Topical All-*trans* Retinoic Acid Augments Ultraviolet Radiation-Induced Increases in Activated Melanocyte Numbers in Mice

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We have previously shown that daily application of 0.05% retinoic acid to the backs of lightly pigmented, hairless HRA:Skh-2 mice increases melanogenesis resulting from exposure to solar-simulated ultraviolet radiation. In this study we show that as early as 1 wk following commencement of treatment, there is a 2-fold increase in the number of epidermal 3,4-dihydroxyphenylalanine positive melanocytes in retinoic acid and ultraviolet radiation treated HRA:Skh-2 mice compared with mice that received ultraviolet radiation only. This increased to a 2.9-fold difference by 6 wk. Retinoic acid also augmented ultraviolet radiation-stimulated melanogenesis, with a 4-fold increase being observed after only 2 wk. These findings were also seen in C57BL mice. Ultraviolet radiation and retinoic acid needed to be applied to the same skin site for the augmentation in melanocyte activation to occur. Ultraviolet B rather than ultraviolet A was mainly responsible for melanogenesis and the retinoic acid primarily increased ultraviolet B-induced melanogenesis. Furthermore, retinoic acid on it's own, in the absence of ultraviolet radiation caused a small but statistically significant increase in 3,4-dihydroxyphenylalanine positive melanocyte numbers and melanogenesis. Thus topical retinoic acid is a potent modulator of melanocyte activation. Alone it is able to increase the number of activated epidermal melanocytes and make melanocytes more sensitive to activation by ultraviolet B. ***Key words: melanin/retinoids/sunlight/tan.***
J Invest Dermatol 112:271–278, 1999

One of the responses of human skin to the ultraviolet (UV) wavelengths of sunlight is melanocyte activation, division, and production of melanin. UV radiation (UVR) is the main known physiologic stimulus for melanocyte activation, melanin production, and the resulting pigmentation of the skin. Melanocytes are situated in the epidermis along the dermoepidermal junction at a ratio of 1 to every 36 keratinocytes, a relationship known as the "epidermal melanin unit." The role of melanin is not completely understood, however; it effectively absorbs both UV and visible light, and may play a protective role against UVR-induced carcinogenesis (Kaidbey *et al*, 1979).

The mechanisms involved in the control of melanin production are not fully understood. Tyrosinase, a copper-binding transmembrane glycoprotein, is the rate-limiting enzyme that catalyzes three steps in the biosynthesis of melanin (Korner and Pawelek, 1982). Tanning has been shown to be associated with increased tyrosinase activity (Gange, 1971). The mechanisms by which UVR upregulates tyrosinase activity have recently been reviewed (Gilchrest *et al*, 1996). In humans, tyrosinase activity can be correlated with skin color, with tyrosinase activity being generally higher in black than white skin (Pomeranz and Ances, 1975; Iwata *et al*, 1990).

The retinoids are metabolic or synthetic derivatives of vitamin A, which have proven to be useful clinically in the treatment of a variety of dermatoses (Peck and DiGiovanna, 1993) and chemoprevention of some cutaneous malignancies (Lippman *et al*, 1987). Retinoids are hormone-like agents that control a number of cellular processes by binding to nuclear receptors and exerting pleiotypic effects on gene expression (reviewed in Rees, 1993). In mammals, vitamin A activity is fulfilled by three major compounds: retinol, retinal, and retinoic acid (RA; vitamin A acid); and perhaps by a number of their metabolites. RA has been found to modulate many of the effects of UVR on the skin, including clinical and histologic improvement of photodamaged skin (Gilchrest, 1992; Griffiths *et al*, 1993a), and protection from UVR-induced reduction of Langerhans cell numbers (Ho *et al*, 1991), and function (Dunlop *et al*, 1994).

In humans, several clinical studies have shown that topical application of RA can improve the irregular hyperpigmentation associated with previous UVR exposure (Rafal *et al*, 1992), melasma (Griffiths *et al*, 1993b), and inflammation (Bulengo-Ransby *et al*, 1993). The mechanism by which RA causes lightening of these hyperpigmented lesions is not fully understood.

Apparently contradictory findings, however, have also been reported. Topical 0.1% RA, when applied to the flank twice daily, appears to stimulate melanogenesis in the Yucatan micropig.[1] When applied for 4 d under occlusion to the skin of white, but not black

Manuscript received May 6, 1998; revised October 6, 1998; accepted for publication November 19, 1998.

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Abbreviations: L-DOPA, 3,4-dihydroxyphenylalanine; RA, retinoic acid.

[1]Zheng P, Kligman AM: Topical tretinoin stimulates melanogenesis in the Yucatan micropig. *J Invest Dermatol* 96:576, 1991 (abstr.)

humans, 0.1% RA induced tyrosinase activity (Talwar *et al*, 1993). Using a mouse strain (HRA:Skh-2), which develops increased pigmentation in response to UVR, we have previously reported that topically applied RA greatly enhanced melanogenesis when applied during a 4 wk UV irradiation regimen (Ho *et al*, 1992). This finding was confirmed in two of three human volunteers. Thus the outcome of RA action on melanocytes *in vivo* is complex and seems to depend on their prior state of activation. RA appears to activate resting melanocytes and augment UVR stimulation of melanogenesis, but does not alter (Talwar *et al*, 1993), or reduce (Weiss *et al*, 1988), melanogenic activity in pigment cells, which are already maximally stimulated.

To examine further the regulatory role of RA on melanocytes we used the lightly pigmented HRA:Skh-2 murine model and examined whether the increase in epidermal melanin, seen after exposure to both RA and solar-simulated UV irradiation, is associated with a concomitant increase in activated melanocyte numbers. The effects of solvent constituents, UVR wavelength, and site of application of the RA were also investigated.

MATERIALS AND METHODS

Animals HRA:Skh-2 mice are inbred, hairless, lightly pigmented and capable of developing a tan in response to UVR. This strain was established at the Skin Cancer and Photobiology Unit, Temple University, Philadelphia, U.S.A. Female mice, 9–11 wk old, were used in all experiments and were purchased from Worksafe Australia, Camperdown, NSW. C57BL/6 mice possess a thick black coat but nonpigmented skin. Females aged 8–10 wk were purchased from Little Bay Animal Holding Facility, Little Bay, Sydney, Australia. The hair on their dorsal trunk was shaved using a Remington Microscreen shaver on the first day of irradiation and subsequently throughout the irradiation period as required. Mice were grouped six per cage, and fed by free access to standard mouse pellets (Standard Rat and Mouse Cubes, Doust and Rabbidge, Sydney, Australia).

Ultraviolet irradiation

Source Mice were irradiated unrestrained in their cages, 30 cm from the light source. Simulated solar UV radiation was provided by a bank of six 100 W cosmolux RA + A1-14-100 W UVA tubes flanking a single central LSFS72T12 UVB tube housed in a planar arrangement in a reflective batten. The spectral output of this light source was designed to simulate the solar spectrum as closely as possible and has been previously described (Bestak *et al*, 1995). Radiation was filtered through a 0.5 mm layer of cellulose triacetate film (Kodacel; Eastman Chemical Products, Kingsport, TN), which reduced radiation sharply below 295 nm, therefore eliminating any UVC. In experiments using UVA wavelengths alone the UVB tube was not used and 0.05 mm thick plastic Mylar filters (Cadillac Plastics, Sydney, Australia) were used to eliminate any UVB irradiation. In experiments using UVB, the UVB tubes only were used without filtering, other than with the cellulose triacetate. The integrated irradiance of the solar-simulated light source was 3.4×10^{-3} W per cm^2 UVA and 1.7×10^{-4} W per cm^2 UVB. Output of the bank of tubes was monitored weekly using an IL 1350 radiometer (International Light, Newburyport, MA), with an SED 038 UVA, and an SED 240 UVB detector.

Procedure Mice were irradiated 5 d per wk (Monday to Friday) with the minimal (o)edemal dose, previously determined to be 10 min for the HRA:Skh-2 mice. Exposure times were increased each wk by 20% of the initial exposure time to overcome acquired tolerance and maintain the minimal (o)edemal dose. The average cumulative doses over a 1 wk period were 0.51 J per cm^2 UVB and 10.2 J per cm^2 UVA and over a 4 wk period, 2.74 J per cm^2 UVB and 54.75 J per cm^2 UVA.

Retinoid treatment of mice

All-trans-*retinoic acid* Mice were treated topically, on their backs, or abdomens, depending on the experiment, with all-*trans*-RA type XX (Sigma, St Louis, MO) dissolved at 0.5 mg per ml in a solvent consisting of ethanol, dimethylsulfoxide, and acetone (1:1:6, vol/vol/vol). Thirty microliters (51 nmol) was delivered per mouse per treatment. In one set of experiments RA was used at 0.0375 mg per ml in the same solvent. Thirty microliters (3.8 nmol) was delivered per mouse per treatment. Control mice were treated with solvent alone. Mice were treated 24 h prior to irradiation to avoid photo-isomerization and photodegradation of the RA. In some experiments RA was dissolved in ethanol alone at a final concentration of 0.5 mg per ml.

Figure 1. RA visibly augments UVR induced melanogenesis. Both mice have been irradiated with UVR for 4 wk. The top mouse was also treated topically with solvent, the bottom mouse with RA.

Quantitation of melanin and melanocytes in epidermal sheets

Preparation of epidermal sheets Dorsal or abdominal trunk skin was excised from the treated area of mice, 72 h after the last treatment, and dermal fat and connective tissue scraped off using a scalpel. The skin was cut into $\approx 1 \times 1$ cm^2 pieces and incubated in Tris-buffered isotonic saline containing 20 mM ethylenediaminetetraacetic acid, pH 7.3, for 3 h at 37°C. The epidermis was then mechanically separated from the dermis using a pair of fine forceps and a dissecting microscope (Halliday *et al*, 1988).

Melanin Melanin in epidermal sheets was stained using the Masson–Fontana method (Stevens, 1982), and quantitated by image analysis (Chromatic Image Analysis System, Leitz, Sydney, Australia) as we have previously described (Ho *et al*, 1992). The melanin content was estimated in a total area of 3.91 mm^2 for each mouse and expressed as the integrated optical density per mm^2. This is a measure of the relative absorption of the stained melanin, and is in arbitrary (nonstandard) units. In additional experiments epidermal sheets were prepared from groups of six mice, pooled and solubilized in phosphate-buffered saline containing 8.0% sodium dodecyl sulfate (Pharmacia Biotech, Uppsala, Sweden) and 8 mM HCl at 37°C overnight. OD at 475 nm were determined and converted to μg melanin per cm^2 epidermis by comparison with a standard curve of melanin (Sigma) (Lotan and Lotan, 1980).

Melanocytes Epidermal sheets were placed in a solution containing 10 mg of 3,4-dihydroxyphenylalanine (L-DOPA; Sigma) in 10 ml of 0.2 M phosphate buffer, pH 6.8, and incubated at 37°C overnight (Warren, 1986). The skin was then fixed in 10% formalin, dehydrated through successive alcohol baths, cleared with xylene and mounted on glass slides, dermal side up, with Histomount (National Diagnostics, NJ) and coverslips. Melanocyte density was counted using a light microscope, with respect to area, using the same image analysis system as described above and expressed as cell number per mm^2. For each mouse the melanocytes in a total area of 2.6–2.9 mm^2 was counted.

Statistics Comparisons between groups was made using a two-tailed unpaired Student's t test; $p < 0.01$ was regarded as significant.

RESULTS

Development of macroscopically visible pigmentation HRA:Skh-2 mice were treated with solvent, RA, UVR, UVR plus solvent, or UVR plus RA for 1, 2, 4, 5, or 6 wk. Visually, mice receiving RA and UVR developed an even tan earlier than the other groups (**Fig 1**). This was observable after only five treatments (1 wk), and became progressively darker over the 6 wk treatment period. After approximately eight treatments, the mice receiving UVR alone or UVR and solvent developed small brown macules that gradually coalesced to confluent pigmentation by 4–5 wk. Thus the pattern of pigmentation differed in the UVR plus RA-treated animals. By 2–3 wk mice treated with RA

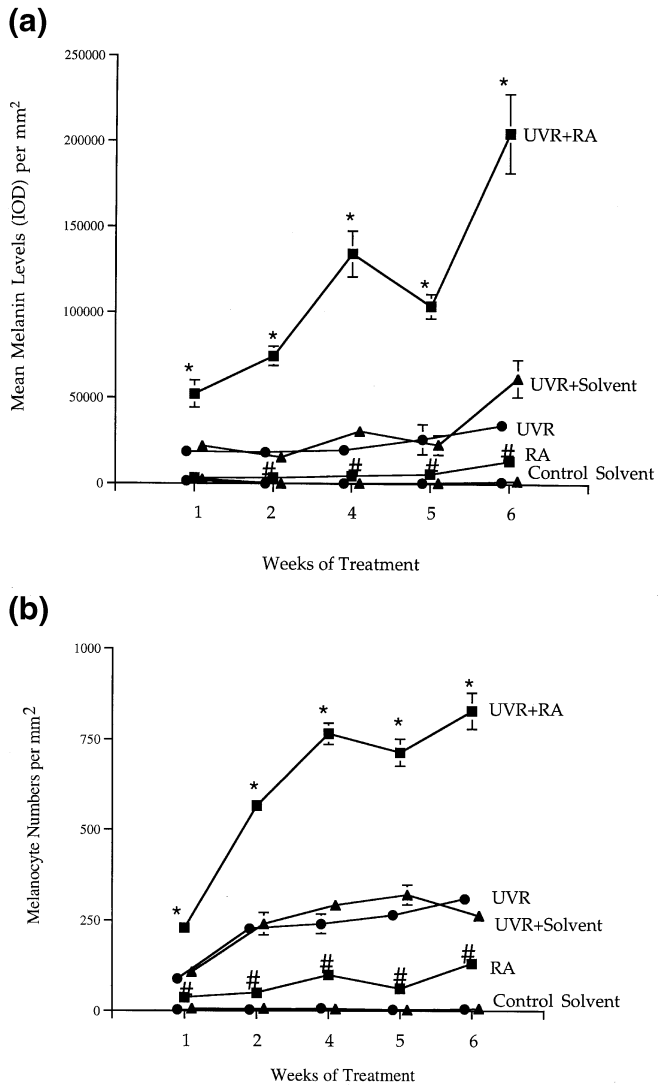

Figure 2. RA increases epidermal melanin and number of melanocytes in the presence and absence of UVR. (a) Melanin levels (integrated optical density, IOD), (b) L-DOPA positive melanocytes. Control (●), untreated; Solvent (▲), solvent only; RA (■), retinoic acid, UVR, solar-simulated UV irradiation. Groups of six HRA:Skh-2 mice were treated 5 d per wk for the number of weeks indicated. Epidermal melanin content was quantitated using image analysis. Each point represents the mean ± SEM of six mice, where no SEM is obvious it is too small to be visualized. *$p < 0.01$ compared with UVR plus solvent groups; #$p < 0.01$ compared with solvent alone treated groups (unpaired Student's t-test) for each time point.

alone developed a faint but distinct even tan compared with mice receiving solvent alone.

All mice receiving RA, whether in conjunction with UVR or not, developed some degree of irritation (erythema) and scaling. This did not seem to be exacerbated by the UVR, in fact mice receiving RA plus UVR stopped scaling after about 2 wk of treatment. Mice receiving RA alone had some persisting scale over the 6 wk period; however, with increasing duration of treatment this became less prominent.

Topical RA augments melanogenesis At all time points examined epidermal melanin content [as measured by integrated optical density per mm^2] was statistically significantly greater ($p < 0.01$) in RA + UVR-treated mice than mice treated with UVR plus solvent (**Fig 2*a***). This difference was observed after only five treatments (1 wk). There were no significant differences between mice treated with UVR alone and those receiving UVR plus solvent at any time point. Over the 6 wk period all UVR-treated mice developed a gradual increase in epidermal melanin content. The rate of increase, however, was greatest for those treated with RA in association with UVR.

An increase in epidermal melanin was also seen in mice receiving RA in the absence of UVR (**Fig 2*a***). This became significantly different from solvent-treated mice after 2 wk of treatment ($p < 0.01$). Although both RA and UVR induced melanogenesis on their own, the combined effect of RA plus UVR treatment far exceeded the summation of each individual treatment. Thus the effects of RA plus UVR were not additive, RA augmented the UVR-induced melanogenesis.

In further experiments, dorsal trunk epidermis from groups of six mice that were irradiated with or without RA for 4 wk were pooled, epidermal melanin was extracted and measured by comparison with purified melanin. Unirradiated mice contained 0.15 μg per cm^2 melanin, which was increased by UVR to 0.44 μg per cm^2. Mice receiving RA and UVR had an increased melanin content of 0.91 μg per cm^2, confirming that RA augmented UVR-induced melanogenesis.

As was noted macroscopically, microscopically the pattern of pigmentation differed between treatment groups. In UVR plus RA-treated animals melanin was dispersed evenly throughout the epidermal sheets. In groups treated with UVR plus solvent or UVR alone, melanin was found to be concentrated primarily around large follicular orifices. With cumulative UVR doses, particularly after 4 wk of treatment, melanin also became prominent in the interfollicular epidermis. Mice receiving RA in the absence of UVR developed an increase in melanin, which was evenly distributed between follicular and interfollicular epidermis.

Topical RA increases the number of activated melanocytes Epidermal melanocyte numbers reflected the changes seen in epidermal melanin content. An increase in epidermal L-DOPA positive melanocyte numbers was observed in all groups with cumulative UVR exposure (**Fig 2*b***).

At all time points examined, when combined with UVR, RA caused at least a 2-fold increase in the number of L-DOPA positive melanocytes ($p < 0.01$ in all groups) over that achieved with UVR plus solvent. By 6 wk of treatment this had increased to almost a 3-fold difference. In all irradiated groups the greatest rate of increase in cell numbers was observed between 1 and 2 wk of UVR exposure. In the UVR alone and UVR plus solvent groups there was a 2.4-fold increase in melanocyte numbers between 1 and 2 wk of irradiation with no difference between these groups. Thereafter the cell numbers became more stationary with melanocyte numbers at 6 wk being similar to 2 wk. In the UVR plus RA group, a 2.5-fold increase in melanocyte numbers was seen between 1 and 2 wk, but they continued to increase with ≈1.5-fold higher numbers by week 6 (828 per cm^2) compared with week 2 (567 per cm^2). Thus, initially, in the UVR plus RA group melanocyte numbers increased at a faster rate than in the UVR only group. After 2 wk of UVR treatment melanocyte numbers plateaued in the absence of RA; however, in the presence of RA they continued to increase up to week 4 of treatment before plateauing. This indicates that RA did not only accelerate, but also prolonged the UVR-induced increase in melanocyte numbers, resulting in a greater total number of epidermal melanocytes compared with that achieved with either RA or UVR alone or the summation of these.

No significant difference was found between mice treated with UVR alone and those that received UVR plus solvent. Similarly, there was no significant difference between control mice (no treatment) and those that received solvent alone.

RA treatment in the absence of UVR also significantly increased L-DOPA positive epidermal melanocyte numbers compared with solvent treated controls at all time points measured ($p < 0.01$ in all groups). Over the 6 wk treatment period RA (in the absence of UVR) treated groups developed a steady increase in the number of activated melanocytes so that by week 6 there was a 3.6-fold greater number of melanocytes compared with week 1. Whereas both RA and UVR alone each increased the number of activated

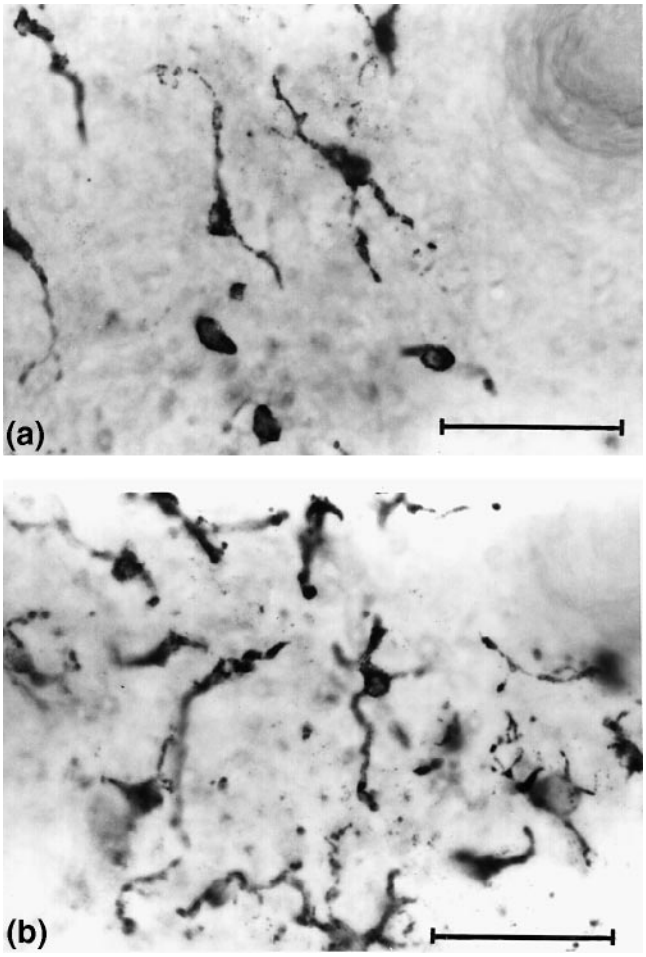

Figure 3. L-DOPA stained melanocytes in epidermal sheets of UVR plus solvent (*a*) and UVR plus RA (*b*) treated mice. Mice had been treated for 4 wk. Melanocytes in the UVR plus RA epidermis often have a larger number of dendrities and an increased cell body. *Scale bar*: 50 μm.

epidermal melanocytes, the summation of these two groups, at each time point examined, only reached slightly over half of that attained with the combination of RA plus UVR. Thus RA augmented the UVR-induced upregulation of activated melanocytes.

The pattern of distribution of activated melanocytes in the epidermal sheets was similar to that of melanin. UVR plus RA-treated groups had melanocytes distributed evenly throughout the epidermis and those treated with UVR in the absence of RA had a predominantly perifollicular pattern with increased numbers of activated interfollicular melanocytes with increased UVR exposure. Melanocytes in RA-treated groups in the absence of UVR showed activation of melanocytes in both perifollicular and interfollicular areas. The few lightly L-DOPA stained cells that could be found in control mice were always located around follicular openings.

When stained with L-DOPA in epidermal sheets, melanocytes of RA- and UVR-treated mice were hypertrophied and more highly dendritic than those from mice treated with UVR and solvent (**Fig 3**). In vertical sections L-DOPA-stained melanocytes were seen in both basal and suprabasal locations within a thickened epidermis in the RA and UVR group. In mice treated with UVR, UVR plus solvent, or RA alone, melanocytes were seen in the basal layer only.

Effect of solvent The same pattern of change in epidermal melanin (**Fig 4*a***) and melanocyte numbers (**Fig 4*b***) were found when RA was dissolved in ethanol alone, thus eliminating any contribution of dimethylsulfoxide or acetone from this response.

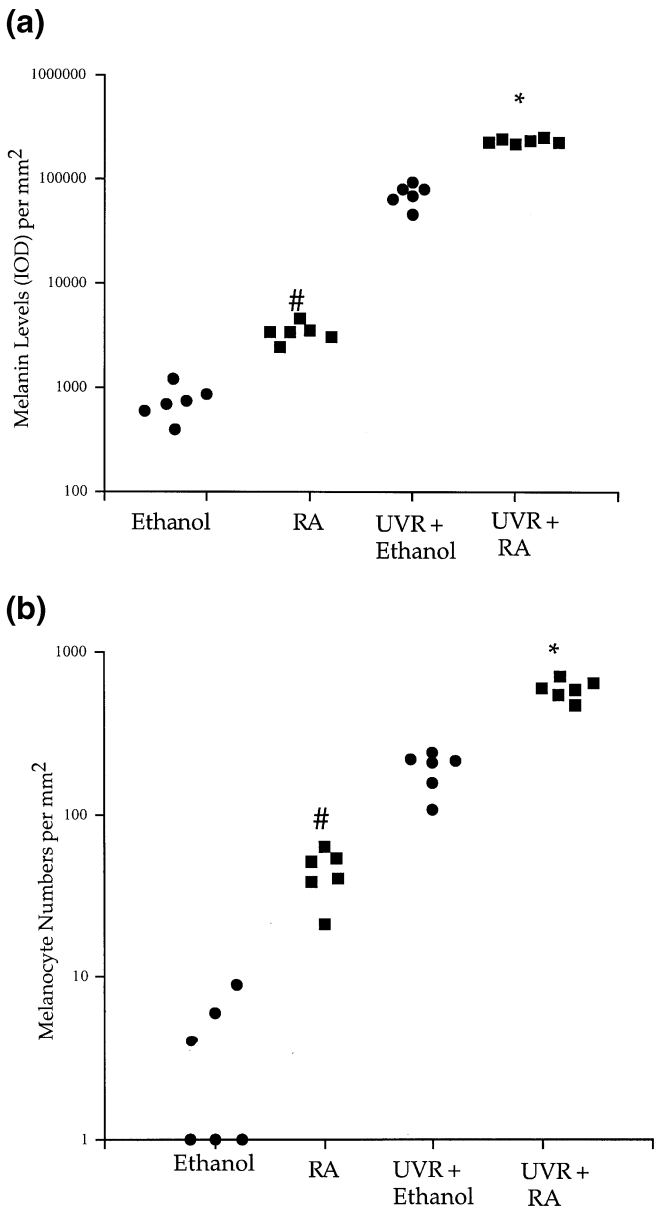

Figure 4. RA in ethanol increases epidermal melanin and number of melanocytes in the presence and absence of UVR. (a) Melanin levels (integrated optical density, IOD), (b) L-DOPA positive melanocytes. Groups of six HRA:Skh-2 mice were treated 5 d per wk for 4 wk. Epidermal melanin content was quantitated using image analysis. Each point represents the integrated optical density or melanocyte numbers per mm^2 of a single mouse. *$p < 0.01$ compared with UVR plus ethanol group; #$p < 0.01$ compared with the ethanol-treated group (unpaired Student's t-test).

Mice were treated for 4 wk. Mean epidermal melanin was significantly higher in the RA and UVR group compared with the UVR- and ethanol-treated group. Mean melanocyte numbers in mice receiving UVR and ethanol were 189 per mm^2 compared with 582 per mm^2 in the RA and UVR group. In the absence of UVR, solvent-treated mice had a mean of three L-DOPA positive melanocytes per mm^2 compared with 44 per mm^2 in RA-treated animals. Thus the response was not affected by the absence of dimethylsulfoxide and acetone.

Effect of ultraviolet wavelength – UVA *versus* UVB Mice were treated with UVA or UVB radiation in the presence and absence of RA. Melanin (**Fig 5*a***) and melanocyte numbers (**Fig 5*b***) were quantitated after 4 wk of treatment. UVB had the larger effect on both melanin levels ($p < 0.01$) and melanocyte numbers

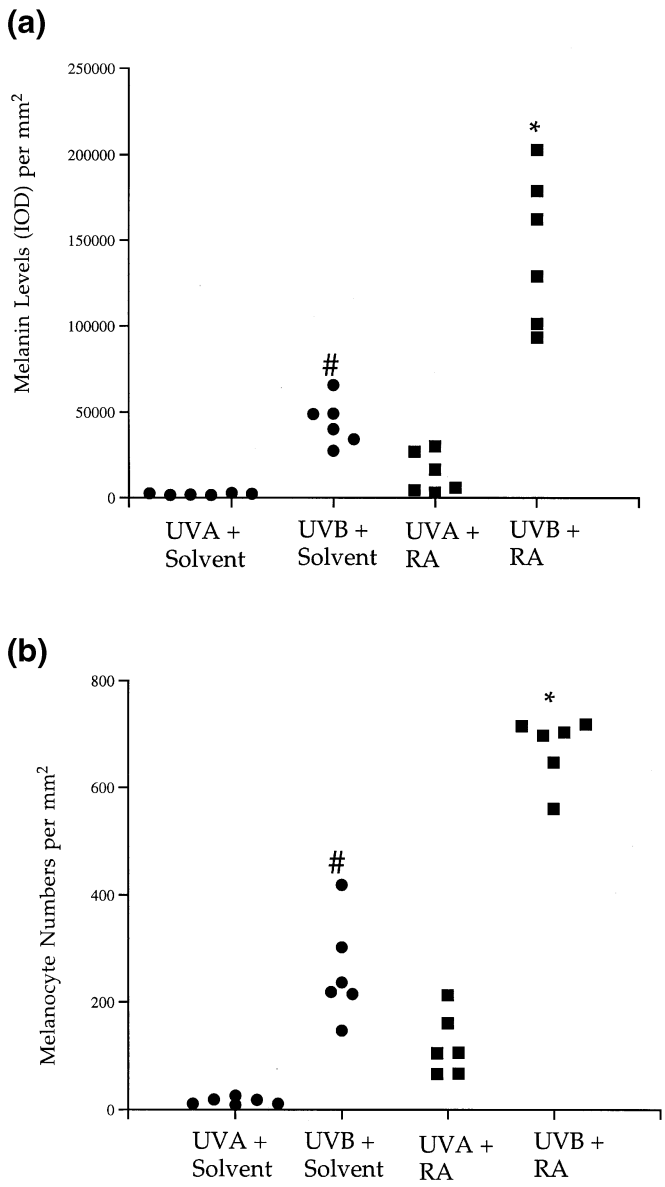

Figure 5. UVB increases epidermal melanin and melanocyte numbers to a greater extent than UVA in the presence and absence of RA. (a) Melanin levels (integrated optical density, IOD), (b) L-DOPA positive melanocytes. Groups of six HRA:Skh-2 mice were treated 5 d per wk for 4 wk. Each point represents a single mouse. *$p < 0.01$ compared with UVA plus RA group; #$p < 0.01$ compared with UVA + solvent-treated group (unpaired Student's t-test).

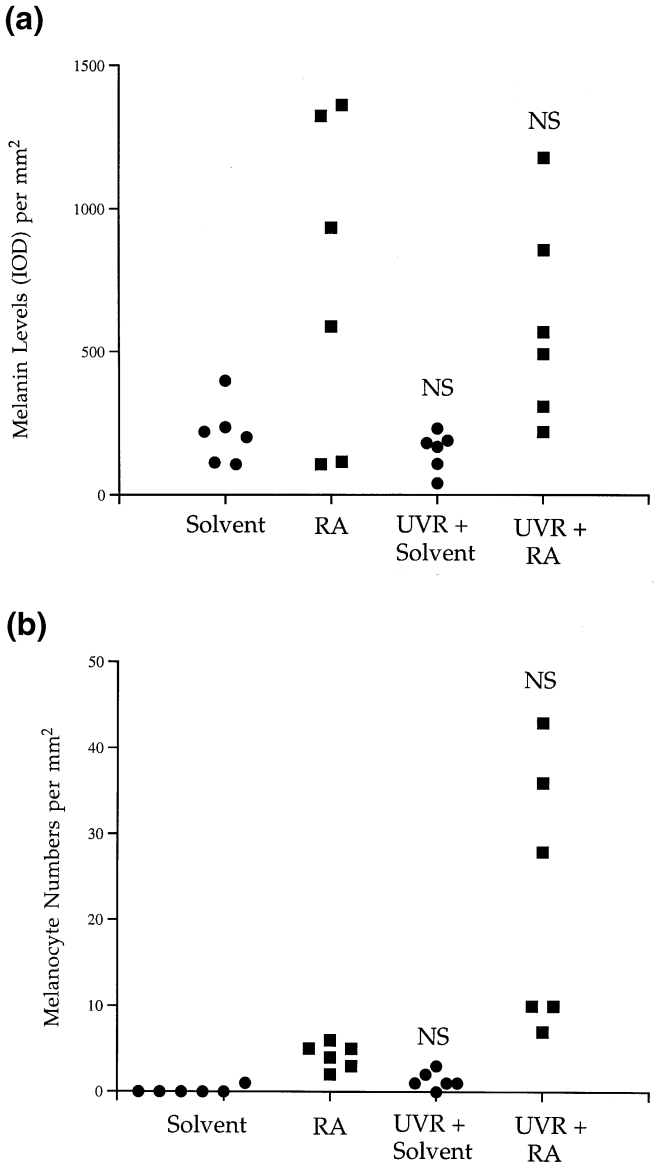

Figure 6. Melanin and melanocyte numbers in abdominal epidermis of mice treated locally with RA was not affected by UVR exposure of dorsal trunk skin. (a) Melanin levels (integrated optical density, IOD), (b) L-DOPA positive melanocytes. UVR, solar-simulated UV irradiation. Groups of six HRA:Skh-2 mice were treated 5 d per wk for 4 wk. Each point represents a single mouse. NS, not significantly different to the group that did not receive UVR (unpaired Student's t-test).

($p < 0.01$) in the presence or absence of RA. Mice treated with UVA alone (15 L-DOPA positive melanocytes per mm^2) had only slightly more activated melanocytes after 4 wk than unirradiated mice (5 per mm^2, **Fig 2*b***). Mice treated with UVA plus RA possessed a similar number of activated melanocytes as mice receiving RA alone (**Fig 2*b***) for the same time period (119 per mm^2 and 98 per mm^2, respectively). Thus the increase in L-DOPA positive melanocytes in UVA plus RA-treated animals can be accounted for by the RA alone, with very little or no contribution from the UVA irradiation.

At 4 wk the combination of UVB plus RA caused a 2.63-fold increase in the number of L-DOPA positive melanocytes compared with UVB plus solvent controls (674 per mm^2 compared with 256 per mm^2, respectively). This was identical to the increase caused by RA plus solar-simulated UVR over UVR plus solvent controls at 4 wk (764 per mm^2 compared with 291 per mm^2, respectively, **Fig 2*b***). Thus RA augmented UVB but not UVA-induced melanogenesis.

RA and UVR need to be applied to the same site To examine whether the same skin site needs to receive both RA and UVR, RA was applied to the abdomen and UVR to the dorsum for 4 wk after which abdominal skin was quantitated for melanin content and melanocyte numbers (**Fig 6**). Dorsal trunk skin could not be examined because radioactive tracer studies (not presented) indicated that some RA reached the dorsal trunk, probably due to grooming by the mice. When back skin was UVR irradiated and solvent applied to the abdomen, epidermal melanin and melanocyte numbers in the abdominal skin were not significantly different to mice that received solvent on the abdomen with no UVR. Thus UVR did not have a systemic effect on melanocytes. Epidermal melanin and melanocyte numbers were increased locally on the abdomen after RA treatment; however, dorsal UVR did not significantly augment the effect of local RA on abdominal melanocytes.

Thus these experiments indicate that distal UVR was not able

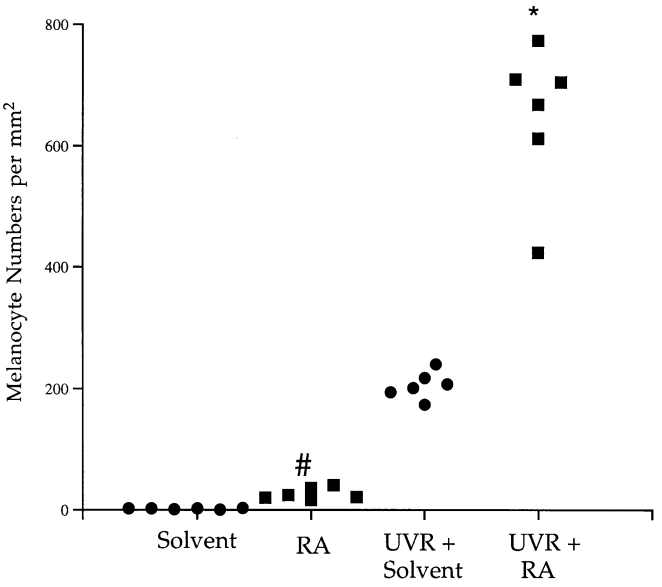

Figure 7. RA at 0.0375 mg per ml augments UVR-induced melanocyte numbers. Solvent, solvent only; UVR, solar-simulated UV irradiation. Groups of six HRA:Skh-2 mice were treated 5 d per wk for 4 wk. Melanocyte numbers were quantitated by image analysis. Each point represents a single mouse. *$p < 0.01$ compared with UVR + solvent group; #$p < 0.01$ compared with the solvent-treated group (unpaired Student's t-test).

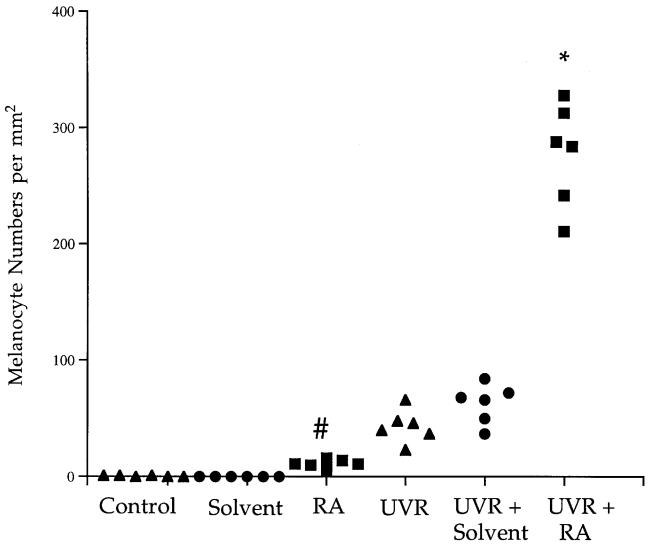

Figure 8. RA increases the number of L-DOPA positive epidermal melanocytes in the presence and absence of UVR in C57BL/6 mice. Solvent, solvent only; UVR, solar-simulated UV irradiation. Groups of six C57BL/6 mice were treated 5 d per wk for 4 wk. Melanocyte numbers were quantitated using image analysis. Each point represents the melanocyte number per mm^2 of epidermis for a single mouse. *$p < 0.01$ compared with UVR plus solvent group; #$p < 0.01$ compared with the solvent-treated group (unpaired Student's t test).

to augment RA effects on L-DOPA positive melanocytes or epidermal melanin content.

Effect of a reduced RA concentration An experiment was conducted using 0.0375 mg per ml RA (7.5% of the concentration used in the other experiments). This lower concentration of RA upregulated melanocyte numbers in the presence and absence of UVR to a similar extent as 0.5 mg per ml (**Fig 7**).

Genetic restriction of the response To determine whether the responses observed were restricted to HRA:Skh-2 mice, C57BL/6 mice were treated for 4 wk. In the C57BL/6 mice RA also augmented the response to UVR. Mice receiving UVR in the absence of RA were noted to have a greater density of activated melanocytes centred around large follicular orifices. In mice receiving UVR in combination with RA activated melanocytes were more evenly distributed throughout the epidermis. UVR plus RA-treated mice had a 3-fold increase in the number of L-DOPA-positive epidermal melanocytes compared with those treated with either UVR alone or UVR plus solvent (**Fig 8**). RA alone was also found to significantly increase melanocyte numbers (mean = 11) compared with solvent-treated mice (mean = 0).

DISCUSSION

This study has demonstrated that topically applied 0.05% or 0.00375% RA augments UVR-induced increases in activated melanocyte numbers in two unrelated mouse strains, HRA:Skh-2 and C57BL/6. RA enhanced the number of activated melanocytes in UVR-irradiated epidermis 2–3-fold. This caused up to a 4-fold increase in melanin production, and a greatly augmented tan, supporting earlier work by our group (Ho *et al*, 1992). These results suggest that the previously reported effect of RA on UVR-induced tanning results from augmented activation of melanocytes and/or increased melanocyte division. RA alone also increased the number of activated melanocytes, and epidermal melanin, although not to the levels achieved in combination with UVR. The UVB wavelengths, not UVA, appeared primarily responsible for this effect. RA resulted in a more evenly distributed melanocyte activation and altered melanocyte morphology resulting in greatly hypertrophied, highly dendritic cells, which occupied both basal and suprabasal positions within a thickened epidermis. This altered melanocyte morphology is consistent with them being activated by RA.

The melanocytes of mice are largely dormant and located in the dermis, epidermis, and outer root sheath of hair follicles (Reynolds, 1954; Uesugi *et al*, 1979; Kligman *et al*, 1982). In control mice only two to three weakly DOPA positive melanocytes were found per mm^2 of epidermis. These were located primarily around the orifices of large tylotrich hair follicles, which are highly specialized for tactile function (Straile, 1960).

In this study melanocytes were detected by staining with L-DOPA. As this method requires the presence of active tyrosinase only melanocytes producing melanin, not resting melanocytes, were detected with this stain. The pattern of melanocyte activation in response to UV irradiation differed in the presence and absence of RA. In the absence of RA, melanocytes located at the orifices of the large tylotrich hair follicles were the first to be activated by UVR. With ongoing UV irradiation the number of perifollicular melanocytes increased, this still being the dominant pattern at 2 wk. After 4 wk of UVR treatment melanocyte density still remained greatest around tylotrich follicles; however, a large number of interfollicular melanocytes had become L-DOPA positive. This correlated with the clinical observation of a tan developing from the coalescence of gradually enlarging pigmented macules.

In contrast, UVR in combination with topical RA caused simultaneous activation of both perifollicular and interfollicular epidermal melanocytes without an obvious follicularly based pattern. Hence a greater number of melanocytes were activated at an earlier time point resulting clinically, in a darker, more even tan. RA may have augmented UVR-induced melanogenesis by partially activating, or causing differentiation, of relatively UVR-resistant interfollicular melanocytes at an earlier time point, possibly making them more sensitive to UVR-induced melanogenesis factors. Alternatively, RA may have caused melanocyte division or redistribution from the dermis or hair follicles to the epidermis.

In all UVR-treated animals, with and without RA, the increase in L-DOPA-positive melanocyte numbers was greatest between weeks 1 and 2 of treatment. After this time, in UVR controls, ongoing exposure resulted in a plateauing in melanocyte numbers. This time course for UVR-induced increases in melanocyte num-

bers has been previously reported in mice (Uesugi *et al*, 1979; Jimbow and Uesugi, 1982; Nair and Tramposch, 1989) and rhesus monkeys (Erickson and Montagana, 1975). Uesugi *et al* (1979) correlated this with the number of mitotic DOPA-positive melanocytes after thymidine labeling. At day 3 there was no dividing DOPA-positive melanocytes. Thymidine-labeled melanocytes rose to 2.15% at day 6 and had fallen to 1.65% at day 10. This suggests that only a small proportion of melanocytes divide, but this could contribute to the dramatic increase in cell numbers seen between weeks 1 and 2 of UVR irradiation. Mitotic figures have been demonstrated in melanocytes in normal epidermis of mice and humans without the presence of external stimuli (Jimbow *et al*, 1975). It is most likely that new melanogenesis after repeated UVR exposure involves both activation of dormant epidermal melanocytes and cell division. It is also possible that redistribution of melanocytes from the outer root sheath of hair follicles could have contributed to the increased number of epidermal melanocytes. Why the melanocyte population becomes relatively "fixed" after about eight to 14 exposures is unknown.

RA altered the pattern of melanocyte activation. Soon after commencement of RA plus UVR treatment, activated melanocytes were evenly distributed throughout the epidermis, in contrast to the predominantly perifollicular pattern induced by UVR alone. Additionally, the accelerated rate of increase in cell numbers seen between 1 and 2 wk of UVR treatment was prolonged to 4 wk with the addition of RA before a plateau phase was attained. RA alone caused some of these effects to a lesser extent with an evenly distributed population of activated melanocytes, which continually increased in number over a 6 wk period. UV radiation then augmented this pattern caused by the RA alone.

At the end of 2 wk of UV irradiation alone it is most likely that maximal activation of dormant melanocytes has been achieved as no great increase in melanocyte numbers occurred after this time. RA + UVR-treated melanocytes, however, continued to increase in number at an accelerated rate for a further 2 wk suggesting that the RA may have increased melanocyte division. Alternatively RA may have been continuing to lead to melanocyte redistribution from the outer root sheath of hair follicles and priming them for activation.

It remains unknown why, after a 2 wk period, no further increases in melanocyte numbers occur in mice receiving UVR alone. The factors regulating the size of the epidermal melanocyte population, during different conditions are not known. The relationship of keratinocyte to melanocyte numbers in the epidermis is tightly controlled probably by keratinocyte-derived factors (Yaar and Gilchrest, 1991). The moderate thickening of the epidermis seen after UVR may therefore only accommodate a small increase in melanocyte numbers. RA, however, induces a marked thickening due to increased numbers of epidermal cell layers, principally to an expansion of the spinous and granular cell layer zones (Connor *et al*, 1986). On vertical sections melanocytes were found at both basal and suprabasal locations in RA plus UVR-treated mice suggesting that an expanded epidermal cell compartment could be occupied by a larger number of melanocytes.

Dimethylsulfoxide was used in these experiments as a vehicle constituent. It has previously been found to increase the number of L-DOPA-positive pigment cells in the ears of mice *in vivo* (Nordlund *et al*, 1981). In tissue culture of human melanoma cells, dimethylsulfoxide inhibits the growth of cells and augments melanin synthesis (Wood and Wood, 1975). We showed, however, that dimethylsulfoxide did not contribute significantly to the action of RA in augmenting melanogenesis. UVB is the main inducer of tanning in humans and was found to be primarily responsible for the tanning response and its augmentation by RA in these studies. UVA did not augment the tanning response above that seen when RA was used alone.

One other study has examined pigmentation following RA plus UVR treatment using the HRA:Skh-2 mouse model. Starting on the second week of a 6 wk irradiation protocol, one side of the UVB-exposed back was treated with 20 μl of 0.1% RA in propylene glycol/ethanol (50:50). RA reduced UVB-induced tanning but caused patches of hyperpigmentation to develop (Nair and Tramposch, 1989). These authors suggested the gross depigmentation could be partially due to RA-induced skin desquamation. The hyperpigmented areas in the RA plus UVR treated mice suggest some degree of RA augmentation of UVB-induced tanning, which is consistent with our studies.

UVR was found to act locally in upregulating melanocyte numbers. The number of melanocytes in abdominal skin was the same whether or not UVR had been delivered to the back. Repeated UVB irradiation has been reported to have a systemic effect on increasing the number of L-DOPA-positive melanocytes in the C57BL mouse (Rosdahl, 1979). Whether this discrepancy is due to differences in the UV spectra or another factor is not known.

The molecular effects of RA on UVR-induced melanogenesis remain unresolved. Topical retinoids have been reported to increase tyrosinase activity in adult human skin by a mechanism which does not involve alterations in tyrosinase mRNA or protein levels but which might involve suppression of an inhibitor of tyrosinase or modification of existing tyrosinase molecules by phosphorylation or glycosylation (Talwar *et al*, 1993). Melanogenesis and tyrosinase activity are also regulated by a number of intracellular signals including cyclic adenosine monophosphate (Abdel-Malek *et al*, 1992), protein kinase C (Allan *et al*, 1995), and nitric oxide (Romero-Graillet *et al*, 1997), with evidence suggesting that the latter two pathways may be important in upregulating tyrosinase activity after UV exposure (Allan *et al*, 1995; Romero-Graillet *et al*, 1997). In view of reports that retinoids can influence protein kinase C activity and isoform expression (Pailler-Rodde *et al*, 1991; Cho *et al*, 1997), enhance cyclic adenosine monophosphate-dependent responses (Mira-y-Lopez, 1991) and nitric oxide synthase activity (Motomura *et al*, 1997) in a variety of cell types, it is possible that the enhancement of UVR-induced melanogenesis in the presence of RA is the result of augmentation of more than one pathway. Alternatively, it is possible that the larger number of activated melanocytes shown in this study may be sufficient to explain RA-augmented melanogenesis, and therefore RA may not augment UVR-induced melanin production by an individual cell, but instead increase the number of active melanocytes.

In conclusion, we have shown RA to augment both constitutive and UVR-induced pigmentation in a melanocyte population with negligible baseline tyrosinase activity. In this setting RA was found to induce melanocyte differentiation modestly (by increasing tyrosinase activity and melanin production) when used alone. When combined with solar simulated UVR, RA: (i) altered the pattern of UVR-induced melanocyte activation; (ii) increased by 2–3-fold the maximal number of melanocytes capable of being detected after exposure to UVR; and (iii) augmented melanogenesis such that even after melanocyte numbers had reached their peak, melanin was continuing to be produced at a rate exceeding that of melanocytes stimulated to differentiate by UVR alone. The mode of action of RA in this system is unknown, but RA and UVR needed to be applied to the same site, and UVB was effective whereas UVA was not. It is probable that complex synergistic interactions are occurring between UVR, RA, and keratinocyte-derived paracrine factors to enhance tyrosinase activity and cell division in these quiescent melanocytes.

We would like to thank Mr Bryan Robertson, Ms Angela Nikolic, and Dr. Nalini Dissanayake for their excellent technical assistance. Financial assistance was provided by Janssen-Cilag Pty Ltd through a fellowship to Dr. Welsh provided by the Australasian College of Dermatologists.

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