{2+} RA-treated	15/20 (75)*	8/20 (40)*
High-Ca^{2+} control	12/20 (60)	7/20 (35)
High-Ca^{2+} RA-treated	20/20 (100)*	19/20 (95)*
Adult, sun-exposed (day 12)		
Low-Ca^{2+} control	2/7 (29)	0/7 (0)
Low-Ca^{2+} RA-treated	7/7 (100)*	4/7 (57)*
High-Ca^{2+} control	6/7 (85)	2/7 (29)
High-Ca^{2+} RA-treated	7/7 (100)	6/7 (85)*
Neonatal foreskin (day 12)		
Low-Ca^{2+} control	7/8 (88)	5/8 (63)
Low-Ca^{2+} RA-treated	5/7 (63)	7/7 (100)
High-Ca^{2+} control	6/7 (85)	7/7 (100)
High-Ca^{2+} RA-treated	5/7 (85)	7/7 (100)
Neonatal foreskin (day 20)		
Low-Ca^{2+} control	13/17 (76)	4/17 (24)
Low-Ca^{2+} RA-treated	14/15 (93)	7/15 (47)*
High-Ca^{2+} control	13/16 (81)	10/16 (63)
High-Ca^{2+} RA-treated	17/22 (77)	13/22 (60)

Cell recovery from organ-cultured tissue was assessed as described in Methods. Values shown represent the number of tissue pieces from which cells were isolated divided by the total number of tissue pieces examined. Statistical significance was determined using the Scheffe method. * Statistical difference between the treatment groups and their respective control groups at the $P < 0.05$ level.

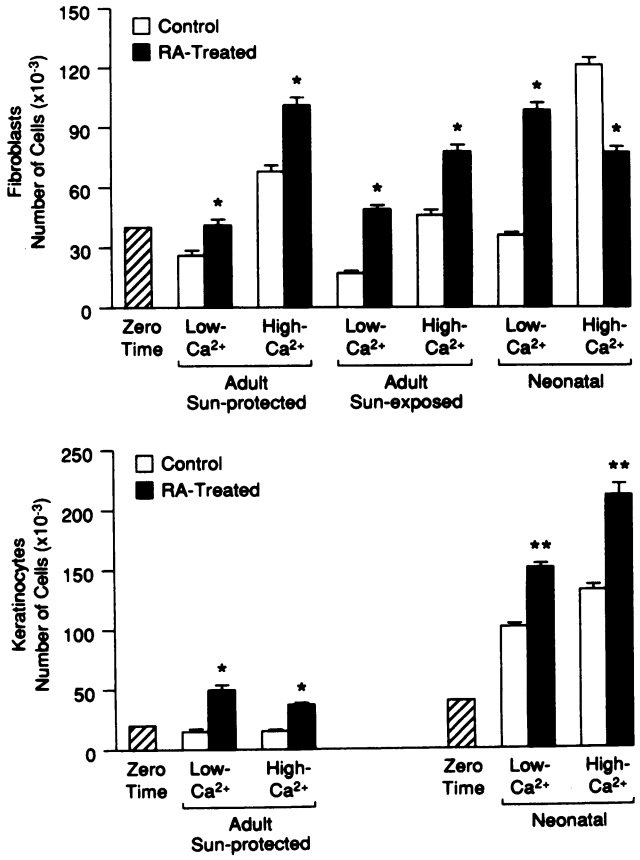

Figure 5. Keratinocyte and fibroblast proliferation in monolayer culture. Values shown represent mean number of cells present in the wells on day 0 and after 48 h of incubation. 48-h values are means±standard deviations based on triplicate samples with a single isolate for each group. Total number of isolates examined with similar results include 7 for adult sun-protected keratinocytes, 12 for neonatal keratinocytes, 14 for adult sun-protected fibroblasts, 10 for adult sun-exposed fibroblasts, and 18 for neonatal fibroblasts. *Statistical significance at the $P < 0.05$ level; **Statistical significance at the $P < 0.01$ level.

may be useful for counteracting the effects of intrinsic aging as well as sun exposure.

How RA acts at the cellular level is not fully understood. One possibility is that both the epidermis and dermis are targets and that RA independently stimulates events in each population to bring about clinical improvement. Consistent with this (and consistent with past observations [51, 57–60]), both adult fibroblasts and keratinocytes responded to RA with increased growth in monolayer culture. Since it is well known from previous studies that both populations have diminished responses to a number of growth factors as a function of age (1–12), the capacity of RA to act as a growth promoter for both cell types may underlie its ability to bring about repair of aged skin in vivo. This may be especially relevant for the epidermis since both neonatal and adult keratinocytes responded to RA much better under growth factor–deficient conditions than in growth factor–supplemented medium (reference 57 and this report) and since production of endogenous growth factors (as well as growth factor responsiveness) is lower in adult keratinocytes than in neonatal cells (11). We realize, of course, that it is difficult to extrapolate from in vitro models directly to what occurs in intact skin. However, the organ culture model is likely to mimic the in vivo situation more closely than either keratinocytes or fibroblasts in monolayer culture.

Another possibility is that, although both keratinocytes and fibroblasts are retinoid responsive, it is the dermal fibroblast that is the critical cell in maintaining the overall structure and function of skin in organ culture and that the effects of RA are mediated through its effects on this cell. The degeneration of organ-cultured skin in low-Ca^{2+} culture medium and the ability of RA to preserve epidermal and dermal structure and function in organ culture under low-Ca^{2+} conditions are consistent with the requirements for extracellular Ca^{2+} in maintaining fibroblast function in monolayer culture (13–16, 51, 61, 62) and with the ability of RA to compensate for inadequate extracellular Ca^{2+} (63). Likewise, the ability of RA to improve structural and functional features of adult skin (but not neonatal skin) in culture medium containing a physiological level of extracellular Ca^{2+} is consistent with monolayer observations (13–16) showing that adult fibroblasts are less growth responsive than neonatal fibroblasts to extracellular Ca^{2+}. In contrast, keratinocytes proliferate under both low-Ca^{2+} and high-Ca^{2+} conditions in monolayer culture (48–50), and retinoid stimulation of these cells is not Ca^{2+} concentration dependent (57). Thus, there is a strong correlation between conditions that preserve fibroblast function in monolayer culture and conditions that preserve organ culture structure/function, while conditions that favor kera-

tinocyte growth in monolayer culture and conditions that preserve structure and function in organ culture are not as closely correlated. This suggestion that retinoids preserve structure and function in organ-cultured epidermis primarily through an effect on dermal fibroblasts is consistent with information from developmental models. Developmental studies have shown that retinoids influence parenchymal organ morphogenesis primarily through effects on mesenchymal cells (64–69).

How fibroblasts preserve structure and function in organ-cultured skin is not fully understood. In addition to production of extracellular matrix components that constitute the dermal connective tissue (Fig. 4 and references 43, 47, and 51), dermal fibroblasts are also a source of growth factors for both keratinocytes and fibroblasts (70–72). Thus, RA could act in some manner to prevent fibroblast death and to activate these cells, with the activated fibroblasts then being responsible for preservation of both dermis and epidermis.

Regardless of how RA acts to preserve structure/function in organ-cultured skin, it appears that effects observed after RA treatment are reflective of retinoid action. Similar histological changes were seen with retinol as with RA. In contrast, vitamin D and SLS did not duplicate these effects when used over a wide range of concentrations.

Acknowledgment

This study was supported in part by a grant from the R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ.

References

1. Ohno, T. 1979. Strict relationship between dialyzed serum concentration and cellular lifespan in vitro. *Mech. Ageing Dev.* 11:179–183.

2. Harley, C. B., S. Goldstein, B. I. Posner, and H. Guyda. 1981. Decreased sensitivity of old and progeric human fibroblasts to a preparation of factors with insulinlike activity. *J. Clin. Invest.* 68:988–994.

3. Plisko, A., and B. A. Gilchrest. 1983. Growth factor responsiveness of cultured human fibroblasts declines with age. *J. Gerontol.* 38:513–518.

4. Kaji, K., and M. Matsuo. 1983. Responsiveness of human lung diploid fibroblast ageing in vitro to epidermal growth factor: saturation density and lifespan. *Mech. Ageing Dev.* 22:129–133.

5. Phillips, P. D., K. Kaji, and V. J. Cristofalo. 1984. Progressive loss of the proliferative response of senescing WI-38 cells to platelet-derived growth factor, epidermal growth factor, insulin, transferrin, and dexamethasone. *J. Gerontol.* 39:11–17.

6. Tamm, I., T. Kikuchi, E. Wang, and L. M. Pfeffer. 1984. Growth rate of control and β-interferon-treated human fibroblast populations over the course of their in vitro life span. *Cancer Res.* 44:2291–2296.

7. Tsuji, Y., T. Ide, S. Ishibashi, and K. Nishikawa. 1984. Loss of responsiveness in senescent human TIG-1 cells to the DNA synthesis-inducing effect of various growth factors. *Mech. Ageing Dev.* 27:219–232.

8. Colige, A., B. Nusgens, and C. M. Lapiere. 1990. Response to epidermal growth factor of skin fibroblasts from donors of varying age is modulated by the extracellular matrix. *J. Cell. Physiol.* 145:450–457.

9. Gilchrest, B. A. 1983. In vitro assessment of keratinocyte aging. *J. Invest. Dermatol.* 81(Suppl. 1):184s–189s.

10. Stanulis-Praeger, B. M., and B. A. Gilchrest. 1986. Growth factor responsiveness declines during adulthood for human skin-derived cells. *Mech. Ageing Dev.* 35:185–198.

11. Sauder, D. N., B. M. Stanulis-Praeger, and B. A. Gilchrest. 1988. Autocrine growth stimulation of human keratinocytes by epidermal cell-derived thymocyte-activating factor: implications for skin ageing. *Arch. Dermatol. Res.* 280:71–76.

12. Stanulis-Praeger, B. M., M. Yaar, G. Redziniak, A. Meybeck, and B. A. Gilchrest. 1988. An extract of bovine thymus stimulates human keratinocyte growth in vitro. *J. Invest. Dermatol.* 90:749–754.

13. Praeger, F. C., and V. J. Cristofalo. 1986. Modulation of WI-38 cell proliferation by elevated levels of CaCl2. *J. Cell. Physiol.* 129:27–35.

14. Praeger, F. C., and B. A. Gilchrest. 1986. Influence of increased extracellular calcium concentration and donor age on density-dependent growth inhibition of human fibroblasts. *Proc. Soc. Exp. Biol. Med.* 182:315–321.

15. Praeger, F. C., and V. J. Cristofalo. 1980. Effects of elevated levels of extracellular Ca^{++} on young and old WI-38 cells. *In Vitro (Rockville).* 16:239–249.

16. Praeger, F. C., and V. J. Cristofalo. 1984. Age-related loss of response to elevated [$CaCl_2$] by WI-38 cells. *The Gerontologist.* 24:226–227.

17. Lavker, R. M. 1979. Structural alterations in exposed and unexposed aged skin. *J. Invest. Dermatol.* 73:59–66.

18. Pieraggi, M. T., M. Julian, and H. Bouissou. 1984. Fibroblast changes in cutaneous ageing. *Virchows Arch. A. Pathol. Anat. Histopathol.* 402:275–287.

19. Kligman, A. M., and R. M. Lavker. 1988. Cutaneous aging. The difference between intrinsic aging and photoaging. *Journal of Cutaneous Aging Cosmetic Dermatology.* 1:5–12.

20. Grove, G. L. 1986. Physiologic changes in older skin. *Dermatologic Clinics.* 4:425–432.

21. Kligman, A. M., D. Dogadkina, and R. M. Lavker. 1993. Effects of topical tretinoin on non-sun-exposed skin of the elderly. *J. Am. Acad. Dermatol.* 29:25–33.

22. Gilchrest, B. A. 1979. Relationship between actinic damage and chronological aging in keratinocyte cultures of human skin. *J. Invest. Dermatol.* 72:219–223.

23. Gilchrest, B. A. 1980. Prior chronic sun exposure decreases the lifespan of human skin fibroblasts in vitro. *J. Gerontol.* 35:537–541.

24. Smith, J. G., Jr., E. A. Davidson, W. M. Sams, Jr., and R. D. Clark. 1962. Alterations in human dermal connective tissue with age and chronic sun damage. *J. Invest. Dermatol.* 39:347–353.

25. Constantine, V. S., and M. W. Hartley. 1966. Collagen and elastic fibers in normal dermis and severe actinic (senile) elastosis. A light and electron microscopic study. *Ala. J. Med. Sci.* 3:329–335.

26. Berger, H., D. Tsambaos, and G. Mahrle. 1980. Experimental elastosis induced by chronic ultraviolet exposure. Light-and-electron microscopic study. *Arch. Dermatol. Res.* 269:39–47.

27. Cole, C. A., R. E. Davies, P. D. Forbes, and L. C. D'Aloissio. 1983. Comparison of action spectra for acute cutaneous responses to ultraviolet radiation: man and albino hairless mouse. *Photochem. Photobiol.* 37:623–630.

28. Poulsen, J. T., B. Staberg, H. C. Wulf, and H. Brodthagen. 1984. Dermal elastosis in hairless mice after UV-B and UV-A applied simultaneously, separately or sequentially. *Br. J. Dermatol.* 110:531–536.

29. Plastow, S. R., C. R. Lovell, and A. R. Young. 1987. UVB-induced collagen changes in the skin of the hairless albino mouse. *J. Invest. Dermatol.* 88:145–150.

30. Bissett, D. L., D. P. Hannon, and T. V. Orr. 1987. An animal model of solar-aged skin: histological, physical, and visible changes in UV-irradiated hairless mouse skin. *Photochem. Photobiol.* 46:367–373.

31. Schwartz, E. 1988. Connective tissue alterations in the skin of ultraviolet irradiated hairless mice. *J. Invest. Dermatol.* 91:158–162.

32. Kligman, L. H., F. J. Akin, and A. M. Kligman. 1982. Prevention of ultraviolet damage to the dermis of hairless mice by sunscreens. *J. Invest. Dermatol.* 78:181–189.

33. Kligman, L. H., F. J. Akin, and A. M. Kligman. 1983. Sun-screens promote repair of ultraviolet radiation-induced dermal injury. *J. Invest. Dermatol.* 81:98–102.

34. Kligman, A. M., G. L. Grove, R. Hirose, and J. J. Leyden. 1986. Topical tretinoin for photoaged skin. *J. Am. Acad. Dermatol.* 15:836–859.

35. Weiss, J. S., C. N. Ellis, J. T. Headington, T. Tincoff, T. A. Hamilton, and J. J. Voorhees. 1988. Topical tretinoin improves photoaged skin: a double-blind, vehicle-controlled study. *JAMA (J. Am. Med. Assoc.).* 259:527–532.

36. Kligman, L. H. 1986. Effects of all-trans retinoic acid on the dermis of hairless mice. *J. Am. Acad. Dermatol.* 15:779–785.

37. Kligman, L. H., C. H. Duou, and A. M. Kligman. 1984. Topical retinoic acid enhances the repair of ultraviolet damaged dermal connective tissue. *Connect. Tissue Res.* 12:139–150.

38. Feldman, D., G. F. Bryce, and S. S. Shapiro. 1991. Ultrastructural effects of UVB radiation and subsequent retinoic treatment on the skin of hairless mice. *J. Cutaneous Pathol.* 18:46–55.

39. Chen, S., I. Kiss, and K. M. Tramposch. 1992. The priming effect of ultraviolet B radiation on retinoic acid-stimulated collagen synthesis in the mouse photo-damage model. *Photoderm. Photoimmunol. & Photomed.* 9:104–108.

40. Moon, R. C., and L. M. Itri. 1984. Retinoids and cancer. *In* The Retinoids. Volume 2. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Academic Press Inc., New York. 327–371.

41. Varani, J., S. E. G. Fligiel, L. Schuger, P. Perone, D. R. Inman, C. E. M. Griffiths, and J. J. Voorhees. 1993. Effects of all-trans retinoic acid and Ca^{++} on human skin in organ culture. *Am. J. Pathol.* 142:189–198.

42. Varani, J., S. E. G. Fligiel, P. Perone, D. R. Inman, and J. J. Voorhees. 1993. Effects of sodium lauryl sulfate on human skin in organ culture: comparison with all-trans retinoic acid and epidermal growth factor. *Dermatology.* 187:19–25.

43. Varani, J., B. K. Larson, P. Perone, D. R. Inman, S. E. G. Fligiel, and J. J. Voorhees. 1993. All-trans retinoic acid and extracellular Ca^{2+} differentially influence extracellular matrix production by human skin in organ culture. *Am. J. Pathol.* 142:1813–1822.

44. Duell, E. A., A. Åström, C. E. M. Griffiths, P. Chambon, and J. J. Voorhees. 1992. Human skin levels of retinoic acid and cytochrome P-450–derived 4-hydroxyretinoic acid after topical application of retinoic acid in vivo compared to concentrations required to stimulate retinoic acid receptor–mediated transcription in vitro. *J. Clin. Invest.* 90:1269–1274.

45. Varani, J., E. J. Lovett, J. P. McCoy, S. Shibata, D. E. Maddox, I. J. Goldstein, and M. Wicha. 1983. Differential expression of a laminin-like substance by high and low metastatic tumor cells. *Am. J. Pathol.* 111:27–34.

46. Liu, S. C., and M. Karasek. 1978. Isolation and growth of adult human epidermal keratinocytes in cell culture. *J. Invest. Dermatol.* 71:157–162.

47. Phan, S. H., J. Varani, and D. Smith. 1985. Rat lung fibroblast collagen metabolism in bleomycin-induced pulmonary fibrosis. *J. Clin. Invest.* 76:241–247.

48. Hennings, H., D. Michael, C. Cheng, P. Steinert, K. Holbrook, and S. H. Yuspa. 1980. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell.* 9:245–254.

49. Boyce, S. T., and R. G. Ham. 1983. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J. Invest. Dermatol.* 81:33–40.

50. Milstone, L. M. 1987. Calcium modulates growth of human keratinocytes in confluent cultures. *Epithelia.* 1:129–140.

51. Varani, J., R. S. Mitra, D. Gibbs, S. H. Phan, V. M. Dixit, B. J. Nickoloff, and J. J. Voorhees. 1990. All-trans retinoic acid stimulates growth and extracellular matrix production in growth-inhibited cultured human skin fibroblasts. *J. Invest. Dermatol.* 94:717–723.

52. Angeles, A. M., V.-M. Kahari, Y. Q. Chen, F. Ramirez, and J. Uitto. 1990. Enhanced collagen gene expression in fibroblast cultures treated with all-trans retinoic acid: evidence for up-regulation of the a2(1) promoter activity. *J. Invest. Dermatol.* 94:504*a*. (Abstr.)

53. Griffiths, C. E. M., A. Russman, G. Majmudar, R. S. Singer, T. A. Hamilton, and J. J. Voorhees. 1993. Restoration of collagen formation in photodamaged human skin by tretinoin (retinoic acid). *N. Engl. J. Med.* 329:530–535.

54. Schwartz, E., F. A. Cruikshank, J. A. Mezick, and H. Kligman. 1991. Topical all-trans retinoic acid stimulates collagen synthesis in vivo. *J. Invest. Dermatol.* 96:976–978.

55. Bauer, E. A., J. L. Seltzer, and A. Z. Elsen. 1982. Inhibition of collagen degradative enzymes by retinoic acid in vitro. *J. Am. Acad. Dermatol.* 6:603–607.

56. Clark, S. D., D. K. Kobayashi, and H. G. Welgus. 1987. Regulation of the expression of tissue inhibitor of metalloproteinases and collagenase by retinoids and glucocorticoids in human fibroblasts. *J. Clin. Invest.* 80:1280–1288.

57. Varani, J., B. J. Nickoloff, V. M. Dixit, R. S. Mitra, and J. J. Voorhees. 1989. All-trans retinoic acid stimulates growth of adult human keratinocytes cultured in growth factor-deficient medium, inhibits production of thrombospondin and fibronectin and reduces adhesion. *J. Invest. Dermatol.* 93:449–454.

58. Harper, R. A., and R. Savage, Jr. 1980. Vitamin A potentiates the mitogenic effect of epidermal growth factor on cultures of normal adult human skin fibroblasts. *Endocrinology.* 107:2113–2114.

59. Harper, R. A., and T. Burgoon. 1982. Differential effects of retinoic acid on the growth of normal fibroblast-like cells in vitro from human, swine and rabbit skin. *Cell Biol. Int. Rep.* 6:163–170.

60. Stanulis-Praeger, B. M., and B. A. Gilchrest. 1989. Effect of donor age and prior sun exposure on growth inhibition of cultured human dermal fibroblasts by all trans-retinoic acid. *J. Cell. Physiol.* 139:116–124.

61. Boynton, A. L., J. F. Whitfield, R. J. Isaacs, and H. J. Morton. 1974. Control of 3T3 cell proliferation by calcium. *In Vitro (Rockville).* 10:12–17.

62. Boynton, A. L., J. F. Whitfield, R. J. Isaacs, and R. Tremblay. 1977. The control of human WI-38 proliferation by extracellular calcium and its elimination by SV-40 virus-induced proliferative transformation. *J. Cell. Physiol.* 92:241–248.

63. Varani, J., J. Shayevitz, D. Perry, R. S. Mitra, B. J. Nickoloff, and J. J. Voorhees. 1990. Retinoic acid stimulation of human dermal fibroblast proliferation is dependent on suboptimal extracellular Ca^{2+} concentration. *Am. J. Pathol.* 136:1275–1281.

64. Obinata, A., M. Kawada, and H. Endo. 1987. Induction of epidermal mucous metaplasia by culture of recombinants of undifferentiated epidermis and retinol-treated dermis in a chemically defined medium. *Dev. Biol.* 123:59–62.

65. Hardy, M., D. Dhouailly, H. Torma, and A. Vahlquist. 1990. Either chick embryo dermis or retinol-treated mouse dermis can initiate glandular morphogenesis from mammalian epidermal tissue. *J. Exp. Zool.* 256:279–289.

66. Covant, H. A., and M. H. Hardy. 1990. Excess retinoid acts through the stroma to produce mucous glands from newborn hamster cheek pouch in vitro. *J. Exp. Zool.* 253:271–279.

67. Sanquer, S., B. Coulomb, C. Lebreton, and L. Debertret. 1990. Human dermal fibroblasts modulate the effects of retinoids on epidermal growth. *J. Invest. Dermatol.* 95:700–704.

68. Viallet, J. P., E. Ruberte, S. Du Manoir, A. Krust, A. Zelent, and D. Dhouailly. 1991. Retinoic acid-induced glandular metaplasia in mouse skin is linked to the dermal expression of retinoic acid receptor β mRNA. *Dev. Biol.* 114:424–428.

69. Schuger, L., R. Mitra, J. Varani, and K. Gilbride. 1993. Effect of all-trans retinoic acid on mouse lung branching activity. *Dev. Biol.* 159:462–473.

70. Saiag, P., B. Coulomb, and C. Lebreton. 1985. Psoriatic fibroblasts induce hyperproliferation of normal keratinocytes in a skin equivalent model in vitro. *Science (Wash. DC).* 230:669–672.

71. Karasek, M. A. 1972. Dermal factors affecting epidermal cells in vitro. *J. Invest. Dermatol.* 59:99–101.

72. Yeager, P. C., C. D. Stiles, and B. J. Rollins. 1991. Human keratinocyte growth-promoting activity on the surface of fibroblasts. *J. Cell. Physiol.* 149:110–116.