Granulocyte/macrophage colony-stimulating factor is an intrinsic keratinocyte-derived growth factor for human melanocytes in UVA-induced melanosis

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Recently we demonstrated that endothelins secreted from human keratinocytes act as intrinsic mitogens and melanogens for human melanocytes in UVB-induced melanosis. We show here that UVA-induced melanosis is associated with other keratinocyte-derived growth factors, secretion of which is specifically stimulated after exposure of human keratinocytes to UVA. Medium conditioned by UVA-exposed human keratinocytes elicited a significant increase in DNA synthesis by cultured human melanocytes in a UVA dose-dependent manner. Analysis of endothelin-1 and interleukin (IL)-1α in the conditioned medium by ELISA, both of which are major keratinocyte-derived cytokines involved in UVB-associated melanocyte activation, revealed that UVA exposure did not cause human keratinocytes to stimulate the secretion of the two cytokines. In contrast, the levels of several other cytokines such as IL-6, IL-8 and granulocyte/macrophage colony-stimulating factor (GM-CSF) were significantly increased in the conditioned medium of human keratinocytes after exposure to UVA at a dose of 1.0 J/cm^2. The gel chromatographic profile of UVA-exposed keratinocyte-conditioned medium demonstrated that there were two factors (P-1 and P-2) with molecular masses of approx. 20 and 1 kDa respectively that stimulate DNA synthesis in human melanocytes, and the larger species (P-1) also increased melanization as assessed by [^{14}C]thiouracil incorporation. Quantitative analysis of cytokines in chromatographic fractions by ELISA revealed the P-1 fraction to be consistent with the molecular mass profile of GM-CSF. Furthermore the stimulatory effect of the P-1 fraction on DNA synthesis in human melanocytes was neutralized by antibodies to GM-CSF, but not to basic fibroblast growth factor or stem cell factor. Binding and proliferation assays with recombinant GM-CSF demonstrated that human melanocytes possess specific binding sites for GM-CSF(K_d 2.11 nM; binding sites, 2.5–3.5 $\times$ 10^4 per cell), and recombinant GM-CSF at concentrations of more than 10 nM significantly stimulated DNA synthesis and melanization. These findings suggest that GM-CSF secreted by keratinocytes plays an essential role in the maintenance of melanocyte proliferation and UVA-induced pigmentation in the epidermis.

INTRODUCTION

Skin colour is an inherited trait maintained by several regulatory mechanisms in which melanocytes distributed within the bottom layer of the epidermis produce melanin polymer at a steady rate by the action of the specific enzyme tyrosinase [1–3]. Melanin synthesis occurs within unique biological organelles, melanosomes, that are transferred via dendrites of melanocytes into epidermal keratinocytes, with subsequent distribution throughout the epidermis, undergoing degradation during keratinization [4,5]. Thus skin pigmentation is associated primarily with the ability of melanocytes to produce melanin. Under the influence of stimuli such as UV exposure [6–9] and allergic contact dermatitis [10], melanocytes proliferate and produce elevated levels of melanin polymer, resulting in cutaneous hyperpigmentation. Recent reports [11–13] that some growth factors produced by human keratinocytes can stimulate the proliferation and melanogenesis of cultured human melanocytes suggest that keratinocytes serve a central role in controlling melanocyte function in a paracrine fashion by providing several growth-factor-like materials. Recently we reported that human keratinocytes can produce and secrete the vasoconstrictive peptides endothelins (ETs) [14], which act as strong mitogens for human melanocytes through specific receptors involved with the activation of the protein kinase C (PKC)-related signal transduction pathway [15]. Of considerable interest is the finding that the above biological process is considerably accentuated when keratinocytes are exposed to UVB [14], implying that ETs are intrinsic mitogens for human melanocytes in UVB-induced melanosis.

Unlike the effects of UVB, little is yet known about how UVA exposure affects autocrine or paracrine linkage of cytokines between epidermal cells. UVA has been documented to elicit completely different biological and histological responses of epidermal cells and skin tissue despite the fact that UVA-induced pigmentation includes melanocyte responses identical, although to a smaller extent, with that evoked by UVB with regard to accentuated proliferation and melanization [9]. Therefore it is of considerable interest to clarify the cellular mechanisms involved in cutaneous pigmentation induced by UVA: if UVA-exposed human keratinocytes produce soluble factors that can stimulate DNA synthesis or melanogenesis of cultured human melanocytes,

Abbreviations used: bFGF, basic fibroblast growth factor; BPE, bovine pituitary extract; EGF, epidermal growth factor; ET, endothelin; GM-CSF, granulocyte/macrophage colony-stimulating factor; KCM, keratinocyte-conditioned medium; IL, interleukin; LT, leukotriene; MGM, serum-free melanocyte medium; PBS(−), calcium- and magnesium-free PBS; PG, prostaglandin; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SCF, stem cell factor; SFM, serum-free keratinocyte medium; TCA, trichloroacetic acid; TX, thromboxane.

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it will be worth while to determine the nature of the cytokines directly associated with melanocyte activation. Aside from studies focusing on the biological effects of exogenously added cytokines on human melanocytes, the approaches using the paracrine linkage of cytokines between keratinocytes and melanocytes would provide new insight into the cellular mechanisms involved with intrinsic cytokines that eventually take part in UVA-induced melanosis. Our previous study on allergy-induced melanosis [16] suggested that different stimuli, including UV irradiation, trigger human keratinocytes in different ways to produce and secrete completely different spectra of growth factors. In contrast, it is likely that human melanocytes undergo proliferation and differentiation in a similar fashion in response to different growth factors secreted from human keratinocytes. In this study, therefore, we determined whether the exposure of human keratinocytes to UVA stimulated the secretion of growth factors that can increase DNA synthesis and melanization of cultured human melanocytes, and identified their biological properties.

MATERIALS AND METHODS

Materials

Normal human keratinocytes, melanocytes and serum-free melanocyte medium (MGM) were obtained from Sankou Pure Chemicals (Tokyo, Japan). ET derivatives, ET ELISA kits, and interleukin (IL) derivatives were purchased from International Reagents Corp. (Kobe, Japan). IL-1α ELISA kits were purchased from Otsuka Pharmaceutical Corp. (Tokyo, Japan). Other ELISA and EIA kits were obtained from Amersham. Recombinant granulocyte/macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) were obtained from Research and Diagnostics Systems. Anti-GM-CSF and anti-basic fibroblast growth factor (anti-bFGF) were purchased from Oncogene Science, Inc. Anti-stem cell factor (anti-SCF) was purchased from Immunobiological Laboratories Co., Ltd. (Gunma, Japan). Prostaglandin (PG), thromboxane (TX) and leukotriene (LT) assay kits were purchased from Cayman Chemical Company. Serum-free keratinocyte medium (SFM), bovine pituitary extract (BPE) and epidermal growth factor (EGF) were obtained from Gibco Labs. Other chemicals were of reagent grade.

Cell cultures

Human keratinocytes were maintained in modified MCDB 153 (SFM) supplemented with 5 ng/ml EGF and 50 μg/ml BPE at 37 °C under a 5 % CO_2 atmosphere. IL derivatives or some growth factors were added to the culture medium as appropriate. Human melanocytes were maintained in modified MCDB 153 (MGM) supplemented with 1 ng/ml recombinant bFGF, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10 ng/ml phorbol 12-myristate 13-acetate (PMA), antibiotics (50 μg/ml gentamicin and 0.25 μg/ml amphotericin B), and 0.2 % (v/v) BPE at 37 °C with 5 % CO_2 as described previously [14]. PMA and bFGF were omitted from the medium as appropriate (control medium).

Determination of cytokine concentrations

To assay cytokines, human keratinocytes were seeded in T-75 flasks (Greiner) at a density of 5×10^4 to 1×10^5 cells per ml and cultured for 24–96 h. The medium was aspirated off and exchanged for fresh SFM containing 5 ng/ml EGF and 0.5 % BPE before adding other reagents, or exposure to UVB or UVA irradiation. The cells were irradiated with UVB or UVA at a dose of 25–200 mJ/cm^2 or 0.1–5 J/cm^2 after the medium was exchanged for calcium- and magnesium-free PBS [PBS(−)]. Immediately after phototreatment, the transferred medium was returned to each flask. After human keratinocytes were cultured in SFM medium supplemented with 0.5 % BPE for 4 days, the keratinocyte-conditioned medium (KCM) was collected and ET-1 was measured in 100 μl per well, and GM-CSF, G-CSF, IL-1α, IL-6 and IL-8 in 200 μl per well, by ELISA. The ET ELISA kit used was a solid-phase enzyme immunoassay method based on the 'multiple antibody sandwich' principle. A human purified polyclonal antibody specific for human ET-1, and human purified monoclonal antibodies specific for GM-CSF, G-CSF, IL-1α, IL-6 or IL-8, were attached to 96-well microtitre plates. Cytokines present in standards and unknowns were captured by the solid-phase antibody. Horseradish peroxidase-labelled rabbit anti-(human cytokines) IgG was added, which bound to multiple epitopes on the cytokines attached to the solid phase. Levels of immunoreactive cytokines were measured by absorbance at 490 nm on an ELISA plate reader (Bio-Rad, Model 3550). The amounts of cytokines in cultures were measured by comparing their absorbance with that produced by standards. The standard curve was linear from 5.0 to 1000 pg/ml for ET derivatives, GM-CSF, G-CSF, IL-6 and IL-8, and from 5.0 to 500 pg/ml for IL-1 derivatives. PGs, LTs and TX were measured by enzyme immunoassay by using antiserum against each PG, LT and TX.

UV irradiation

One or two days after plating the human keratinocytes, culture medium was removed from the cells. After three washes with 10 ml of PBS(−), 5 ml of PBS(−) was added to the cells. The cultures were irradiated once with UVB (Toshiba SE lamp) at doses ranging from 25 to 200 mJ/cm^2 (most of the energy was emitted within the UVB range 295–315 nm with a peak at 305 nm), or with UVA (Toshiba BLB lamp) at doses ranging from 0.1 to 5 J/cm^2 (most of the energy was emitted within the UVA range 310–400 nm obtained by removing contaminating UVB with a glass filter 0.5 cm thick, with a peak at 365 nm). Immediately after phototreatment, the transferred medium was returned to each flask. The irradiated cells were maintained in SFM medium supplemented with 0.5 % BPE at 37 °C in a 5 % CO_2 atmosphere.

Effects of KCM or GM-CSF on thymidine incorporation by melanocytes

Melanocytes were seeded in 24-well trays at a density of 4×10^4 to 1×10^5 cells per ml and cultured overnight in MGM (without PMA and bFGF) supplemented with 0.2 % BPE for 24 h as described previously [14]. Half of the medium (500 μl) was aspirated off and mixed with 200 μl of 10-fold concentrated medium conditioned by UVA-exposed keratinocytes or chromatographic fractions. The KCM was concentrated by freeze-drying. To determine the effects of anti-GM-CSF or other antibodies, 2.0 μg/ml anti-(human GM-CSF) IgG (or antibodies against other cytokines) was added to the culture medium. The cells were labelled with 1.0 μCi/ml [^{3}H]thymidine for the last 4–12 h of a 24 h incubation or for 24 h. After three washes with PBS(−), the cells were lysed with 2 M NaOH, then neutralized with 2 M HCl. Acid-insoluble material was precipitated with 4 vol. of 10 % (v/v) trichloroacetic acid (TCA), collected on glass filters, washed three times with 10 % TCA and once with ethanol, then dried. The radioactivity on the filters was measured in a liquid-scintillation counter [17].

Assay of melanogenic activity

Exogenous thiouracil has been reported to become part of the melanin polymer by binding to the quinones generated during the synthesis of melanin. This binding is dependent on tyrosinase activity [18,19] and there is a close correlation between the extent of [^{14}C]thiouracil incorporation and the tyrosine hydroxylase activity that is measured by the release of ^3H_2O as the result of the formation of L-3,4-dihydroxyphenylalanine from L-[3,4-^{3}H]tyrosine [20] in living mammalian pigment cells [14,15] and pigmented skin tissue [21]. Therefore the incorporation of [^{14}C]thiouracil into the TCA-insoluble fraction of melanocytes was measured as an indicator of tyrosinase activity. As a result of its preferential incorporation into melanin [18] and the rare presence of quinones in other eukaryotic cells, it is possible to use thiouracil incorporation as an indicator of melanin synthesis in pigment cells.

Melanocytes were seeded in 24-well culture trays at a density of $(3–8) \times 10^4$ cells per ml and cultured overnight in MGM (without PMA and bFGF) containing 0.2% BPE. After treatment with medium fractions (each 200 μl) or GM-CSF, the cells were labelled by incubation for 24 h with 0.5–1.0 μCi/ml [^{14}C]thiouracil. To assay the thiouracil incorporation, after three washes with PBS the cells were lysed with 2 M NaOH at 37 °C for 15 min and neutralized with 2 M HCl. The mixtures were mixed with scintillation fluid, and the radioactivity in each was measured in a liquid-scintillation counter.

Gel chromatography

Conditioned medium (200 μl) was applied to a TSK-2000SW column (7.5 mm × 600 mm) equilibrated with PBS. The medium was eluted with the same buffer at a flow rate of 1.0 ml/min, and 0.5 ml fractions were collected with a Bio-Rad HPLC system. The melanogenic and mitogenic activities of each fraction (200 μl) were evaluated by measuring [^{3}H]thymidine and [^{14}C]thiouracil incorporation respectively for 24 h at a dilution of 2:5 as described above.

Measurement of [^{125}I]GM-CSF binding

For GM-CSF binding assays, human melanocytes were seeded in 24-well culture trays at a density of $(5–8) \times 10^4$ cells per ml and cultured for 24 h. The medium was aspirated off and MCDB 153 medium containing 0.1% BSA was added, followed by culture overnight at 37 °C. After washing, binding studies were performed at 4 °C for 4 h with 0.5 μCi [^{125}I]GM-CSF (2000 Ci/mmol) in the presence or absence of unlabelled GM-CSF (0–1 μM). Binding was terminated by washing twice with ice-cold medium containing BSA, bound [^{125}I]GM-CSF radioactivity was measured and specific binding was calculated as total binding minus non-specific binding in the presence of 1 μM unlabelled GM-CSF [15].

RESULTS

Stimulation of DNA synthesis in melanocytes by UVA-exposed KCM

The culture medium conditioned for 4 days after exposing human keratinocytes to UVA significantly stimulated DNA synthesis of human melanocytes after incubation for 24 h (Table 1). DNA synthesis was markedly stimulated at UVA doses of 0.2 and 1.0 J/cm^2 when the conditioned medium was concentrated 10-fold and added at a 2:5 dilution to melanocyte cultures. In preliminary experiments on various dilutions of the conditioned medium added to melanocyte culture, the 2:5 dilution was optimal for expression of the stimulatory effect on human melanocyte DNA synthesis (results not shown).

Table 1 Stimulatory effect of the conditioned medium from UVA-exposed human keratinocytes on the DNA synthesis of cultured human melanocytes

The conditioned medium was concentrated 10-fold and added at a 2:5 dilution to cultured melanocytes. DNA synthesis was evaluated by measuring [^{3}H]thymidine incorporation for the last 12 h of a 24 h incubation. In non-irradiated experiments, PBS(−) was replaced in keratinocyte culture for the same period of irradiation and the conditioned medium was collected in the same manner as in the irradiation experiments. The values are means ± S.D. derived from three wells.

UVA (J/cm^2)	DNA synthesis (d.p.m. per well) (Irradiated)	(Non-irradiated)
0	1209 ± 371	1195 ± 274
0.1	1578 ± 323	1162 ± 142
0.2	5032 ± 803	1344 ± 282
1.0	4964 ± 503	1149 ± 144
3.0	4067 ± 419	1043 ± 279

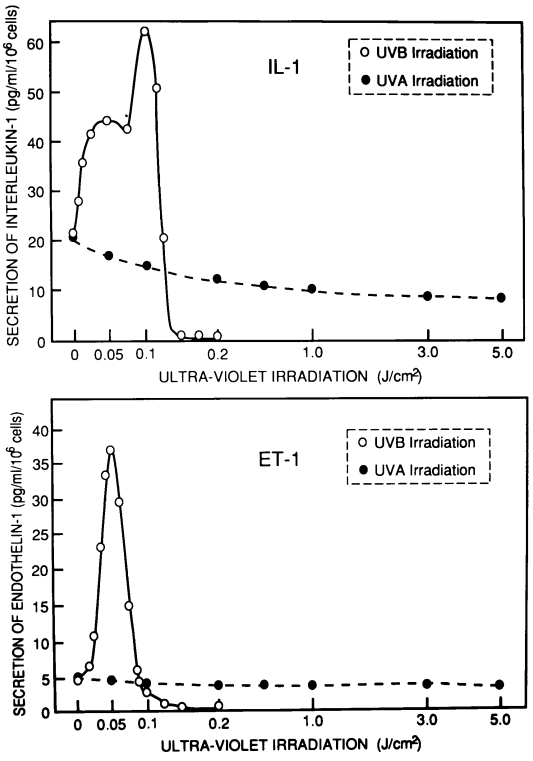

Figure 1 Effects of UVB or UVA exposure on the secretion of IL-1α (top panel) and ET-1 (bottom panel) into the culture medium of human keratinocytes

Keratinocytes were exposed once to the indicated dose of UVB or UVA light. The secreted levels of ET-1 and IL-1α in conditioned medium collected after 4 days of culture were measured with an immunoassay and are expressed as pg/ml per 10^6 cells (integrated secretion over 4 days).

Effects of UVA exposure on the secretion of ET-1 and IL-1α

Because ET-1 and IL-1α are cytokines essential for the stimulatory effect of the conditioned medium from UVB-exposed

Table 2 Effects of UVA exposure on the secretion of several cytokines into the medium of human keratinocytes

Keratinocytes were exposed once to UVA at a dose of 1.0 J/cm^2. The secreted levels of several cytokines in the conditioned medium collected after 4 days of culture were measured with an immunoassay and are expressed as pg/ml (integrated secretion over 4 days). The values are means ± S.D. derived from three wells. N.D., not detectable.

	Cytokine excretion		
Factors	Control (pg/ml)	UVA (pg/ml)	Percentage of control
IL-1α	20.2 ± 3.7	18.1 ± 6.2	89.6
IL-6	0.27 ± 0.18	0.35 ± 0.21	129.6
IL-8	20.5 ± 4.3	28.5 ± 4.6	139.0
GM-CSF	57.0 ± 7.6	114.3 ± 6.7	200.5
ET-1	25.3 ± 4.6	22.8 ± 8.0	90.1
G-CSF	N.D.	N.D.	—
PGE_2	677.0 ± 24.9	780.0 ± 20.8	115.2
$PGF_2\alpha$	170.9 ± 5.5	107.3 ± 4.6	62.8
TXB_2	3.3 ± 1.8	3.4 ± 3.9	103.0
LTB_4	7.5 ± 1.3	7.5 ± 1.5	100.0
LTC_4	N.D.	N.D.	—

keratinocytes on DNA synthesis of melanocytes [14], we tested whether UVA exposure also induces an increase in their secretion at concentrations high enough to elicit cellular responses to human melanocytes. As shown in Figure 1, when human keratinocytes were exposed to increasing doses of UVA irradiation, the integrated secretion (pg/ml per 10^6 cells) of ET-1 and IL-1α into medium throughout 4 days in culture was not stimulated even at a UV dose of 5.0 J/cm^2. In contrast, UVB irradiation induced marked stimulation of both ET-1 and IL-1α secretion with a maximal effect at doses of 0.05 and 0.1 J/cm^2 respectively.

Cytokines secreted from UVA-exposed keratinocytes

To clarify which cytokines are secreted on UVA irradiation, we measured the levels of several cytokines in the medium of keratinocyte cultures 4 days after a single UVA exposure at a dose of 1.0 J/cm^2. Secretion of IL-6 and IL-8 was slightly, and of GM-CSF significantly, increased, but no such increases in IL-1α or ET-1 secretion were seen (Table 2). Furthermore there were no significant increases in the secretion of several eicosanoids.

Gel chromatography of conditioned medium

To obtain the molecular profiles of the cytokines responsible for stimulating DNA synthesis in melanocytes, gel chromatography of UVA-exposed KCM was performed and each fraction was tested for ability to stimulate DNA synthesis in cultured human melanocytes. Gel chromatography demonstrated the presence of two different factors (P-1 and P-2), with molecular masses of about 20 and 1 kDa respectively, capable of stimulating DNA synthesis in human melanocytes (Figures 2a–2c). Whereas the larger molecule (P-1) was effective in increasing melanization (2286 ± 231 d.p.m. per well compared with 1224 ± 93 d.p.m. per well in the control) as measured by [^{14}C]thiouracil incorporation for 24 h when 200 μl was added at a 2:5 dilution to melanocyte cultures, the smaller species (P-2) had no stimulatory effect on melanization (1564 ± 52 d.p.m. per well compared with 1224 ± 93 d.p.m. per well in the control). Quantitative analysis of

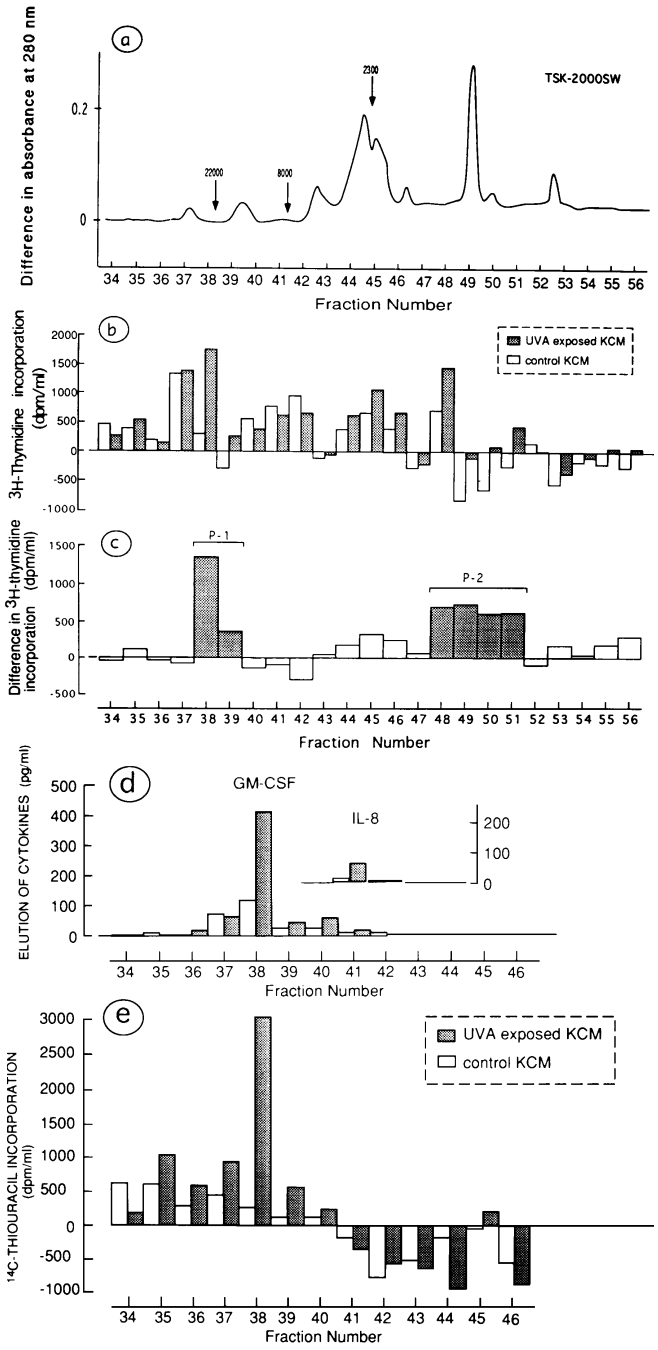

Figure 2 Gel chromatography (TSK-2000SW) of UVA-exposed KCM

(**a**) Differences in absorbance at 280 nm between UVA- and non-UVA-exposed (control) KCM. (**b**) [^{3}H]Thymidine incorporation in each chromatographic fraction. (**c**) Differences in [^{3}H]thymidine incorporation in each fraction (with the same fraction number) between UVA- and non-UVA-exposed (control) KCM. (**d**) Quantitative analysis of GM-CSF and IL-8 in each chromatographic fraction by ELISA. (**e**) [^{14}C]Thiouracil incorporation in each chromatographic fraction. The stimulatory effects of chromatographic fractions from UVA-exposed and non-exposed KCM on DNA synthesis were compared by measuring the incorporation of [^{3}H]thymidine into cultured melanocytes after incubation at a 2:5 dilution for 24 h (**b**). The stimulation of DNA synthesis in each fraction due to UVA irradiation was expressed as the difference in [^{3}H]thymidine incorporation between the fractions (with the same fraction number) from UVA-exposed and non-exposed (control) KCM (**c**). Quantitative analyses of GM-CSF and IL-8 in chromatographic fractions from UVA-exposed and non-exposed (control) KCM were performed by ELISA (**d**) and compared with the fractions (P-1 and P-2) containing activities stimulatory for DNA synthesis and/or melanization with regard to molecular mass estimated by the fraction number. The stimulatory effect of each chromatographic fraction (200 μl) on melanin synthesis was measured by incorporation of [^{14}C]thiouracil into cultured melanocytes after incubation at a 2:5 dilution for 24 h (**e**).

Table 3 Stimulatory effects of recombinant GM-CSF on DNA synthesis and melanization in cultured human melanocytes

Recombinant GM-CSF was added at indicated concentrations to human melanocyte cultures for 24 h. DNA synthesis and melanine synthesis were assayed by measuring [^{3}H]thymidine incorporation during the last 4 h of the 24 h incubation and [^{14}C]thiouracil incorporation during the 24 h incubation respectively. The values are means ± S.D. derived from three wells.

[GM-CSF] (nM)	DNA synthesis (d.p.m. per well)	Melanin synthesis (d.p.m. per well)
0	933 ± 68	1657 ± 124
1	995 ± 106	1783 ± 194
10	1270 ± 197	2250 ± 142
50	1501 ± 148	—
100	1862 ± 109	3131 ± 217
250	1892 ± 237	—

Table 4 Steady-state binding profile of GM-CSF on human melanocytes

Confluent cells were incubated at 4 °C for 4 h with [^{125}I]GM-CSF in the presence or absence of unlabelled GM-CSF at various concentrations. Specific cell-associated [^{125}I]GM-CSF binding was measured in melanocytes when incubated at 4 °C for 4 h in assay medium containing various concentrations of [^{125}I]GM-CSF.

IC_{50} (nM)	0.1
Maximum binding capacity (pmol per mg protein)	0.13
K_d (nM)	2.11
Number of specific binding sites per cell	$(2.5–3.5) \times 10^4$

cytokines throughout the chromatographic fractions by ELISA revealed that the active fraction (P-1), which stimulated both DNA synthesis and melanization in cultured human melanocytes, was consistent with GM-CSF in molecular mass profile (Figures 2b–2e). The stimulatory effect of P-1 on DNA synthesis ([^{3}H]thymidine incorporation) in human melanocytes was neutralized by antibodies to GM-CSF [1313 ± 39 d.p.m. per well in antibody/P-1(+) and 2408 ± 105 d.p.m. per well in control/P-1(+), compared with 539 ± 21 d.p.m. per well in antibody/P-1(−) and 641 ± 68 d.p.m. per well in control/P-1(−)], but not by those to bFGF [2229 ± 59 d.p.m. per well in antibody/P-1(+) and 510 ± 76 d.p.m. per well in antibody/P-1(−)] or SCF [2373 ± 119 d.p.m. per well in antibody/P-1(+) and 678 ± 54 d.p.m. per well in antibody/P-1(−)].

Effects of recombinant GM-CSF on DNA synthesis and melanization of human melanocytes

To verify the stimulatory effect of GM-CSF on human melanocytes, we measured the potential of recombinant GM-CSF to increase DNA synthesis and melanization in cultured human melanocytes. Recombinant GM-CSF caused significant stimulation of DNA synthesis and melanization in human melanocytes at concentrations of more than 10 nM (Table 3)

Steady-state binding studies

Receptor binding assays (Table 4) showed that unlabelled GM-CSF competitively inhibited binding of [^{125}I]GM-CSF to human melanocytes with an IC_{50} of 0.1 nM. [^{125}I]GM-CSF binding to human melanocytes was saturable, and Scatchard-plot analysis indicated the presence of a single class of high-affinity sites and a maximum binding capacity of 0.13 pmol/mg of protein. The dissociation constant (K_d) was calculated to be 2.11 nM and the number of sites for the specific binding of GM-CSF in these cells was $(2.5–3.5) \times 10^4$ per cell.

DISCUSSION

Little is known about the mechanisms by which melanocytes are activated to proliferate and produce increased amounts of melanin polymer in pigmentary disorders or after exposure to several exogenous stimuli such as UV or allergic reactions. Recent reports [12,22] that the lysate or the conditioned medium of normal cultured human keratinocytes (exposed or non-exposed to UVB) contains factors that stimulate DNA synthesis and/or melanization in human melanocytes, suggested that the epidermal cells surrounding melanocytes in a 30:1 ratio play an essential role in regulating melanocyte function by secreting various growth factors in response to stimuli. Using the recently developed system for human melanocyte culture [23], the potential of several known growth factors to boost the proliferation and differentiation of human melanocytes has been tested. Normal cultured human melanocytes are unresponsive to an extremely large number of growth factors such as insulin-like growth factor, IL-1, IL-6, platelet-derived growth factor and α-melanocyte-stimulating hormone [22]. bFGF was discovered to be a unique growth factor that stimulates the growth of human melanocytes in culture [11]. Because the unique feature of human melanocytes is that they cannot survive in the absence of bFGF even when supplemented with serum or other growth factors, and because UVB irradiation causes epidermal cells to stimulate the production of bFGF within the cells [11], it was proposed that bFGF is an intrinsic keratinocyte-derived growth factor for human melanocytes in UVB-induced melanosis. Despite evidence of bFGF production within keratinocytes and the stimulatory effect of the cell extract on human melanocytes, recent studies [13] demonstrated that bFGF cannot be secreted by classical exocytosis into the medium by cultured human keratinocytes under any conditions because of the lack of a signal peptide. This is consistent with the failure of anti-bFGF antibody to neutralize the mitogenic effect induced by conditioned medium from keratinocytes [12], suggesting that other, as yet unidentified, growth factors play an essential role in melanocyte growth stimulation. The roles of other cytokines such as eicosanoids remain unclear with regard to the involvement of UV melanogenesis or the maintenance of skin colour, because although LTB_4 or LTC_4 is mitogenic for human melanocytes *in vitro* [24], human keratinocytes do not secrete those factors in amounts sufficient to increase DNA synthesis in human melanocytes under normal or UVB-exposed conditions [25]. PGE_2 does not stimulate melanocyte proliferation and its melanogenic effect remains controversial despite the observation that it is secreted from keratinocytes after UVB exposure [25].

Recently we demonstrated that human melanocytes are up-regulated by ET derivatives (potent vasoconstrictive peptides), that stimulate melanocyte proliferation and melanization via a receptor-mediated signal transduction pathway [15,26]. Furthermore keratinocytes trigger melanocyte activation by secreting ETs in response to several stimuli such as UVB or IL-1α and β, resulting in regulation of the function of melanocytes [14]. Because UVB causes keratinocytes to produce IL-1 [27] and because ET secretion that is increased by UVB exposure, the level of which was comparable with that induced by exogenously added IL-1α, was cancelled by anti-IL-1α antibody [14], it is likely that UVB light indirectly increases ET production through an autocrine linkage of IL-1α. Direct evidence for the involvement

of ET-1 in UVB melanogenesis was represented by the finding that the stimulatory effect on DNA synthesis of cultured human melanocytes by UVB-exposed KCM was neutralized by anti-ET-1 antibody [14].

The biological effect of UVA exposure on human keratinocytes is poorly documented compared with that of UVB despite considerable evidence showing cellular damage to the epidermis [25]. UVA exposure induces definite skin pigmentation, although to a lesser extent than UVB exposure, accompanied by the accentuated function of melanocytes [8]. Therefore it is of considerable interest to clarify the biological mechanisms by which UVA exposure elicits cutaneous pigmentation, with special reference to the paracrine linkage of cytokines between keratinocytes and melanocytes. The present study demonstrated that UVA- and UVB-associated melanocyte activation are mediated through completely different biological pathways. Thus, unlike the effect of UVB on human keratinocytes, UVA at any dose tested did not stimulate the secretion of cytokines such as ET-1 and IL-1α, which are intrinsically involved in UVB-associated melanocyte activation. Instead the secretion of other cytokines such as GM-CSF, IL-6 and IL-8 was augmented by UVA irradiation in cultured human keratinocytes.

A gel chromatography study with conditioned medium demonstrated that the fraction detected by ELISA as GM-CSF is comparable in molecular profile with those that stimulated DNA synthesis as well as melanization ([^{14}C]thiouracil incorporation) of cultured human melanocytes. The incorporation of [^{14}C]thiouracil into pigment cells occurs in parallel with the activity of tyrosine hydroxylase in living cells [14,15,21], and is a convenient indicator of melanin synthesis owing to its selective incorporation into melanin-synthesizing cells [18,19] as well as its specific inhibitory or stimulatory profile by the tyrosinase inhibitor phenylthiourea and the melanogenic stimulus PGE_2 respectively [16,21]. Because, in UVA-induced cutaneous pigmentation, melanocytes are stimulated to undergo both proliferation and melanogenesis, the biological properties of the GM-CSF-containing active fraction in UVA-exposed KCM eliciting both mitogenic and melanogenic effects strongly suggest that GM-CSF is an intrinsic stimulatory component for human melanocytes in UVA-induced melanization in the epidermis. In contrast, the nature of the low-molecular-mass fraction (P-2) active only in mitogenesis is presently unknown owing to its unstable properties, although materials other than peptides such as eicosanoids might be separated into these chromatographic fractions. Among eicosanoids, LTC_4/LTB_4 are the most likely candidates for the low-molecular-mass active substances because of their known mitogenic effects on human melanocytes [24]. However, LTB_4 was not increased and LTC_4 was undetectable in the KCM after UVA exposure, suggesting that there is no involvement of LTC_4/LTB_4 in the mitogenic effect of the P-2 fraction.

GM-CSF is a glycoprotein identified by its ability to promote the maturation of haematopoietic progenitor cells to granulocytes and macrophages [28,29]. A variety of cells, including mononuclear phagocytes, T lymphocytes, vascular endothelial cells and fibroblasts, are known to secrete GM-CSF [30,31]. Keratinocytes have also been reported to produce GM-CSF [32,33], and this secretion is significantly augmented by UVB irradiation through the production of IL-1α in an autocrine fashion [34,35]. In our culture system with human keratinocytes, UVB at a dose of 50 mJ/cm^2 (twice) did not enhance the secretion of GM-CSF into the medium (results not shown), whereas the secretion of ET-1 and IL-1α were increased significantly. In contrast, secretion of GM-CSF into the medium was stimulated 2-fold 4 days after a single exposure to UVA at a dose of 1 J/cm^2 although the secretion of IL-1α was not increased. This discrepancy was probably due to differences in sources of keratinocytes (i.e. Pam 212 keratinocytes [34]) or insufficient screening of UVA light in the UVB study [35]. There have so far been no reports describing the stimulatory effect of GM-CSF itself on cultured human melanocytes. This may be because of some difficulties in detecting their mitogenic activity by using recombinant GM-CSF, which has about one-tenth the potency of natural GM-CSF in melanocyte activation, probably because of the lack of carbohydrate moieties in the recombinant form. As expected, in this study we confirmed the stimulatory effect of recombinant GM-CSF on human melanocytes at concentrations higher than those estimated by studies with UVA-exposed KCM.

That anti-GM-CSF antibody neutralized the stimulatory effect of the active chromatographic fraction (P-1) on DNA synthesis in cultured human melanocytes represents direct evidence for the involvement of GM-CSF as an intrinsic growth factor for human melanocytes in UVA melanogenesis. Other antibodies such as anti-bFGF and anti-SCF failed to abolish the stimulatory effect, suggesting that GM-CSF is the only UVA-associated keratinocyte-derived mitogen in human melanocytes.

On the basis of our observation that there are specific receptors for GM-CSF on human melanocytes, several lines of evidence for the action via tyrosine kinase in other cells [30,31] indicated that, as with the action of bFGF on cultured human melanocytes, GM-CSF can act as a mitogenic stimulator on human melanocytes. Both belong to the bFGF family, which express their function via the tyrosine kinase system. Because a relatively high level (70 pg/ml per 10^6 cells) of GM-CSF is secreted into the medium even under normal conditions, the stimulation of DNA synthesis of cultured human melanocytes by the conditioned medium even from non-UV-exposed human keratinocytes [12] may be ascribed to GM-CSF.

In summary, this study provides new insight into the biological mechanisms underlying UVA-associated pigmentation. UVB and UVA activate human keratinocytes in different ways to produce and secrete completely different spectra of growth factors. Human melanocytes undergo proliferation and differentiation in a similar fashion in response to different growth factors secreted from human keratinocytes. Most importantly, the findings of the present study suggest that different UV wavelengths, and probably also other stimuli have different impacts on the expression of the growth-factor stimulation cascade. These observations may facilitate a fundamental understanding of the melanization mechanism underlying UVA melanogenesis and the homoeostatic maintenance of skin colour.

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