Modulation of skin cell functions by transforming growth factor- β 1 and ACTH after ultraviolet irradiation

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Abstract

The adaptive responses in skin to ultraviolet (UV) radiation include increased cornification of keratinocytes and increased synthesis and distribution of melanin by melanocytes.

The possible involvement of paracrine factors in the generation of these responses was studied in a novel two-stage culture model. Human melanocytes or keratinocytes were first irradiated or sham-irradiated and then the conditioned media collected from these cells after 24 h were used to treat unirradiated skin cells.

Immunofluorescent staining for transforming growth factor (TGF)- β 1 was increased in UV-irradiated keratinocytes compared with sham-irradiated cells. Increased TGF- β 1 was also detected in the culture media of irradiated keratinocytes. Treatment of unirradiated keratinocytes with conditioned media collected from UV-irradiated keratinocytes resulted in increased absolute numbers and percentages of cornified envelopes per well compared with treatment with conditioned media from sham-irradiated keratinocytes. The magnitude of this

effect increased with increased dose of initial irradiation. The effects of conditioned media from UVR-treated cells were mimicked by authentic TGF- β 1. Treatment of conditioned media from irradiated cells with an antibody shown to neutralise the effects of TGF- β 1 but not with a non-immune antibody of similar isotype, abolished this bioactivity of the conditioned media from UV-irradiated cells. Immunofluorescent staining for ACTH was also increased in UV-irradiated keratinocytes. Conditioned media from UV-irradiated keratinocytes increased tyrosinase activity of unirradiated melanocytes, an effect which was mimicked by authentic ACTH. This bioactivity of conditioned media from irradiated keratinocytes was abolished in the presence of an antibody which neutralised the activity of ACTH but not MSH. These results provide evidence to support the involvement of TGF- β 1 and ACTH in the cornification and pigmentary responses respectively of skin cells after UV exposure.

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Introduction

Exposure to ultraviolet (UV) irradiation results in a number of responses in irradiated skin which may be considered adaptive, that is, they are likely to provide some protection against further UV damage (Fitzpatrick 1986). These responses include increased thickness of the stratum corneum (cornification) (Fitzpatrick 1986) and increased pigment production and distribution (Szabo *et al.* 1982, Pawelek *et al.* 1992). Whilst some of the cellular changes are likely to be the result of direct interaction with UV, for instance through DNA (Gange *et al.* 1985, Gilchrest *et al.* 1993, Robert *et al.* 1996), it is likely that locally released agents, acting in a paracrine or endocrine manner, contribute to the observed responses.

Indeed, there is some evidence that altered cellular function may be found in sites that are not subject to direct irradiation, suggesting the involvement of humoral factors. Stierner *et al.* (1989) reported increased numbers of

melanocytes in UVB-exposed as well as shielded skin of human volunteers. Rosdahl (1979) also described increased melanocyte cell numbers in the shielded ears of mice after exposure of the other ear to UVB radiation. Conditioned media obtained from keratinocytes after exposure to UVB or UVA suppressed the induction of delayed hypersensitivity in mice (Swartz 1984, Aubin *et al.* 1991). These studies indicate that, apart from local effects, there is a possibility that some factors are released from irradiated cells into the circulation. Nevertheless, most released factors are likely to act mainly in a paracrine manner to modulate skin cell function after UV irradiation. Certainly, paracrine modulation of skin cell function has been documented under basal conditions (Halaban *et al.* 1988, Gordon *et al.* 1989, Archambault *et al.* 1995).

Although a large number of hormones, growth factors and cytokines are known to be released by skin cells as a result of solar irradiation, their subsequent involvement in

the responses to this irradiation is somewhat speculative (Halaban *et al.* 1988, Kupper *et al.* 1989, Luger 1989, Kock *et al.* 1990, Kramer *et al.* 1993). To study this problem, a two-stage model using cultured skin cells was developed. In the first step of this model, human keratinocytes or melanocytes were subjected to UV irradiation of various types and the direct effects on cell proliferation, cornification and pigmentation were measured (Dissanayake *et al.* 1993). In the second stage, conditioned media were collected from irradiated skin cells and used to treat unirradiated skin cells in an attempt to determine whether any of the adaptive responses such as increased cornification of keratinocytes or increased pigmentary activity of melanocytes could be induced by this conditioned media.

Our results indicate that adaptive responses could indeed be induced by conditioned media and provide some evidence for the involvement of transforming growth factor beta 1 (TGF- β 1) and adrenocorticotrophin (ACTH) in these processes.

Materials and Methods

Materials

Standard cell culture materials were obtained from CytoSystems (Sydney, Australia), Sigma Chemical Company (St Louis, MO, USA) and CSL (Sydney, Australia) as previously described (Dissanayake *et al.* 1993, McLeod *et al.* 1995). L-[3,5- 3 H]Tyrosine and [methyl- 3 H]thymidine were obtained from Amersham Australia Pty Ltd (Sydney, Australia). Extracellular matrix (ECM)-coated tissue culture plates were purchased from Cell Dynamics (Sydney, Australia). Ultima Gold scintillant was purchased from Packard Australia (Sydney, Australia).

Human ACTH (1–39), alpha melanocyte-stimulating hormone (α MSH) and TGF- β 1 from human platelets were obtained from Sigma Chemical Company. Anti-TGF- β 1 (monoclonal, immunoglobulin (Ig) Y) neutralising antibody raised in chickens and a non-immune antibody of similar isotype were purchased from Immuno Diagnostics (R & D Systems, Minneapolis, MN, USA). The TGF- β 1 antibody neutralised 100% of the bioactivity of human TGF- β 1 but not that of human TGF- β 2 and was stated to have no cross-reactivity with fibroblast growth factor, human platelet-derived growth factor, human interleukin (IL)- α , IL-1 β , IL-2, IL-3, IL-4, IL-6, IL-7, tumour necrosis factor (TNF)- α , TNF- β , GM-colony-stimulating factor (CSF), or G-CSF as determined by enzyme-linked immunosorbent assay. Fluorescein isothiocyanate (FITC)-labelled sheep anti-rabbit IgG was purchased from Silenus Laboratories (Sydney, Australia). Anti-TGF- β 1 (polyclonal) was from Sigma Chemical Company. ACTH antibody (IgG) specific for residues 18–25 of human ACTH (1–39) used for immunohistochemistry studies and Western blot was supplied by

Dr Jean Joss of Macquarie University, Sydney (Joss *et al.* 1990). Nonimmune IgG antibody was purchased from Sigma Chemical Company.

Culture of melanocytes

Human melanocytes were cultured as previously described by Ranson *et al.* (1988). In brief, cultures were routinely maintained in melanocyte growth medium (MGM) which consists of Essential Minimum Eagle Medium (EMEM) with 2 mM glutamine, 0.01 mM non-essential amino acids, 5% (v/v) heat inactivated fetal calf serum (FCS), 10 nM cholera toxin and 16–32 nM phorbol 12-myristate-13-acetate (PMA), pH 7.2. Prior to experiments, melanocytes were plated onto ECM-coated multiwell dishes in melanocyte growth medium and were maintained without PMA or cholera toxin for 48 h (Ranson *et al.* 1988, McLeod *et al.* 1995).

Culture of keratinocytes

Human keratinocytes from neonatal foreskins were propagated in keratinocyte growth medium which consists of Dulbecco's modification of Eagle's Medium (DMEM) base medium with 0.3 mM Ca^{++} pH 7.4 with the following additives: 10% (v/v) heat inactivated FCS, 2.2 g/l NaHCO_3 , 10 mM HEPES buffer, 10 ng/ml epidermal growth factor, 0.5 $\mu\text{g/ml}$ hydrocortisone and 10^{-10} M cholera toxin, penicillin 30 mg/l, and streptomycin 50 mg/l, as previously described (Dissanayake *et al.* 1993).

UV sources

As a source of UV irradiation, one FS20T12 UVB and one FL20SBL UVA (NSW Ultraviolet, Sydney, Australia) tube were mounted on a lamp housing. Two filters, cellulose acetate film and photocopier film (Celcast) were chosen in order to allow for transmission of predominantly UVA or mixed UVA+B irradiation. These filters were subjected to transmission analysis using a Hitachi UV-VIS variable wavelength spectrophotometer (model 100–10). It was found that the cellulose acetate filter had very little transmission of wavelengths below 290 nm but transmitted >80% at wavelengths above 310 nm. The photocopier film showed no transmission below 310 nm, <20% at 315 nm and >50% above 325 nm. Transmittance was also monitored after increased exposure to UVR and the filters were changed on a regular basis. The irradiance of the lamps with filters were measured with an IL 1700 Research Radiometer with tungsten calibration. Irradiances with the cellulose acetate filter were UVB 0.08 mW/cm^2 and UVA 0.20 mW/cm^2 . With the photocopier film the corresponding values were UVB 0.002 mW/cm^2 and UVA 0.37 mW/cm^2 . The solar simulator used in these experiments, which is comprised of a multiple lamp array configured to simulate the full spectrum of terrestrial

sunlight at the equator at mid-summer noon and variable throughout each major spectral bandwidth, has previously been described in detail (Dissanayake *et al.* 1993). It should be noted that the use of the cellulose acetate filters with all UV sources mimics the action of natural atmospheric UV absorbers, but in so doing almost completely blocks the most biologically active wavelengths of the UV range, below 290 nm, so that a relatively high total UVB dose is required for a biological effect.

Irradiation of cells

Second or third passage keratinocytes were used for the experiments. Keratinocytes were plated onto uncoated plastic in keratinocyte growth medium (KGM) and melanocytes were plated onto bovine corneal endothelial-derived extracellular matrix-coated wells in MGM (Ranson *et al.* 1988, McLeod *et al.* 1995). Cells were irradiated 24 h after seeding. The doses of UV irradiation used in this study were based on our previous study (Dissanayake *et al.* 1993).

Before irradiation, the medium was aspirated and replaced with 3–4 drops of phosphate-buffered saline (PBS) (without calcium or magnesium). The appropriate filter was fitted and the cells were kept under the UV lamps or the solar simulator with the lid open. Immediately after the irradiation, the PBS was aspirated, 500 µl growth medium was added into each well and the cells incubated for 24 h except where stated, before collection of conditioned media. Sham-irradiated cells were handled in an identical manner but were covered with black cardboard rather than the filters at the time of irradiation.

Studies with conditioned medium

After the irradiation, cells were incubated with KGM or MGM for 24 h unless otherwise stated. At the end of the incubation, conditioned media were collected into sterile tubes and stored in a -80°C freezer until use. Unirradiated keratinocytes were seeded into 24-well plates in KGM. Twenty-four hours after seeding, UV-conditioned medium was added to each well at a concentration of 30% (v/v) in a total volume of 1 ml. This concentration of conditioned medium had been shown in preliminary studies to produce maximal changes in the parameters measured in target cells. Cells were incubated with conditioned media for 36 h. Conditioned medium from sham-irradiated cells was used at the same concentration as a control. Unirradiated melanocytes were plated on ECM-coated multiwell plates in MGM. Twenty-four hours after seeding, the medium was changed to growth medium without PMA or cholera toxin. After a further 48 h, UV-conditioned medium was added to the cells at a concentration of 30% (v/v) in a total volume of 1 ml and the cells were incubated for 36 h. Conditioned medium from sham-irradiated cells was used as a control.

Antibody studies

Test agents, TGF- β 1, ACTH, and α MSH were directly added in an aqueous vehicle. For studies with neutralising antibodies, the antigen and antibody were allowed to react in a small volume of culture media before being added to the cells. Similarly, UV-conditioned media or sham-irradiated conditioned media were allowed to react with neutralising antibody before being added to the cells.

Cell counts

Cells were counted after detachment with trypsin in a haemocytometer or Model B Coulter counter (Coulter Electronics, Hialeah, FL, USA).

Cornified envelopes

Cornified envelope formation was measured according to the protocol of McLane *et al.* (1990). Cells were detached with trypsin and a known volume of cell suspension was dispensed into Eppendorf tubes and centrifuged. A solution of 2% sodium dodecyl sulphate and 10 mM dithiothreitol was added to the cell pellets. After incubation at room temperature for 30 min, cornified envelopes were counted in a haemocytometer.

Tyrosinase activity

Tyrosinase activity was measured in cell lysates by a modification of the method of Pomerantz (1966), as previously described by Ranson *et al.* (1988) and McLeod *et al.* (1995). Briefly, aliquots of cell lysate in sodium phosphate buffer (pH 6.8) were incubated with 50 mM dihydroxyphenylalanine (DOPA) and L-[3,5- ^3H]tyrosine for 4 h. At the end of the incubation, unreacted [^3H]tyrosine was removed by adsorption onto 10% (w/v) charcoal in 0.1 M citric acid followed by treatment with 50% (w/v) Dowex resin solution. The supernatant containing [^3H]H $_2$ O was mixed with Ultima Gold scintillant and counted in a beta counter (Packard, Sydney, Australia).

Immunofluorescence studies

These experiments were carried out 24 h after UV irradiation. Following fixation of cells with methanol for 30 s, cells were incubated in 3% (v/v) goat serum in 0.1% BSA in PBS for 30 min at 37°C to reduce non-specific staining. The cells were subsequently incubated overnight at 4°C with TGF- β 1 (IgG) or ACTH (IgG) antibody, diluted in PBS, supplemented with 3% goat serum and 0.1% BSA. The cells were then treated with a 1:50 dilution of anti-rabbit IgG-conjugated FITC for 1 h in the dark. All steps were separated by washes with PBS containing 0.1% BSA. Finally, the cells were mounted in

3% glycerol in PBS. In control experiments, the TGF- β 1 or ACTH antibody was replaced with rabbit non-immune (similar isotype) IgG.

Immunoassays, Western blot and Northern blot analysis

TGF- β 1 in cell culture conditioned media was measured by Predicta ELIZA kit (Genzyme Diagnostics, Cambridge, MA, USA) formulated for use with tissue culture media. According to the manufacturer, there was no significant cross-reactivity with TGF- β 2 or - β 3 and 6.9% cross-reactivity with TGF- β 5. A double antibody RIA kit (ICN Pharmaceuticals, Costa Mesa, CA, USA) was used in an attempt to measure ACTH in tissue culture media. Neither this kit, nor others available, were formulated for use with tissue culture media.

Proteins from lyophilised samples of culture media from sham- and UV-irradiated media were separated by SDS-PAGE and transferred to nitrocellulose by semi-dry electrophoresis. Blots were incubated with the ACTH antibody (Joss *et al.* 1990) and signals were detected using a 125 I-labelled rabbit anti-mouse second antibody (New England Nuclear, Boston MA, USA). RNA was extracted from sham- and UV-irradiated keratinocytes, separated by electrophoresis and transferred to a nylon membrane (Genescreen, Dupont, Wilmington, DE, USA) by Northern blot as previously described (Nelson *et al.* 1996). The membranes were then hybridised with a cDNA probe to TGF- β 1 (ATCC, Rockville, MD, USA) and with a riboprobe to the housekeeping gene 36B4 (Chambon *et al.* 1984) and scanned using a Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical methods

Results are expressed as means $\pm$ S.D. for cells from one donor or means $\pm$ S.E. for pooled results from several donors. The means for each point for any one donor were generated from triplicate or quadruplicate determinations. The effects of treatments with one dependent variable such as the cell numbers per well were compared using Student's *t*-test for unpaired samples or where appropriate, a one-way analysis of variance coupled with a Ryans Q test. For tyrosinase activity, where results were corrected for cell numbers, the standard deviations of the ratios were calculated using the method of Colquhoun (1971) before application of the other tests.

Results

Keratinocytes

Incubation of unirradiated keratinocytes for 36 h with conditioned media (CM) collected from keratinocytes 24 h after exposure to solar-simulated irradiation

Figure 1 Effects of keratinocyte-conditioned media collected after solar-simulated irradiation (S-KCM) on cell numbers (a) or cornified envelope formation (b) in unirradiated keratinocytes cultures. Cells were incubated with S-KCM for 36 h. Absolute numbers of cornified envelopes per well were 146 ± 18 (Sham), 191 ± 9 (1.3 J/cm²), and 199 ± 4 (3.9 J/cm²). Values shown are means $\pm$ S.D. of triplicate determinations. *** $P < 0.005$, **** $P < 0.001$ compared with control keratinocytes incubated with sham-irradiated cells.

(S-KCM) resulted in a dose-dependent inhibition of keratinocyte cell numbers compared with control keratinocytes exposed to conditioned medium from sham-irradiated cells (Fig. 1A). The cell numbers decreased to

Table 1 Effects of conditioned media collected after UVA+B or UVA irradiation on keratinocyte cell numbers and cornified envelope formation. Values shown are the means $\pm$ S.D. of triplicate determinations

UV treatment (mJ/cm ²) ^a	Cell numbers per well	Cornified envelopes per well
UVB		
Sham-irradiated	35 682 $\pm$ 3153	941 $\pm$ 20
105 (filtered UVB)	29 670 $\pm$ 1462*	1335 $\pm$ 45***
210 (filtered UVB)	21 233 $\pm$ 1294***	1735 $\pm$ 39***
UVA		
Sham-irradiated	34 666 $\pm$ 1449	864 $\pm$ 38
1100	29 050 $\pm$ 1457**	1118 $\pm$ 108**
3400	22 750 $\pm$ 2182***	1631 $\pm$ 20***

^aThe UV doses given in this table are the UV doses of irradiation given to keratinocytes from which conditioned media were collected. With the cellulose acetate filter, the filtered UVA doses for these cells were 240 mJ/cm² and 480 mJ/cm².

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$, significantly different from control values.

84 $\pm$ 2% ($P < 0.001$) of control after treatment with S-KCM collected after 1.3 J/cm² total irradiation (which includes filtered UVB and UVA) and to 72 $\pm$ 1.7% ($P < 0.001$) of control with conditioned medium collected after 3.9 J/cm² irradiation. In a similar experiment repeated with cells from a different donor, the corresponding drops in cell numbers were to 84 $\pm$ 11% ($P < 0.001$) and 69 $\pm$ 4.5% ($P < 0.001$) of control respectively after treatment with 1.3 J/cm² and 3.9 J/cm² S-KCM. Of cell lines from 5 different donors, treatment with S-KCM (3.9 J/cm²) caused an average drop in cell numbers to 82 $\pm$ 6 (S.E.)% ($P < 0.001$) of control.

Exposure of unirradiated keratinocytes for 36 h to S-KCM stimulated cornified envelope formation (Fig. 1B). Incubation in conditioned media harvested from keratinocytes after exposure to 1.3 J/cm² solar-simulated irradiation increased cornified envelope formation to 150 $\pm$ 16% ($P < 0.01$) of control while S-KCM (3.9 J/cm²) caused an increase to 167 $\pm$ 12% ($P < 0.005$). In cell lines from 4 different donors, an average increase in cornified envelopes to 207 $\pm$ 17 (S.E.)% ($P < 0.005$) of control was observed after treatment with S-KCM (3.9 J/cm²).

Conditioned media collected from keratinocytes after exposure to UVA+B (UVA+B-KCM) or UVA (UVA-KCM) produced similar effects when used to treat unirradiated keratinocytes (Table 1). Dose-dependent inhibition of unirradiated keratinocyte numbers was seen with conditioned media collected from cells exposed to either radiation type. Furthermore, dose-dependent increases in cornified envelope formation were observed when unirradiated keratinocytes were incubated with conditioned media collected after UVA+B or UVA irradiation (Table 1).

Figure 2 demonstrates the immunofluorescence stain of keratinocytes for TGF- β 1. There is a clear, widespread staining for TGF- β 1 in UV-irradiated cells (a) compared with sham-irradiated cells (c). Use of a non-specific antibody of a similar isotype to that of the TGF- β 1 antibody with this protocol resulted in little fluorescence background in irradiated or non-irradiated cells (b and d). Measured by ELIZA kit, TGF- β 1 in the culture media increased significantly with UV exposure in cultures from 3 separate donors. In one cell line, TGF- β 1 concentrations were < 0.1 ng/ml (limit of detection of assay) in media from sham-irradiated cells increasing to 0.17 ± 0.04 ng/ml after exposure to 25 mJ/cm² UVB and to 0.25 ± 0.02 ng/ml after 75 mJ/cm²; in media from keratinocytes of a second donor, TGF- β 1 was again undetectable in sham-irradiated cultures but increased to 0.14 ± 0.04 ng/ml after 50 mJ/cm² and to 0.5 ± 0.03 ng/ml after 100 mJ/cm² UVB. In the third cell strain there was a 2.5-fold increase from 0.29 ± 0.06 ng/ml in sham-irradiated cultures to 0.73 ± 0.12 ng/ml after 100 mJ/cm² UVB. TGF- β 1 message was detected on Northern blots from both sham- and UV-irradiated keratinocytes from two cell strains. After correction for loading, however, no significant increase in TGF- β 1 message was seen in samples from irradiated cultures.

Since TGF- β 1 is known to increase cornified envelope formation (Reiss & Sartorelli 1987) and since TGF- β 1 detection increased after UV irradiation, the effects of authentic TGF- β 1 alone on keratinocyte cell numbers and cornified envelope formation were tested. A concentration of 10 ng/ml TGF- β 1 was used in accord with previous work (Pietenpol *et al.* 1990). Cell numbers decreased by $25 \pm 5.5\%$ ($P < 0.005$) while there was a $180 \pm 11\%$ ($P < 0.005$) increase in cornified envelope formation after incubation of keratinocytes with TGF- β 1 for 36 h. These effects of TGF- β 1 on keratinocytes were abolished when the keratinocytes were incubated with both TGF- β 1 and an anti-TGF- β 1 neutralising antibody (control: cell numbers $83 \pm 9 \times 10^3$ /well, cornified envelopes 310 ± 20 /well; TGF- β 1: cell numbers $58 \pm 6 \times 10^3$ /well, cornified envelopes 557 ± 33 /well; TGF- β 1+antibody: cell numbers $83 \pm 5 \times 10^3$ /well, cornified envelopes 370 ± 11 /well).

In view of these results, the UV-conditioned media were tested with an anti-TGF- β 1 neutralising antibody, to see whether the antibody would inhibit the bioactivity of UV-KCM. Cells were incubated with S-KCM alone or S-KCM with TGF- β 1 antibody for 36 h before assay. Growth medium and sham-irradiated conditioned medium were treated in a similar manner. As shown in Fig. 3, the presence of TGF- β 1 antibody had no significant effect on cornified envelope formation in cells incubated in growth medium or media harvested from sham-irradiated cells. In contrast, the increase in cornified envelope formation noted in unirradiated keratinocytes incubated with S-KCM was abolished in the presence of

Figure 2 Direct immunofluorescent staining of keratinocytes for TGF- β 1 after exposure to UVR from a UVA+B source. (a) UV-irradiated keratinocytes stained with TGF- β 1 antibody. (b) UV-irradiated keratinocytes stained with a non-specific antibody of a similar isotype to the TGF- β 1 antibody. (c) Sham-irradiated keratinocytes stained for TGF- β 1. (d) Sham-irradiated keratinocytes stained with a non-specific antibody of a similar isotype to the TGF- β 1 antibody. Original magnification $\times 100$. Scale bars represent 30 μ m.

anti-TGF- β 1 neutralising antibody. Similarly, the presence of TGF- β 1 antibody had no effect on cell numbers incubated in growth medium or media harvested from sham-irradiated cells. In contrast an 84 ± 3 (s.d.)% inhibition of cell numbers observed after treatment with S-KCM was abolished in the presence of anti-TGF- β 1 neutralising antibody. Similar results were also obtained in a second experiment using cells from a different donor. A non-immune antibody of similar isotype to the TGF- β 1 antibody did not inhibit the bioactivity of UV-KCM on keratinocytes and had no significant effect on cell numbers or cornified envelope formation of keratinocytes in the presence of growth media or media from sham-irradiated cells.

Melanocytes

Incubation for 36 h with conditioned media harvested from keratinocytes or melanocytes 24 h after solar, UVA+B or UVA irradiation stimulated tyrosinase activity of melanocytes, compared with incubation with conditioned media from sham-irradiated cells (Table 2). In four different cell lines after treatment with S-KCM (3.9 J/cm^2), the average increase in tyrosinase activity was

151 ± 16 (s.e.)% ($P < 0.005$) of control. In two cell lines treated with CM from melanocytes irradiated with the solar simulator (1.3 J/cm^2), the average increase in tyrosinase activity was 134 ± 18 (range)%.

Figure 4 illustrates staining for ACTH in sham- and UV-irradiated keratinocytes. A bright fluorescent staining of keratinocytes for ACTH was observed in UV-irradiated cells (a) compared with sham-irradiated cells (c). Use of a non-specific antibody of a similar isotype to that of the ACTH antibody resulted in little fluorescence stain in irradiated or non-irradiated cells (b and d). Measurable concentrations of ACTH were not detected in culture media from sham- or UV-irradiated keratinocytes by commercial kits, although none were designed for use with tissue culture fluid, but ACTH was present by Western blot in culture media from both sham- and UV-irradiated keratinocytes.

In view of these results, unirradiated melanocytes were incubated with authentic ACTH. Incubation of melanocytes for 36 h with ACTH at a concentration of 10^{-10} M (McLeod *et al.* 1995), resulted in an increase in tyrosinase activity from 133 ± 10 (counts per minute (c.p.m.)/1000 cells) to 210 ± 21 ($P < 0.005$). This stimulation of tyrosinase was abolished in the presence of an antibody to the

Figure 3 Neutralizing effect of antibody to TGF- β 1 on cornified envelope formation in unirradiated keratinocytes treated with S-KCM. Keratinocytes were incubated with keratinocyte growth medium (KGM), sham-irradiated conditioned medium (KCM) or solar-simulated conditioned medium (S-KCM) with or without antibody to TGF- β 1 (ab) for 36 h before the assay. Values shown are the means $\pm$ S.D. of triplicate determinations. *** $P < 0.005$ compared with conditioned medium from sham-irradiated cells and from keratinocyte growth medium.

18–24 region of ACTH (147 ± 16 c.p.m./1000 cells). The cross reactivity of the ACTH antibody to α MSH was tested. Incubation of melanocytes for 72 h with α MSH at a concentration of 10^{-6} M (McLeod *et al.* 1995) caused a $127 \pm 18\%$ increase in tyrosinase activity and the ACTH antibody (Ab) failed to abolish this effect (MSH+ACTH Ab $132 \pm 8\%$ of control, $P < 0.005$).

When the neutralising antibody to ACTH was used with conditioned media from irradiated keratinocytes, the antibody had no effect on tyrosinase activity in the presence of melanocyte growth medium, had a small stimulatory effect on tyrosinase in the presence of conditioned medium from sham-irradiated keratinocytes, but it abolished the $230 \pm 24\%$ ($P < 0.001$) stimulation of tyrosinase seen when these melanocytes were incubated with solar-KCM (Fig. 5). In a second experiment, using conditioned media from irradiated or sham-irradiated melanocytes, the anti-ACTH antibody abolished the small but significant increase in tyrosinase activity in melanocytes exposed to conditioned media from irradiated cells ($122 \pm 4\%$, $P < 0.005$). Use of a non-immune antibody of similar isotype to that of ACTH had no effect on bioactivity of UV-KCM in melanocytes.

Table 2 Effect of conditioned media collected after solar, UVA+B or UVA irradiation, on tyrosinase activity of melanocytes^a. Values shown are the means $\pm$ S.D. of triplicate determinations

UV treatment (mJ/cm ²) ^b	Tyrosinase activity (% of control) ^d
UV-KCM	
S-KCM (3900)	229 $\pm$ 2***
UVB-KCM (210) ^c	115 $\pm$ 2.8*
UVA-KCM (3400)	177 $\pm$ 23****
UV-MCM	
S-MCM (1300)	158 $\pm$ 23**
UVB-MCM (210) ^c	172 $\pm$ 6****
UVA-MCM (560)	141 $\pm$ 7.2***
UVA-MCM (3400)	159 $\pm$ 7.5****

^aMelanocytes plated on ECM were maintained in the absence of PMA and cholera toxin for 48 h prior to the addition of UV conditioned media.

^bThe UV doses given in this table are the UV doses of irradiation given to keratinocytes and melanocytes from which conditioned media were collected.

^cWith the cellulose acetate filter, the filtered UVA doses for these cells were 525 mJ/cm².

^dControl tyrosinase activities in cells from different donors ranged from 1676–2021 c.p.m./1000 cells/4 h.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$, significantly different from control values.

Discussion

Ultraviolet radiation exposure produces thickening of the stratum corneum and increased melanin pigmentation in the skin, which act as two defensive barriers against further UVR damage (Gange *et al.* 1985, Fitzpatrick 1986). These effects have been well documented after direct exposure to UVR. The possible novel signal pathways that induce pigmentation after exposure to direct UVR have been addressed in recent years. Gilchrist *et al.* (1993) reported that when human melanocytes were treated with a DNA repair enzyme, T4 endonuclease V, at the time of irradiation a marked increase in tyrosinase activity compared with control groups was noted. Involvement of protein kinase C-dependent pathways in increased melanogenesis has been described by Gordon and Gilchrist (1989), Park *et al.* (1993), McLeod *et al.* (1995) and Oka *et al.* (1996).

The idea that skin cells release soluble factors which have autocrine and paracrine effects has been described in previous studies (Rosdahl 1979, Halaban *et al.* 1988, Kupper *et al.* 1989, Kratz *et al.* 1991). Indeed a large number of growth factors and other cytokines have been shown to be released as a result of UVR (Swartz 1984, Kupper *et al.* 1989, Luger 1989, Aubin *et al.* 1991, Kramer *et al.* 1993). In this study, we extended these observations to show that conditioned media collected from irradiated skin cells were able to induce, *in vitro*, effects in unexposed skin cells which mimic the *in vivo* responses. Conditioned media harvested from keratinocytes after UV irradiation of

Figure 4 Direct immunofluorescent staining of keratinocytes for ACTH after exposure to UVR from an UVA+B source. (a) UV-irradiated keratinocytes stained with ACTH antibody. (b) UV-irradiated keratinocytes stained with a non-specific antibody of a similar isotype to that of the ACTH antibody. (c) Sham-irradiated keratinocytes stained for ACTH. (d) Sham-irradiated keratinocytes stained with a non-specific antibody of a similar isotype to that for ACTH. Original magnification $\times 100$. Scale bars represent 30 μm .

various types increased pigmentary activity of unirradiated melanocytes and increased cornification of unirradiated keratinocytes. Similarly, conditioned media harvested from UV-irradiated melanocytes increased tyrosinase activity of melanocytes.

Increased epidermal thickness following UV exposure is brought about partly by increased proliferation of keratinocytes and particularly by increases in the cornified layer (Fitzpatrick 1986). Even in the absence of melanocytes, vitiliginous skin showed high values of sun protection factor after repeated exposure to UV radiation (Morison 1985). Whilst our model does not provide an explanation for the hyperproliferation of keratinocytes noted *in vivo* (Pearse *et al.* 1987, Hashimoto *et al.* 1991) and *in vitro* (Dissanayake *et al.* 1993) after direct exposure to UV irradiation, the results show that UV exposure causes keratinocytes to release a soluble factor or factors which promote cornification.

The possibility that this factor might be TGF- β 1 was investigated. TGF- β 1 is a known potent growth inhibitor for many cell types, including epithelial cells (Shipley *et al.* 1986, Pietenpol *et al.* 1990). TGF- β 1 has been reported to cause a reversible growth arrest of keratinocytes at the G1

phase of the cell cycle (Moses *et al.* 1987, Pietenpol *et al.* 1990). Arrest of cell growth seems to be a prerequisite for initiation of differentiation of keratinocytes (Fuchs 1990). It has also been reported that TGF- β 1 enhanced formation of cornified envelopes in normal human keratinocytes which were maintained in low calcium growth medium supplemented with epidermal growth factor (EGF) and this effect was not seen in the absence of EGF (Reiss & Sartorelli 1987). The study by Matsumoto *et al.* (1990) reported, however, that TGF- β 1 enhanced formation of cornified envelopes in normal human keratinocytes in high calcium conditions, but inhibited this activity in low calcium media. The growth inhibitory effect of TGF- β 1 was seen in both high and low calcium conditions. Mansbridge & Hanawalt (1988) reported that the synthesis of keratins 6 and 16, which are usually associated with hyperproliferation of keratinocytes, was increased after treatment with TGF- β 1, while synthesis of keratin 1, associated with keratinocyte differentiation, was inhibited. In other studies, sodium butyrate enhanced cornified envelope formation in cultured human keratinocytes (Schmidt *et al.* 1989). Neutralising antibodies to TGF- β 1 effectively reduced this effect (Wang *et al.* 1992) while

Figure 5 Neutralising effect of ACTH antibody on tyrosinase response in unirradiated melanocytes treated with S-KCM. Melanocytes were incubated with melanocyte growth medium (MGM), sham-irradiated conditioned medium (KCM) or solar-simulated conditioned medium (S-KCM) with or without ACTH antibody (ab) for 36 h before the assay. Values are the means $\pm$ S.D. of triplicate determinations. *** $P < 0.005$, **** $P < 0.001$ compared with cells incubated with conditioned medium from sham-irradiated cells or with melanocyte growth medium.

additional TGF- β 1 significantly enhanced the effect of sodium butyrate on keratinocyte differentiation. Even though there are some discrepancies among these reports, there are clear indications that TGF- β 1 is an important modulator of keratinocyte maturation.

In the present study, increased production of TGF- β 1 by keratinocytes after UV irradiation was demonstrated by immunofluorescence and by immunoassay. Furthermore, authentic TGF- β 1 mimicked the action of conditioned media collected from UV-irradiated cells on unexposed cells. This effect of UV-conditioned media on cornified envelope formation was abolished by a TGF- β 1 neutralising antibody. The results are consistent with the proposal that TGF- β 1 released by irradiated skin cells is at least partly responsible for the increased cornification which occurs after UV irradiation.

In relation to the other adaptive response to UV irradiation, detection of mRNA for pro-opiomelanocortin (POMC) has been reported in murine and human keratinocytes by Amornisiripanitch and Nordlund (1989), Schauer *et al.* (1994) and Wintzen *et al.* (1996) and in human melanocytes by Farooqui *et al.* (1993). Increased

POMC message levels in keratinocytes after UVR were reported by Wintzen *et al.* (1996). Regulatable secretion of α MSH and ACTH (Schauer *et al.* 1994), a 30 kDa POMC peptide as well as the products β -lipotropic hormone (β LPH) and β -endorphin (Wintzen *et al.* 1996) has been reported. To date, however, in terms of protein production, only α MSH by A431 epidermal carcinoma cells (Schauer *et al.* 1994) and β LPH in human keratinocytes have been reported to be increased by UV exposure. We extended these observations to demonstrate that ACTH immunoreactivity is increased in irradiated keratinocytes. The conditioned media collected from irradiated keratinocytes and melanocytes stimulated tyrosinase activity of unirradiated melanocytes. We also showed that this effect was mimicked by addition of ACTH (1–39) and that the ACTH response was abolished by a neutralising antibody to ACTH. Furthermore, the antibody which binds to the 18–24 region of ACTH abolished the tyrosinase enhancing effect of conditioned media collected from irradiated keratinocytes. The ACTH antibody used in this study did not abolish the effect of MSH on melanocytes. These results are consistent with the proposal that after UV irradiation, keratinocytes release ACTH or an ACTH-like factor(s) that may be at least partly responsible for the subsequent pigmentary response. These results do not preclude the involvement of other mechanisms in generating the responses to UVR, such as the reported increase in MSH receptors in mouse melanoma cells after UVR (Bologna *et al.* 1989).

The two-stage culture model used in the studies presented here, in which human skin cells receive direct UVR and their CM is then used to treat unirradiated skin cells, has proved useful to demonstrate the likely involvement of two paracrine factors – TGF- β 1 and ACTH – in the modification of skin cell activity after UVR. Further studies with this model and keratinocyte–melanocyte co-cultures are likely to provide additional insights into the mechanism of skin adaptation to UVR.

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