The Paracrine Role of Stem Cell Factor/c-kit Signaling in the Activation of Human Melanocytes in Ultraviolet-B-Induced Pigmentation

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The interaction of stem cell factor with its receptor, c-kit, is well known to be critical to the survival of melanocytes. Little is known about the role(s) of the stem cell factor/c-kit interaction in epidermal pigmentation, however. To clarify whether the stem cell factor/c-kit signaling has a paracrine role in ultraviolet-B-induced pigmentation, we determined whether the exposure of human keratinocytes, melanocytes, and the epidermis to ultraviolet B light stimulates the expression of stem cell factor or c-kit at the gene and/or protein levels. We further examined whether interrupting the binding of stem cell factor to c-kit by subepidermal injection of a monoclonal antibody to c-kit affects ultraviolet-B-induced pigmentation in brownish guinea pig skin. When human keratinocytes and melanocytes in culture were exposed to ultraviolet B light, transcripts of stem cell factor and c-kit (as assessed by reverse transcription polymerase chain reaction) and expression of those proteins (by enzyme-linked immunosorbent assay and western blotting) increased significantly and peaked at a dose of 20–40 mJ per cm^2. In ultraviolet-B-exposed human epidermis, stem cell factor transcripts and protein expression were also markedly enhanced compared with the nonexposed epidermis. Immunohistochemistry with antibodies to stem cell factor revealed an increased staining in the ultraviolet-B-exposed epidermis, which was accompanied by a slight epidermal hyperplasia. In the course of ultraviolet-B-induced pigmentation of brownish guinea pig skin, the subepidermal injection of c-kit inhibitory antibodies completely abolished the induction of pigmentation in the ultraviolet-B-exposed area, and there was no increase in the number of dihydroxyphenylalanine-positive melanocytes. These findings indicate that the stem cell factor/c-kit signaling is critically involved in the biologic mechanism of ultraviolet-B-induced pigmentation. ***Key words: c-kit/human melanocytes/pigmentation/stem cell factor/UVB. J Invest Dermatol 116:578–586, 2001***

The murine c-kit receptor tyrosine kinase and its ligand, stem cell factor (SCF), are encoded by the dominant white spotting (*W*) locus and the steel (*Sl*) locus, respectively. Mutations in these loci elicit pleiotropic developmental defects resulting in the loss of neural-crest-derived pigment cells, hematopoietic stem cells, and primordial germ cells (Matsui *et al*, 1990; Orr-Urtreger *et al*, 1990; Bernstein *et al*, 1991; Williams *et al*, 1992; Besmer *et al*, 1993; Galli *et al*, 1993; Halaban and Moellmann, 1993). Phenotype analysis of *W* and *Sl* mice has revealed the involvement of the SCF/c-kit signaling in melanocyte development at embryonal stages. The significance of the SCF/c-kit signaling in the development of murine melanocytes has also been demonstrated through experiments using a monoclonal c-kit antibody (ACK2), an antagonistic blocker of c-kit function (Nishikawa *et al*, 1991; Okura *et al*, 1995; Yoshida *et al*, 1996).

It has also been reported that the SCF/c-kit signaling is deeply associated with the development of human melanocytes. Patients with piebaldism, a disorder presenting at birth with amelanotic patches on ventral and/or acral skin surfaces, but apparently lacking detectable defects in germ cells or the hematologic system, were shown to be heterozygous for c-kit mutations (Giebel and Spritz, 1991; Spritz *et al*, 1992; Ezoe *et al*, 1995). In Type II Waardenburg syndrome (WS), an autosomal dominant disorder characterized by pigmentary abnormalities and sensorineural deafness, patients are heterogeneous and about 15% are heterozygous for mutations in microphthalmia-associated transcription factor (MITF) (Read and Newton, 1997). MITF expression is regulated by SCF through the mitogen-activated protein kinase pathway and stimulates transcription of the key enzyme tyrosinase gene in melanocytic cell lines (Bertolotto *et al*, 1998; Hemesath *et al*, 1998). Type IV WS (Shah–Waardenburg syndrome with Hirschsprung disease) has been reported to be caused by mutations in the genes encoding endothelin-3 (ET-3) or ET_B receptor (Read and Newton, 1997). Such evidence may indicate that SCF and ETs play a coordinated role in regulating epidermal melanogenesis under homeostatic conditions.

In ultraviolet B (UVB) induced pigmentation, ET-1 is upregulated at the transcriptional level in UVB-exposed human skin (Imokawa *et al*, 1995), and ET-1 has been suggested to play an important role as a mitogen (Imokawa *et al*, 1992; Yohn *et al*, 1993) and as a melanogen (Yada *et al*, 1991) for human melanocytes in UVB-induced pigmentation. Little is known about the significance

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Abbreviations: BPE, bovine pituitary extract; SCF, stem cell factor; ET, endothelin.

of other melanogenic cytokines *in vivo*, however, which might be secreted by keratinocytes and which might act as mitogens or melanogens for human melanocytes *in vivo and in vitro*.

Recently, SCF has been reported to stimulate the proliferation of human melanocytes in culture (Imokawa *et al*, 1996) and in xenografts of normal human skin (Grichnik *et al*, 1998). Therefore, it is of particular interest to characterize whether the SCF/c-kit signaling is also involved in the melanocyte activation mechanism during UVB-induced pigmentation, perhaps accompanied by an increased expression of SCF and/or c-kit expression in keratinocytes and melanocytes, respectively. In this study, therefore, we examined whether the exposure of human keratinocytes, melanocytes, or epidermis to UVB light stimulates the expression of SCF or c-kit at the gene and/or protein levels. We further examined whether the interruption of SCF binding to c-kit elicited by the subepidermal injection of a monoclonal antibody to c-kit affects UVB-induced pigmentation in brownish guinea pig skin. Brownish guinea pig is an ideal model for such a study as functional melanocytes exist in the epidermis and they respond well to UVB irradiation to increase the pigmentation (Imokawa *et al*, 1986). Here we report that the SCF/c-kit signaling between keratinocytes and melanocytes in the epidermis plays an important role in the early phase of melanocyte activation during UVB-induced pigmentation.

MATERIALS AND METHODS

Materials Normal human keratinocytes were obtained from Kyokutou (Tokyo, Japan) or Sankou Pure Chemicals (Tokyo, Japan). Normal human melanocytes and serum-free melanocyte medium (MGM) were purchased from Sankou Pure Chemicals. Human SCF enzyme-linked immunosorbent assay (ELISA) kits and polyclonal rabbit antisera against human SCF (SCF-89) were purchased from Immuno-Biological Laboratories (IBL, Gunma, Japan). Serum-free keratinocyte medium (SFM), bovine pituitary extract (BPE), epidermal growth factor (EGF), ACK2 monoclonal antibody, Dulbecco's phosphate-buffered saline (PBS) and RPMI medium 1640 were purchased from Gibco Laboratories. Other chemicals were of reagent grade.

Cell cultures Human keratinocytes were maintained in modified SFM supplemented with 5 ng per ml EGF and 50 μg per ml BPE at 37°C with 5% CO_2. Human melanocytes were maintained in modified MGM supplemented with 1 ng per ml recombinant basic fibroblast growth factor (bFGF), 5 μg per ml insulin, 0.5 μg per ml hydrocortisone, 10 ng per ml phorbol 12-myristate 13-acetate, antibiotics (50 μg per ml streptomycin), and 0.2% (vol/vol) BPE at 37°C with 5% CO_2, as previously described (Imokawa *et al*, 1992). Bone marrow cells were obtained from brownish guinea pigs as described elsewhere (Harrison *et al*, 1981) and were cultured in RPMI medium 1640 with 20% fetal bovine serum at 37°C with 5% CO_2.

Animals Outbred brownish guinea pigs (tortoiseshell guinea pigs), ranging in age from 12 to 20 wk at the beginning of the study, were used. The animals had free access to food and chlorinated water and were housed in individual cages. Prior to UVB irradiation, each guinea pig was shaved with an electric clipper to remove long hair. The remaining stubble was removed with a shaver.

Irradiation with UVB light Cells were seeded in T-75 flasks (Falcon) at a density of 5×10^4–10^5 cells per ml. After cultivation for 24–96 h, the culture medium was transferred and exchanged for PBS. The cells were washed three times with 10 ml PBS and then kept in 10 ml PBS. They were irradiated twice at a 48 h interval with doses of UVB light ranging from 10 to 80 mJ per cm^2, at which dose most of the energy is emitted within the UVB range (295–315 nm) with a peak of 305 nm. Immediately after UVB treatment, the PBS was removed and exchanged for fresh medium. After cultivation for another 24 h, the conditioned medium and cells were harvested.

Irradiation was performed on human volar forearms of normal volunteers after prior determination of their minimal erythema dose (MED). One area (approximately 1.0 cm^2) on the left forearm was irradiated with 2 MED of UVB.

On the dorsal skin of brownish guinea pigs, irradiation test sites of 1 cm^2 were chosen in areas of comparable baseline pigmentation and were delineated with an indelible marker to mark the sites for subsequent treatments. The guinea pigs were irradiated twice at a 24 h interval with a dose of 288 mJ per cm^2 of UVB light.

Suction blisters Human epidermal sheets (blister roofs) were obtained from healthy volunteers using the suction blister technique as described elsewhere (Furukawa *et al*, 1987). Briefly, suction blisters were produced on skin of volar forearms 3 d after exposure to UVB using a 1 ml or 2.5 ml plastic syringe with negative pressure. Within 1 h, blisters 10 mm in diameter were raised, and the epidermal sheet of approximately 0.8 cm^2 each was removed with a surgical blade. Blisters were also recovered from the nonirradiated (control) sites.

Reverse transcription polymerase chain reaction (RT-PCR) After UVB irradiation, SCF expression in human keratinocytes and epidermis and c-kit expression in human melanocytes were investigated using semiquantitative RT-PCR normalized with constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Total RNA from keratinocytes, melanocytes, and the epidermal sheet was isolated using a single-step guanidine thiocyanate-phenol-chloroform method with TRIzol (Gibco BRL, Grand Island, NY). cDNA was then synthesized by reverse transcription of 1 μg total RNA, using oligo-dT and Moloney murine leukemia virus reverse transcriptase (Gibco BRL).

The following sets of oligonucleotide primers were used: SCF (Stratagene), 5′-GAT-GTT-TTG-CCA-AGT-CAT-TGT-TGG-3′, which corresponds to nucleotides 367–390 of SCF cDNA (Martin *et al*, 1990), and 5′-ACT-GAC-TCT-GGA-ATC-TTT-CTC-AGG-3′, which is complementary to nucleotides 694–717 of SCF cDNA; c-kit (Clontech), 5′-TTC-TTA-CCA-GGT-GGC-AAA-GGG-CAT-GGC-TTT-CC-3′, which corresponds to nucleotides 2337–2368 of c-kit cDNA (Yarden *et al*, 1987), and 5′-GTC-ATA-CAT-TTC-AGC-AGG-TGC-GTG-TTC-AGG-GC-3′, which is complementary to nucleotides 2693–2724 of c-kit cDNA; GAPDH (Clontech), 5′-ACC-ACA-GTC-CAT-GCC-ATC-AC-3′, which corresponds to nucleotides 586–605 of GAPDH cDNA (Arcari *et al*, 1984), and 5′-TCC-ACC-ACC-CTG-TTG-CTG-TA-3′, which is complementary to nucleotides 1018–1037 of GAPDH cDNA. Amplification was performed using Taq polymerase (Gibco BRL) over 38 cycles for c-kit in melanocytes and SCF in epidermis, for 33 cycles for SCF in keratinocytes and G3PDH in epidermis, and for 20 cycles for G3PDH in keratinocytes and melanocytes with an automated thermal cycler (MJ Research). Each cycle consisted of the following steps: denaturation at 94°C, annealing at 62°C, and extension for 1.5 min at 72°C. PCR products were analyzed by agarose gel electrophoresis.

ELISA To measure SCF in human keratinocytes, the conditioned medium was collected and quantified (100 μl per well) for SCF by ELISA. The cells were rinsed with PBS and scraped off with a rubber policeman. They were then suspended in 50 mM sodium phosphate buffer (pH 7.8). The cell suspension was sonicated for 5 min with a Bioruptor (Olympus) and centrifuged at $10{,}000 \times g$ for 20 min at 4°C. The supernatant was further centrifuged at $100{,}000 \times g$ for 60 min at 4°C. The resulting precipitate was dissolved in 25 mM sodium phosphate buffer (pH 6.8) containing 0.1% Triton X-100 and quantified (100 μl per well) for SCF by ELISA. The SCF ELISA kit is a solid phase enzyme immunoassay using the multiple antibody sandwich principle. A purified polyclonal rabbit antibody specific for human SCF was attached to 96-well microtiter plates. SCF present in samples and standards was captured by the solid phase antibody. After washing, horseradish peroxidase labeled rabbit antihuman SCF IgG was then added, which bound to multiple epitopes on SCF attached to the solid phase. Levels of immunoreactive SCF were measured by absorbance at 450 nm with an ELISA plate reader (model 550, Bio-Rad Laboratories, Hercules, CA). The amount of SCF in samples was determined by comparing absorbance with that produced by standards. The standard curve was linear from 50 to 3200 pg per ml.

Western blotting To measure c-kit expression after UVB irradiation in human melanocytes, 20 μg protein solubilized in Nonidet P-40/sodium dodecyl sulfate (SDS) buffer consisting of 0.1 M Tris-HCl (pH 7.2), 0.01% SDS, 1% Nonidet P-40, and protease inhibitor cocktail tablet (Boehringer Mannheim; 1 tablet per 50 ml) was separated on 7.5% SDS gel. This was then transferred to polyvinylidene difluoride membrane (Immobilon-p, Millipore, Bedford, MA) and incubated with a purified polyclonal rabbit antibody specific for c-kit (Santa Cruz Biotechnology) diluted at 1:1000. Subsequent visualization of antibody binding was carried out with enhanced chemiluminescence (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

To measure SCF expression 3 d after UVB irradiation of human epidermis, epidermis was removed from the UVB-irradiated volar

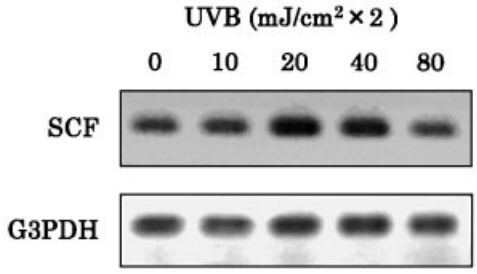

Figure 1. The expression of SCF transcript is stimulated by UVB irradiation in cultured human keratinocytes. Semiquantitative RT-PCR was carried out for 33 cycles using specific primers for SCF in comparison with G3PDH for 20 cycles. Human keratinocytes were exposed twice at a 48 h interval to the indicated doses of UVB light, followed by RT-PCR analysis 24 h after the last irradiation. PCR products were analyzed by agarose gel electrophoresis. Each band shows a representative result of three separately performed RT-PCR analyses.

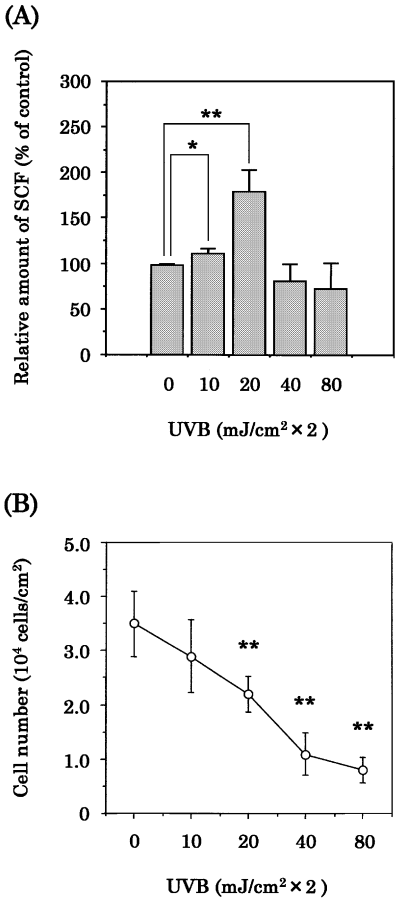

Figure 2. UVB exposure increases the amount of membrane-bound SCF in cultured human keratinocytes. (*A*) ELISA of the content of membrane-bound SCF; (*B*) viable cell number per cm^2 after UVB irradiation. Human keratinocytes were exposed twice at a 48 h interval to the indicated doses of UVB light, followed by ELISA to measure SCF in the particulate fraction of human keratinocytes. The content of membrane-bound SCF in UVB-exposed culture measured by ELISA was divided by the content of total protein and is expressed as a percentage of control relative to the nonexposed control. The values represent means ± SD from three independent experiments. *$p < 0.05$, **$p < 0.01$.

forearm of normal volunteers by suction blister. The epidermal sheets were homogenized with a glass homogenizer in 200 mol of Nonidet P-40/SDS buffer, and then sonicated for 5 min with a Bioruptor

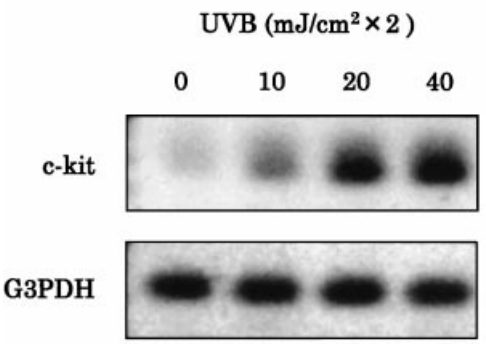

Figure 3. UVB irradiation stimulates the expression of c-kit transcripts in cultured human melanocytes. Semiquantitative RT-PCR was carried out for 38 cycles using specific primers for c-kit in comparison with G3PDH for 20 cycles. Human melanocytes were exposed twice at a 48 h interval to the indicated doses of UVB light, followed by RT-PCR analysis 24 h after the last irradiation. PCR products were analyzed by agarose gel electrophoresis. Each band shows a representative result of three separately performed RT-PCR analyses.

(Olympus). After centrifugation at 15,000 × *g* for 10 min at 4°C, the supernatants were collected and western blotting analysis was performed as described elsewhere (Mizutani *et al*, 1994). Briefly, 5 μg of each sample and 0.075 μg recombinant human SCF (Sigma, St. Louis, MO) were electrophoresed in a 10% SDS gel and electrically transferred to a polyvinylidene difluoride membrane. After blocking with 3% skim milk, the membrane was incubated with polyclonal rabbit antisera against human SCF (SCF-89: IBL) at 5 μg per ml for 1 h as the primary antibody. Subsequent visualization of antibody binding was carried out as described above.

Immunohistology Epidermis from UVB-irradiated and nonirradiated sites on human volar forearms and the skin from brownish guinea pig were fixed in formalin and embedded in paraffin. SCF and c-kit immunoreactivity were investigated using polyclonal rabbit antisera against human SCF (SCF-89: IBL) and human c-kit (Medical and Biological Laboratories, Nagoya, Japan), respectively, and the avidine–biotin peroxidase complex method (ABC-Elite kit; Vecter Laboratories, Burlingame, CA) with ImmunoPure Metal Enhanced DAB Substrate Kit (Pierce, Rockford, IL) or TrueBlue Peroxidase Substrate (Kirkegaard Perry Laboratories, Gaithersburg, MD). Negative controls were performed with normal rabbit IgG (Inter-cell Technologies). As an additional negative control for SCF and c-kit expression, the primary antibody was preincubated with soluble SCF peptides (Sigma).

DNA synthesis Bone marrow cells cultured in 96-well plates were incubated with murine SCF (Sigma) at various concentrations in experiment 1. In experiment 2, they were incubated with the most appropriate dose of murine SCF (as determined in experiment 1) and with 5 μg per ml of ACK2. After 3 d, the cells were labeled for 6 h with 1 μCi per well (100 μl) [^{3}H]-thymidine. After three washes with PBS, the cells were lyzed with 2 M NaOH at 37°C for 15 min and neutralized with 2 M HCl. The acid-insoluble materials were precipitated with 4 volumes of 10% (wt/vol) ice-cold trichloroacetic acid for 30 min at 4°C, collected on a glass fiber filter, washed three times with 10% ice-cold trichloroacetic acid and once with ice-cold ethanol, and then dried. The filter was mixed with scintillation fluid and the radioactivity was determined with a liquid-scintillation counter.

Injection procedure Brownish guinea pigs were injected subepidermally with 5 μg of ACK2, which is not only a marker of c-kit positive cells but also a blocker of c-kit function *in vivo* (Nishikawa *et al*, 1991; Okura *et al*, 1995; Ito *et al*, 1999). This treatment was performed 24 h and 48 h after the last UVB irradiation. Purified rat IgG (5 μg) (Cappel) was used as a control antibody.

Measurement of skin color Prior to the measurement of skin color, the backs of the guinea pigs were shaved with an electric clipper to remove long hair, and the remaining stubble was removed with a shaver. The intensity of UVB-induced pigmentation was measured by a color difference meter (Nippon Denshoku Industries) and is expressed as δL value.

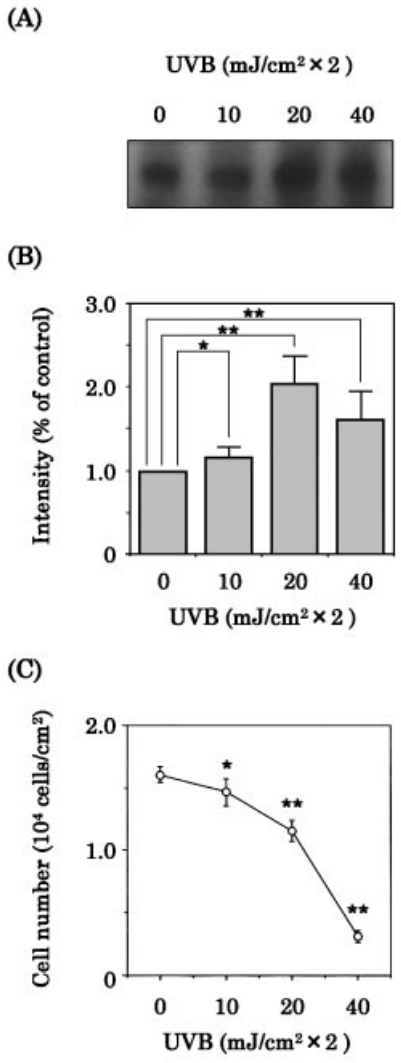

Figure 4. UVB irradiation enhances c-kit expression in cultured human melanocytes as revealed by western blotting. (*A*) Western blotting: each band shows a representative result of western blotting analyses that were repeated four times. (*B*) Densitometric analysis: the values represent means ± SD from four independent experiments. *p < 0.05, **p < 0.01. (*C*) Viable cell number per cm^2 after UVB irradiation. *p < 0.05, **p < 0.01. Human melanocytes were exposed twice at a 48 h interval to the indicated doses of UVB light, followed by western blotting using c-kit antibody in the lysate of cultured human melanocytes, 24 h after the last irradiation.

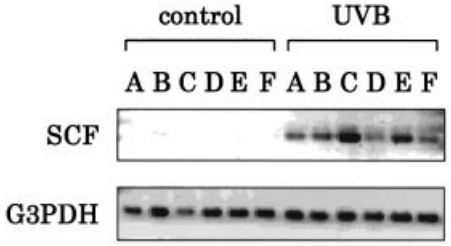

Figure 5. UVB irradiation markedly increases the expression of SCF transcript in human epidermis *in vivo*. Volar forearms of healthy human volunteers were exposed to UVB light at 2 MED, followed by suction blister biopsy for RT-PCR analysis of SCF transcript. Semiquantitative RT-PCR was carried out for 38 cycles using specific primers for SCF in comparison with G3PDH for 33 cycles. PCR products were analyzed by agarose gel electrophoresis. A–F represent different volunteers.

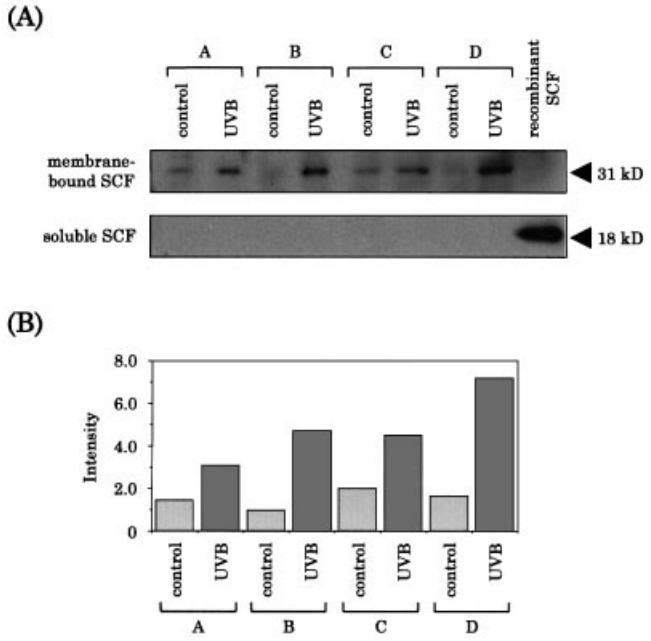

Figure 6. Membrane-bound SCF is markedly increased by UVB irradiation in human epidermis *in vivo*. (*A*) Western blotting. (*B*) Densitometric analysis. Epidermal sheets obtained as blister roofs were solubilized in Nonidet P-40/SDS buffer. Five micrograms protein of each extract were electrophoresed and analyzed by western blotting as described in *Materials and Methods*. A–D represent different volunteers.

Measurement of dihydroxyphenylalanine (DOPA)-positive melanocytes After measurement of skin color on the backs of brownish guinea pigs, skin specimens were removed and treated as previously described (Imokawa *et al*, 1986). Briefly, tissues were rinsed in 0.1 M phosphate buffer (pH 6.8) and incubated in 2 M NaBr for 2 h at 37°C. The epidermal sheets were then separated from the dermis and fixed in 10% cold neutral formalin for 30 min, washed twice with 0.1 M phosphate buffer (pH 6.8), and incubated in 0.1% DOPA in 0.1 M phosphate buffer (pH 6.8) for 3 h. The numbers of DOPA-positive melanocytes per mm^2 were counted using an Olympus-BHA microscope at a magnification of 200×.

Statistics Statistical analysis was carried out by Student's *t* test.

RESULTS

UVB irradiation increases SCF expression in human keratinocytes To clarify the possible involvement of SCF in UVB-induced pigmentation, we determined whether UVB irradiation induces an increase in the expression of SCF at the gene and protein levels. When cultured human keratinocytes were exposed twice at a 48 h interval to UVB irradiation, the expression of SCF transcript increased 24 h following the last irradiation, with a maximal effect at a UVB dose of 20 mJ per cm^2 (**Fig 1**). Using an ELISA assay, increased amounts of SCF were detectable in the particulate fraction again with a peak at 20 mJ per cm^2 (**Fig 2**), whereas SCF was not detected in the culture medium of UVB-exposed keratinocytes (data not shown).

UVB irradiation accentuates c-kit expression in cultured human melanocytes In determining whether the SCF/c-kit signaling is involved in the signaling pathway leading to UVB-induced pigmentation, we examined the expression of c-kit transcript in cultured human melanocytes exposed twice at a 48 h interval to UVB irradiation. c-kit mRNA (**Fig 3**) and protein (**Fig 4**) were markedly increased in a dose-dependent manner with a maximal effect at 40 mJ per cm^2 and 20 mJ per cm^2, respectively.

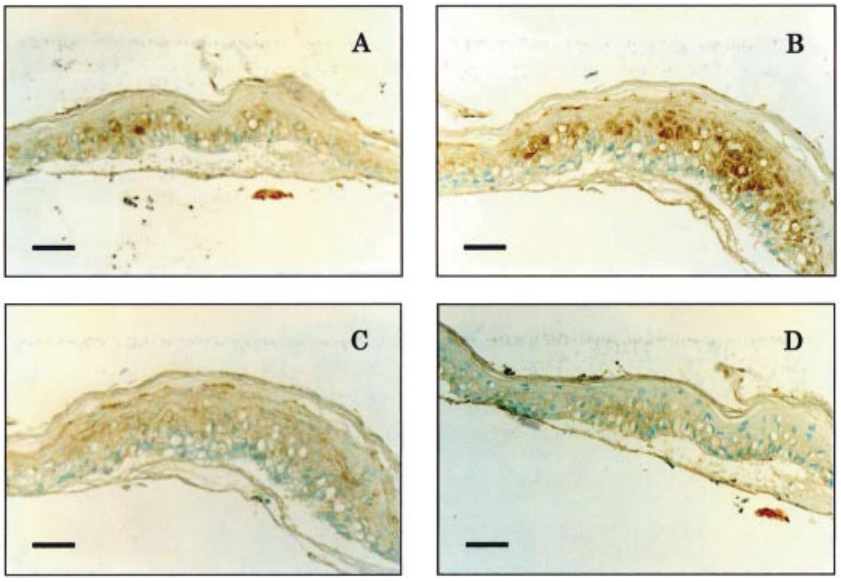

Figure 7. UVB irradiation increases the production of SCF in human epidermis as revealed by immunohistochemistry. (*A*) Immunostaining of nonexposed epidermis with anti-SCF. (*B*) Immunostaining of UVB-exposed epidermis with anti-SCF. (*C*) Immunostaining of UVB-exposed epidermis with nonspecific IgG. (*D*) Immunostaining of nonexposed epidermis with nonspecific IgG. *Scale bar*: 100 μm.

UVB irradiation markedly increases membrane-bound SCF content in human epidermis In order to examine whether SCF expression is enhanced by UVB irradiation at the gene and protein levels *in vivo*, human volar forearms of normal volunteers were exposed to UVB light at 2 MED. Three days after the irradiation, blisters were induced at the irradiated sites by the suction blister technique, and the epidermal sheet was removed for subsequent studies. Blisters were also induced as controls on nonirradiated sites. RT-PCR analysis of the UVB-exposed epidermis showed that there was an increased expression of SCF transcripts compared with the nonexposed epidermis, there being no detectable level of SCF transcript in the nonexposed epidermis (**Fig 5**). Western blotting analysis of membrane-bound SCF showed a marked increase in the UVB-exposed human epidermis compared with the nonexposed epidermis, whereas soluble SCF was not detectable in either type of epidermis (**Fig 6**). Immunohistochemistry of the UVB-exposed human skin with SCF antibodies showed a strong immunostaininig located in the stratum spinosum, accompanied by epidermal hyperplasia, compared with the nonexposed epidermis (**Fig 7**). On the other hand, there was no immunostaining with control nonspecific IgG in the UVB-exposed epidermis.

C-kit inhibitory antibody injection inhibits UVB-induced pigmentation in dorsal skin of brownish guinea pigs Because brownish guinea pigs have functional melanocytes in their epidermis that respond well to several stimuli including UVB light, which induces hyperpigmentation (Imokawa *et al*, 1986), we used this guinea pig as a useful model for human skin pigmentation to determine whether interruption of the SCF/c-kit signaling abolishes the UVB-induced pigmentation. Prior to that inhibitory experiment, we evaluated the cross-reactivity of ACK2 (antimurine c-kit) (Nishikawa *et al*, 1991) to brownish guinea pig c-kit by examining the stimulatory effect of murine SCF on the proliferation of bone marrow cells from brownish guinea pigs and prevention of that effect by ACK2. The addition of murine SCF stimulated bone marrow cells to synthesize DNA in a dose-dependent manner (**Fig 8*A***), and the increase could be completely abrogated by ACK2 (**Fig 8*B***). Control IgG had no such effect, indicating that ACK2 can inhibit c-kit receptor function of brownish guinea pigs. Further, immunohistochemistry of brownish guinea pig skin with antibody to human c-kit revealed that melanocytes in the epidermis of brownish guinea pig express c-kit (**Fig 9**).

In order to interrupt SCF binding to c-kit, guinea pigs were exposed twice to UVB light at a dose of 288 mJ per cm^2 and then were injected subepidermally with 5 μg per 50 μl of ACK2 (or purified rat IgG as a control) 24 h and 48 h after the last UVB irradiation. When observed 6 d after the first UVB irradiation, ACK2 injection remarkably abolished the UVB-induced pigmentation and the color of the UVB-irradiated skin remained similar to the nonexposed area (measured by color difference meter), whereas a similar injection with nonspecific IgG did not elicit such inhibition (**Figs 10**, **11*A***). When observed 10 d after the first irradiation, the inhibitory effect was only partial and a slightly increased skin pigmentation was measured by color difference meter (**Fig 11*B***). In parallel, the numbers of DOPA-positive melanocytes were comparable on day 6 in the ACK2-injected site and in the nonexposed area, in contrast to significantly increased numbers of DOPA-positive melanocytes in the nonspecific-IgG-injected site (**Fig 12*A***). On day 10, the number of melanocytes in the ACK2-treated site had slightly increased but was still significantly less than in the nonspecific-IgG-treated site (**Fig 12*B***). Continuous observations of skin color changes revealed that the significant inhibition of pigmentation remained at a level similar to that at day 10 until several weeks post irradiation (data not shown).

DISCUSSION

The process of UVB-induced pigmentation consists of three main steps, i.e., the proliferation of melanocytes (Rosdahl and Szabo, 1978), the synthesis and activation of tyrosinase (Imokawa and Mishima, 1982; Mishima and Imokawa, 1983), and the transfer of melanosomes to keratinocytes (Okazaki *et al*, 1976). As for melanocyte proliferation, keratinocyte-derived cytokines including bFGF (Halaban *et al*, 1988), ET-1 (Imokawa *et al*, 1992; Yohn *et al*, 1993), and α-melanocyte stimulating hormone (α-MSH) (Schauer *et al*, 1994; Abdel-Malek *et al*, 1995; Chakraborty *et al*, 1996; Wintzen and Gilchrest, 1996; Hedley *et al*, 1998) have been documented to be upregulated in their production and secretion following UVB irradiation and can act as mitogens for human melanocytes. As melanogens, ET-1 (Yada *et al*, 1991; Tada *et al*, 1998), α-MSH (Abdel-Malek *et al*, 1995), and nitric oxide (Roméro-Gaillet *et al*, 1997) have been reported to stimulate melanogenesis in human melanocytes via increased expression of tyrosinase transcripts and proteins. Little is known about the *in vivo* significance of those melanogenic cytokines in UVB pigmentation on human skin, however, except for ET-1, which is upregulated at the transcriptional level in UVB-exposed human skin (Imokawa *et al*, 1995). α-MSH has also been reported to enhance pigmentation in sun-exposed areas of human skin when injected subcutaneously (Lerner and McGuire, 1961; Levine *et al*, 1991), indicating the coordinated role in sun-induced pigmentation.

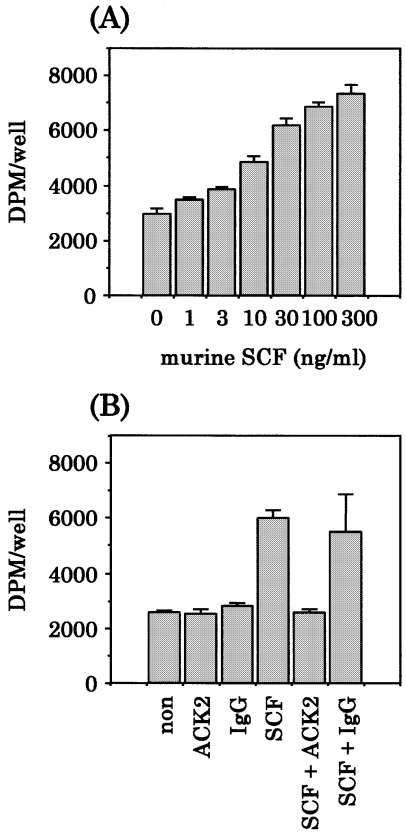

Figure 8. The c-kit inhibitory antibody, ACK2, can inhibit SCF-stimulated DNA synthesis of bone marrow cells from brownish guinea pig. (*A*) Dose-dependent stimulatory effect of murine SCF on DNA synthesis of bone marrow cells. Bone marrow cells were incubated in culture for 78 h with murine SCF at the indicated concentrations, followed by the addition of [^{3}H]-thymidine (1 μCi per well) for the last 6 h. (*B*) Preventive effect of ACK2 on murine SCF-stimulated DNA synthesis in bone marrow cells. Bone marrow cells were incubated for 78 h with murine SCF (100 ng per ml) in the presence of ACK2 (5 μg per ml), followed by the addition of [^{3}H]-thymidine (1 μCi per well) for the last 6 h. The values represent means ± SD from five independent experiments.

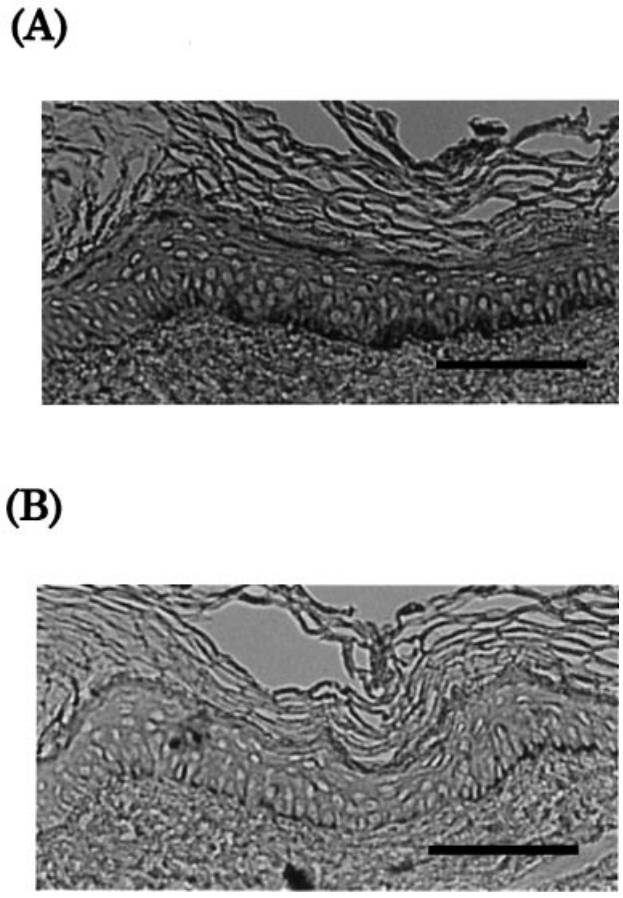

Figure 9. c-kit is expressed on melanocytes in the epidermis of guinea pig. (*A*) Immunostaining of guinea pig skin with anti-c-kit. (*B*) Immunostaining of guinea pig skin with nonspecific IgG. *Scale bar*: 100 μm.

Recent available evidence suggests that there is a complex network in the epidermis for secreting and responding to autocrine and paracrine cytokines by keratinocytes and melanocytes, respectively (Halaban *et al*, 1988; Abdel-Malek *et al*, 1995; Imokawa *et al*, 1996; Tada *et al*, 1998). This is effected via corresponding receptors that are also modulated in their expression by various cytokines, and there is crosstalk in signaling between cytokines to support the accentuated activation of melanogenesis in melanocytes. Recently, we have found that the proliferation of cultured human melanocytes is stimulated by SCF and is synergistically enhanced by ET-1 (Imokawa *et al*, 1996). Consistent with this, it has been reported that intracutaneous injection of SCF increased the number, size, and dendricity of melanocytes in xenografts of normal human skin, whereas interruption of SCF binding to its receptor c-kit by c-kit inhibitory antibodies decreased all those factors (Grichnik *et al*, 1998). In dermatofibroma with epidermal hyperpigmentation, it has been suggested that SCF secreted by dermal fibroblasts plays an essential role in stimulating melanocytes located in the epidermis overlying the dermal fibroblast tumor.[1]

[1]Shishido E, Manaka I, Kadono S, Kawashima M, Imokawa G: The paracrine mechanism of accentuated epidermal pigmentation in dermatofibroma: role of fibroblast-derived melanogenic cytokines. *Pigment Cell Res Sup* 7:80, 1999 (abstr.)

These lines of evidence support the potential role of SCF in regulating epidermal melanogenesis under homeostatic or even stimulatory conditions (including UVB exposure), if the SCF/c-kit signaling was upregulated in epidermal cells by UVB irradiation. Because no data are available on the expression of SCF in human keratinocytes in response to UVB irradiation, it is of particular interest to determine whether UVB light stimulates the expression of SCF in human keratinocytes at the gene and/or protein levels. If so, it would also be important to know whether upregulated production of SCF is substantially relevant to the activation of epidermal melanocytes through its specific receptor c-kit.

In this study, for the first time, we have found that the exposure of cultured human keratinocytes and melanocytes to UVB light upregulates transcription and protein expression of SCF and c-kit, respectively. In parallel, SCF transcripts and membrane-bound SCF were found to increase remarkably in UVB-exposed human epidermis isolated by suction blister, compared with the non-exposed epidermis. Finally, the subepidermal injection of c-kit antibodies into brownish guinea pig skin completely abolished the UVB-induced pigmentation, in strict contrast to the lack of inhibition elicited by the similar injection of nonspecific IgG. This melanogenic suppression following the injection of c-kit antibodies was accompanied by no increase in the number of DOPA-positive melanocytes. These findings strongly suggest that upregulation of the SCF/c-kit signaling between keratinocytes and melanocytes is associated with the increased pigmentation in the UVB-exposed skin.

One of the most important issues addressed in the mode of action of SCF refers to how SCF acts on melanocytes during the

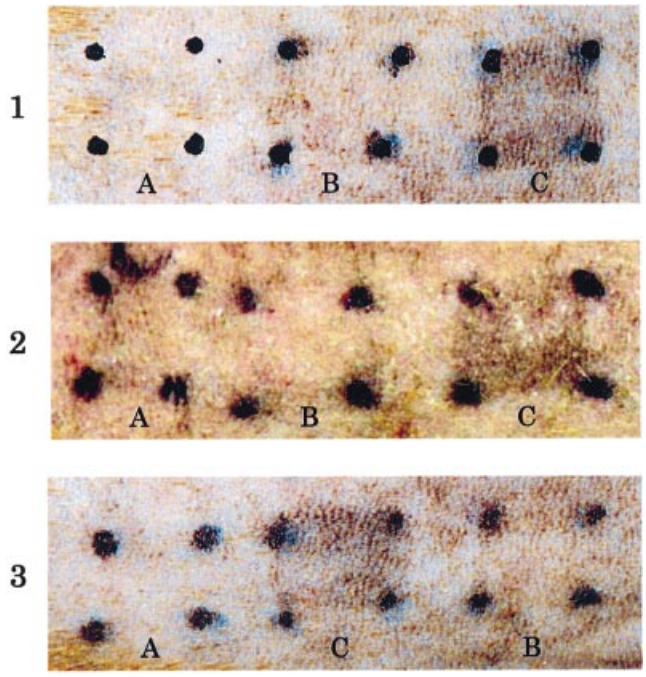

Figure 10. The c-kit inhibitory antibody, ACK2, abolishes the UVB-induced pigmentation on dorsal skin of brownish guinea pig as revealed by photography. Guinea pigs were exposed twice to UVB light and were then injected subepidermally with ACK2 or with purified rat IgG 24 h and 48 h after the final UVB irradiation, as detailed in *Materials and Methods*. The photograph was taken using three representative brownish guinea pigs on day 6. (*A*) Non-irradiation; (*B*) irradiation + ACK2 treatment; (*C*) irradiation + nonspecific IgG treatment. The order of the skin region in the bottom figure was changed to balance site to site variation for the induced pigmentation.

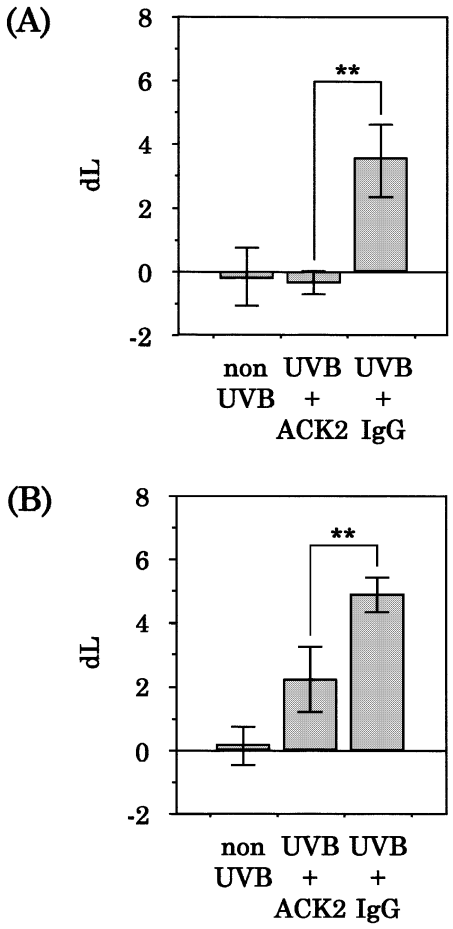

Figure 11. The c-kit inhibitory antibody, ACK2, abolishes the UVB-induced pigmentation on dorsal skin of brownish guinea pig as measured by color difference meter. Guinea pigs were exposed twice to UVB light and were then injected subepidermally with ACK2 or with purified rat IgG 24 h and 48 h after the final UVB irradiation as detailed in *Materials and Methods*. Measurements of color difference were carried out on day 6 (*A*) and on day 10 (*B*). The values represent means ± SD from five independent experiments. $^{**}p < 0.01$.

upregulation of melanogenesis to stimulate their proliferation and melanization. Thus, the observed inhibition of UVB-induced pigmentation by antibodies to c-kit was complete at day 6 but not at day 10, indicating that the action of SCF occurs in an early phase of melanogenic stimulation. This was also corroborated by the increased number of DOPA-positive melanocytes at day 10, even in the antibody-injected site, which was paralleled by the skin color changes, although there was still a significant inhibition in melanocyte number and skin color in the antibody-injected site compared with the control. In our experiments using hairless mouse epidermis, the expression of SCF as measured by immunohistochemistry and ELISA reached a peak as early as 24 h after the second daily irradiation of UVB (data not shown). This contrasts with the late phase of production and secretion for the known UVB-pigmentation-associated intrinsic cytokine ET-1, in similar UVB-exposed conditions, which takes as long as 5 d or more (Imokawa *et al*, 1995). Therefore, it is likely that the incomplete inhibition of pigmentation observed at day 10 following the blockage of SCF/c-kit binding is due to an essential role of the SCF/c-kit signaling in an early phase of melanocyte activation. In the later phases of melanogenesis, ET-1 may play an essential role as described previously (Imokawa *et al*, 1995).

The majority of SCF produced in human epidermis has been reported to be a membrane-bound type that is not secreted into intercellular spaces between keratinocytes (Longley *et al*, 1993; Hamann *et al*, 1995). The cellular localization of UVB-induced SCF expression in the stratum spinosum as revealed by immunohistochemistry suggests the requirement for melanocyte dendrites expanding up to the stratum spinosum layers to interact with membrane-anchored SCF via c-kit receptor. In the human epidermis of lentigo seniles where the expression of SCF in keratinocytes and c-kit in melanocytes is accentuated in addition to the increased expression of epidermal ET cascade,[2] which synergistically leads to their epidermal hyperpigmentation, there is a distinct immunostaining with antibody to c-kit on melanocyte dendrites expanding through the stratum spinosum layers,[3] although the present immunohistochemistry in guinea pig epidermis using antibody to human c-kit did not demonstrate such a clear localization in the dendrite because of its relatively low affinity. This evidence may reflect a possible signaling between membrane-anchored SCF and c-kit even in the stratum spinosum layers. The membrane-bound characteristic that requires no secretory processing within the cells also allows us to speculate that the stimulatory effect of SCF on melanocytes differs from ET-1 in the phase in which it stimulates melanogenesis as the secretion of ET-1 requires multiple steps. This cascade includes the release of the primary cytokine interleukin-1α (IL-1α), IL-1α autocrine action on

[2]Tajima S, Manaka I, Kawashima M, Kobayashi T, Imokawa G: Role of endothelin cascade between keratinocytes and melanocytes in hyperpigmentation of senile freckle. *J Invest Dermatol* 110:571, 1998 (abstr.)

[3]Fushimi H, Shishido E, Kadono S, Manaka I, Kawashima M, Ichikawa M, Imokawa G: The role of stem cell factor (SCF) in hyperpigmentation mechanism involved in senile freckle (SF). *Pigment Cell Res* 13:475, 2000 (abstr.)

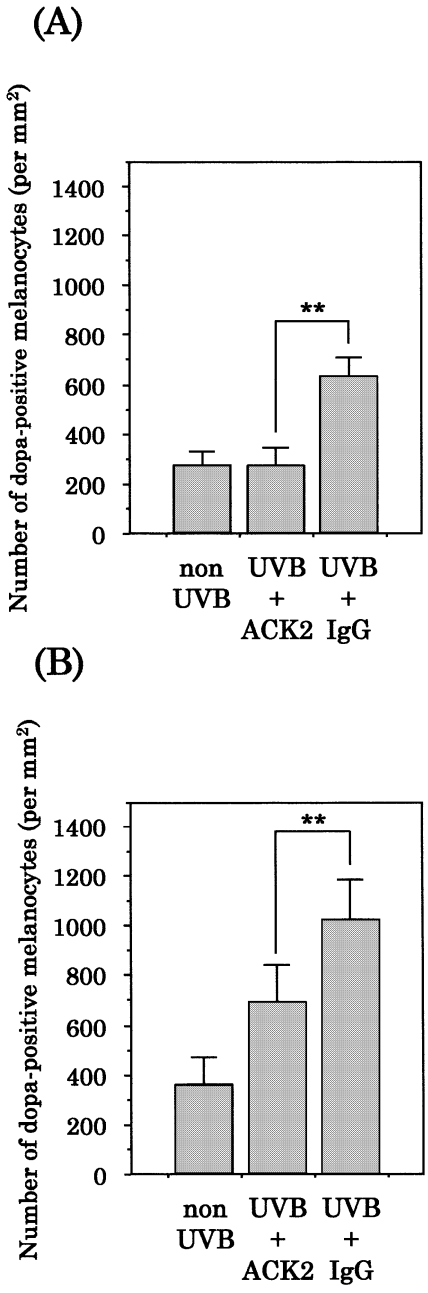

Figure 12. The c-kit inhibitory antibody, ACK2, suppresses the increase in the number of DOPA-positive melanocytes in the epidermis of brownish guinea pig skin. Guinea pigs were exposed twice to UVB light and were then injected subepidermally with ACK2 or purified rat IgG 24 h and 48 h after the final UVB irradiation, as detailed in *Materials* and *Methods*. The numbers of DOPA-positive melanocytes were measured in epidermal sheets on day 6 (*A*) and on day 10 (*B*), and are expressed as the numbers per mm^2. The values represent means ± SD from six (*A*) or eight (*B*) independent experiments. **$p < 0.01$.

keratinocytes, gene expression and protein synthesis of ET, the processing of prepropeptides, and the subsequent processing of big-ET by endothelin-converting enzyme to finally generate the active form, ET (Xu *et al*, 1994).

The major role of SCF in melanogenic phenomena was originally considered to be targeting for c-kit receptor bearing cells, melanoblasts or melanocytes, as seen for the migration of melanoblasts from the neural crest toward hair follicles via the epidermis (Matsui *et al*, 1990; Orr-Urtreger *et al*, 1990). In this regard, the membrane-bound type of SCF seems to play an essential role in such targeting because of its extremely low mobility through tissues. In support of this, overexpression of membrane-bound SCF transgenes in keratinocytes resulted in the postnatal maintenance of epidermal melanocytes in mice (Kunisada *et al*, 1998), whereas in normal mice melanoblasts and melanocytes found at birth in the epidermis decrease in number from postnatal day 4 and then virtually disappear after 1 mo, except for those in hair follicles (Hirobe, 1984). No data are available *in vivo* on the role of SCF as a mitogen and/or a melanogen for human melanocytes, however, except for studies on the melanogenic mechanism involved in dermatofibroma where SCF is secreted as a soluble type. This study provides a new insight into the role of membrane-bound SCF as a mitogen and a melanogen in the initiation of UVB-induced pigmentation.

We have previously reported that ET, a vasoconstrictive peptide, is secreted by keratinocytes and plays an important role in the accentuated proliferation and melanogenesis of melanocytes in UVB-induced pigmentation. The important role of SCF demonstrated in this study would not necessarily be in opposition to, but might be coordinated with, the role of ETs in UVB-induced pigmentation. Thus, in the early phase of UVB-induced pigmentation, SCF production may be stimulated and may be expressed as the membrane-bound form on the plasma membrane of keratinocytes, and then lock onto the c-kit receptor, resulting in persistent c-kit activation without c-kit internalization and degradation within melanocytes, as has been suggested by Longley and Carter (1999). Further, ET secretion by keratinocytes can be stimulated in the later phase and may synergistically activate melanocyte function, probably through the crosstalk mechanism in the signaling pathway with SCF as suggested by Imokawa *et al* (2000).

In conclusion, these findings suggest that, in addition to the ET-$1/ET_B$ receptor signaling, SCF/c-kit signaling is also involved in the biologic mechanism of UVB pigmentation as a mitogen and as a melanogen for human melanocytes. Most importantly, these findings facilitate a fundamental understanding of the melanogenesis mechanism as well as the homeostatic maintenance of skin color.

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