Endothelin-1 as a New Melanogen: Coordinated Expression of Its Gene and the Tyrosinase Gene in UVB-Exposed Human Epidermis

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We previously demonstrated that human keratinocytes produce and secrete endothelins (ET), which can be strong mitogens for human melanocytes. Ultraviolet B (UVB) exposure highly stimulated the paracrine linkage of endothelins between keratinocytes and melanocytes, indicating that they are keratinocyte-derived intrinsic mitogens in UVB-induced pigmentation. In this study, the role of ET-1 as a melanogen in UVB melanogenesis was investigated *in vitro* and *in vivo*. In the conditioned medium of keratinocytes exposed to UVB, melanin synthesis by human melanocytes, as measured by ^{14}C-thiouracil incorporation, was significantly accentuated. This stimulatory effect was reduced by anti–ET-1 to the level of that in the non–UVB-exposed control, suggesting an essential role of ET-1 as an intrinsic melanogen in UVB-induced melanogenesis. In a parallel study, the addition of 10 nM ET-1 induced an increase in tyrosinase activity in cultured human melanocytes and was accompanied by elevated levels of tyrosinase and tyrosinase-related protein-1 mRNA expression as shown by Northern blotting. Reverse transcription–polymerase chain reaction of RNA isolated from the epidermis of human skin exposed to UVB revealed that, whereas in non-exposed sites ET-1, IL-1α, and tyrosinase mRNA signals were scarcely detected, UVB-irradiation, with a dose of twice the minimal erythema dose, caused a significant increase in the expressions of the three genes 5 d after irradiation. These findings suggest that ET-1 is an important mediator for UVB-induced pigmentation in the epidermis *in vivo*. *J Invest Dermatol 105:32–37, 1995*

We previously demonstrated that human keratinocytes produce and secrete endothelins (ET), which can be strong mitogens for human melanocytes [1,2]. Ultraviolet B (UVB) exposure stimulated the paracrine linkage of endothelins between keratinocytes and melanocytes, indicating that they are keratinocyte-derived intrinsic mitogens in UVB melanogenesis [2]. Melanocytes are well-differentiated in the epidermis where a cell-specific product, melanin, is synthesized within unique biologic organelles called melanosomes, by the action of a specific enzyme, tyrosinase [3–7]. They function to provide basal skin color and protect the skin against ultraviolet irradiation, which also results in cutaneous hyperpigmentation through accentuated melanocyte proliferation and differentiation [8–10]. It is likely that increased melanin synthesis, due to an accentuated function of tyrosinase in conjunction with an increased melanocyte reproduction, is deeply associated with UVB-induced pigmentation. Therefore, it is of considerable significance to determine whether UVB-exposed human keratinocytes increase the secretion of factors that, in conjunction with its mitogenic effect, can stimulate tyrosinase activity in human melanocytes and whether such factors can also be ascribed to ET derivatives. If so, it would be worthwhile to determine whether ET-1-induced mitogenesis of human melanocytes is accompanied by an increased melanogenesis. Basic fibroblast growth factor (bFGF) does not stimulate tyrosinase activity, although it has mitogenic action on human melanocytes* [11]. Therefore, we first studied the role of ET-1 in the accentuation of melanogenesis induced by UVB light. We examined the stimulatory effect of the conditioned medium of UVB-exposed keratinocytes, or of ET-1 itself, upon the function and gene expression of tyrosinase in cultured human melanocytes. The physiologic significance of ETs in UVB-induced epidermal pigmentation has remained unclear in the absence of *in vivo* evidence of increased ET production by surrounding keratinocytes in the epidermis. We thus determined, using reverse transcription–polymerase chain reaction (RT-PCR) of RNA isolated from human epidermis, whether human skin exposed to UVB light stimulates human melanocytes *via* up-regulated ET production by human keratinocytes within the epidermis.

MATERIALS AND METHODS

Materials Normal human keratinocytes, melanocytes, and serum-free melanocyte medium (MGM) were obtained from Sankou Pure Chemicals (Tokyo, Japan). Endothelin derivatives and anti–ET-1 antibodies were

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Abbreviations: ET, endothelin, PDBu, phorbol-12,13-dibutyrate; TRP-1, tyrosinase-related protein-1.

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purchased from International Reagents Corp. (Kobe, Japan). Serum-free keratinocyte medium (SFM), bovine pituitary extract (BPE) and epidermal growth factor (EGF) were obtained from Gibco (Tokyo, Japan). 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. In specific experiments, interleukin derivatives or some growth factors were added to the culture medium. 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 under 5% CO_2 as previously described [2]. In several experiments, BPE, PMA, and bFGF were deleted from the medium (control medium).

Irradiation with Ultraviolet Light One or two days after the plating of human keratinocytes, culture medium was removed from the cells. After three washes of cells with 10 ml of phosphate buffered saline (PBS) (−), 5 ml of PBS (−) was added. The cells were irradiated twice at 24 h intervals with a dose of 50 mJ/cm^2 of UVB light (Toshiba SE lamp without any filtering for UVB), which emits most of their energy within the UVB range (295–315 nm) with a peak of 305 nm. Immediately after photoexposure, the medium was returned to each flask. The irradiated cells were maintained at 37°C in a 5% CO_2 atmosphere. In UVB exposure of human skin, back skin was irradiated with a dose of twice the minimal erythema dose (MED) of the same UVB light as described in the culture experiments.

DNA Synthesis Melanocytes grown in 24-well culture trays were incubated with ET-1 at a concentration of 10 nM or keratinocyte-conditioned medium. Twenty hours later the cells were labeled for 4 h with 1.0 μCi/ml [^{3}H] thymidine. After three washes with PBS, the cells were lysed with 2 N NaOH at 37°C for 15 min and neutralized with 2 N HCl. Acid-insoluble materials were precipitated by adding four volumes of 10% ice-cold trichloroacetic acid (TCA), collected on glass microfiber filters, washed three times with 10% TCA and once with ethanol, then dried [12].

Measurements of [^{14}C] Thiouracil Incorporation and ^3H_2O Release Tyrosinase activity in living cells was assayed according to the method of Oikawa *et al* [13]. In addition, because exogenous thiouracil is documented to become part of the melanin polymer by binding to the quinones generated during the synthesis of melanin depending on tyrosinase activity [14,15], and there is a close correlation between the extent of its incorporation and the amount of tyrosine hydroxylated (calculated from the release of ^3H_2O) in mammalian pigment cells [2,16] and pigmented skin tissue [17], the incorporation of ^{14}C-thiouracil into the TCA-insoluble fraction of melanocytes was measured as another indicator of tyrosinase activity. Because of the preferential incorporation into melanin [15] and the rare presence of quinones in other eukaryotic cells, it is possible to designate ^{14}C-thiouracil as a useful indicator of melanin synthesis in pigment cells. Melanocytes were seeded in 24-well culture trays at a density of 3–8 × 10^4 cells/ml and cultured overnight in MGM (without PMA and bFGF) containing low tyrosine (34.1 μg/l), 0.2% BPE, and 10 nM ET-1. The cells were labeled for the last 21 h of the 24 h incubation with 0.5 μCi/ml [^{14}C] thiouracil or 1.0 μCi/ml [^{3}H] tyrosine. To measure ^3H_2O release, 500 μl of the medium was mixed with 500 μl of 20% charoal containing 10% trichloroacetic acid (TCA) (charcoal solution). The mixture was vortex-mixed for 30 seconds and centrifuged for 10 min at 2,000 × *g*. A portion of the supernatant (750 μl) was mixed with 500 μl of charcoal solution. The mixture was vortex-mixed for 30 seconds and centrifuged for 10 min at 2,000 × *g*. One milliliter of the supernatant was transferred and this treatment via charcoal solution was performed twice. The final supernatant was mixed with scintillation fluid, and the radioactivity was determined in a liquid scintillation counter. To assay the thiouracil incorporation, after three washes with PBS, the cells were lysed with 2 N NaOH at 37°C for 15 min and neutralized with 2 N HCl. The mixtures were mixed with scintillation fluid, and the radioactivity was determined in a liquid scintillation counter.

Effect of Keratinocyte-Conditioned Medium on Melanocytes Conditioned medium was collected after keratinocytes were irradiated with UVB light and maintained for 4 d in modified MCDB medium supplemented with 50 μg/ml BPE at 37°C with 5% CO_2. Melanocytes were seeded in 24-well trays at a density of 4 × 10^4–10^5 cells/ml and cultured for 24 h as previously described [12]. Half the medium (500 μl) was aspirated and mixed with 250 μl of ten-fold concentrated conditioned medium. The concentration of keratinocyte-conditioned medium was carried out by lyophilization. To determine the ET-1 antibody effect, 5–10 μg/ml of anti-human ET-1 IgG was added to the culture medium. Three hours later, the cells were labeled for 21 h with 0.5 μCi/ml [^{14}C] thiouracil.

Northern Blots Poly(A)+ RNAs were isolated using a Fast Track mRNA isolation kit (Invitrogen). RNAs (1 μg) were size-fractionated on 1.0% agarose/formaldehyde gels and blotted onto Hybond N+ (Amersham). The blots were prehybridized in 50% formamide/10% Denhardt's solution/5 × SSPE/0.1% sodium dodecylsulfate (SDS)/denatured salmon sperm DNA (100 μg/ml) for 2 h at 42°C and hybridized at 42°C overnight with a 1-kilobase (kb) cDNA probe for tyrosinase, and with a 0.6-kb cDNA probe for tyrosinase-related protein-1 (TRP-1) random prime-labeled with [α-^{32}P]dCTP (Amersham) and with a 40-base oligonucleotide probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) labeled with [gamma^{32}P] adenosine triphosphate. The membranes were washed in 2 × SSPE, 0.1% SDS for 15 min twice at 42°C and then exposed to a phosphor-imaging plate for 2 d. The total radioactivity of hybridized bands was quantified using a Fujix Bio-Image analyzer BAS2000 (Fuji Photo Film Co. Ltd., Kanagawa, Japan).

Detection of ET-1 and Tyrosinase Gene Transcripts in Human Epidermis Exposed to UVB Biopsy specimens were obtained from back skin (Japanese man, aged 28) before and 2 or 5 d after UVB irradiation with a dose of twice the MED. The epidermal sheet was separated by heating for 3 min at 60°C. Total cellular RNA was extracted from the epidermis using acid guanidinium phenolchloroform [1] with only a single precipitation. The RNA was quantified by measuring the optical density at 260 nm. About 1 μg total RNA was obtained from one tissue sample. To synthesize oligo(dT16)-primed cDNA, total RNA was heated to 65°C for 3 min, then chilled on ice. Reverse transcription was performed in 20 μl of a reaction mixture containing 2 μl of 10 × PCR buffer II, 4 μl $MgCl_2$ (25 mM), 1 μl oligo(dT16) (50 μM), 8 μl of deoxynucleotides (2.5 mM each), 1 μl RNase inhibitor (20U/μl), 1 μl Moloney murine leukemia virus reverse transcriptase (50 U/μl), and 3 μl RNA (300 ng in DEPC-treated water), which was incubated at 42°C for 60 min and 52°C for 30 min, then heated to 99°C for 5 min to inactivate the reverse transcriptase. For PCR amplification of the cDNA, 2 μl of the cDNA reaction mixture was added to 8 μl of 80 μl PCR mix containing 4 μl $MgCl_2$ (25mM), 8 μl of 10 × PCR buffer II, 1 μl of both 3′ and 5′ primers (20 μM each), and 0.5 μl Taq polymerase (Cetus). The mixture was overlaid with mineral oil, then amplified using a Perkin-Elmer/Cetus thermal cycler. The PCR cycle conditions were melting for 1 min at 95°C, annealing for 1 min at 60°C, and extension for 2 min at 72°C. Reaction products (5 μl) were resolved on a 1–1.5% agarose gel and visualized by ethidium bromide staining. Primers for interleukin-1α (IL-1α), transforming growth factor α (TGFα), basic fibroblast growth factor (bFGF), and gamma interferon (IFN) were purchased from Clontech. Others were synthesized on DNA synthesizer (Applied Bio System). The sequences of primer pair, 3′ and 5′, used in this study are shown in **Fig 1**.

RESULTS

Stimulation of Melanin Synthesis in Melanocytes by Keratinocyte-Conditioned Medium and Its Inhibition by Anti–ET-1 Antibody The culture medium conditioned by UVB-exposed keratinocytes significantly stimulated melanin synthesis by human melanocytes in 24 h as revealed by ^{14}C-thiouracil incorporation **(Fig 2)**. Markedly stimulated melanin synthesis occurred at doses of 50 mJ/cm^2 of UVB, when the conditioned medium was concentrated ten-fold and added in a 1:2 dilution to melanocyte culture. When the conditioned medium, obtained after exposure to a dose of 50 mJ/cm^2 of UVB, was incubated with anti–ET-1 antibody, the level of melanin synthesis was diminished to that in the non–UVB-exposed control **(Fig 2)**.

Effects of ET-1 on Melanin Synthesis Activity in Cultured Human Melanocytes When incubated for 24 h with 10 nM ET-1, cultured human melanocytes significantly increased tyrosinase activity and melanin synthesis as revealed by ^3H_2O release **(Fig 3*a*)** and ^{14}C-thiouracil incorporation **(Fig 3*b*)**, respectively. This was accompanied by significantly accentuated DNA synthesis as revealed by ^{3}H-thymidine incorporation **(Fig 3*c*)**.

Effects of ET-1 on Tyrosinase Gene Transcripts in Cultured Human Melanocytes Northern blots with a human cDNA probe for tyrosinase and tyrosinase-related protein-1 (TRP-1) revealed that human melanocytes stimulated by ET-1 increased the levels of 2.3-kb tyrosinase and 1.5-kb TRP-1 transcripts 1 and 2 d

	5' primers	3' primers	size of product
IL-1 α	5'-ATGGCCAAAGTTCCAGACATGTTTG-3'	5'-GGTTTTCCAGTATCTGAAAGTCAAGT-3'	808bp
β-Actin	5'-ATGGATGATTATCGCCGCG-3'	5'-CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC-3'	2551bp
TGF-α	5'-ATGGTCCCCTCGGCTGGACAG-3'	5'-GGCCTGCTTCTTCTGGCTGGCA-3'	886bp
IFN-γ	5'-ATGAAATTATTACAAGTTATTAATCTTGGCTTT-3'	5'-GATGCTCTTCGACCTCGAAACAGCAT-3'	494bp
GAPDH	5'-GAAGGTGAAGGTCGGAGTCAACG-3'	5'-AGTCCTTCCACGATAACCAAAGTTG-3'	516bp
Tyrosinase*	5'-CCGGAATTCAGAATGCTCCTGGCTGTTTTGTA-3'	5'-GCAAGCTTAGATGTGCAAGGCATTGTGCA-3'	1122bp
TRP-1	5'-CCTGGGAACACTTTGTAACAGC-3'	5'-TCATATTCTTTCTTCAGCAATAGCATTG-3'	691bp
ET-1	5'-TTTCAGAATGGATTATTTGCTCATG-3'	5'-GACAGGCCCCGAAGTCTGTCA-3'	664bp
bFGF	5'-CCACTTCAAGGACCCCAAGC-3'	5'-AGCTCTTAGCAGACATTGGAAG-3'	395bp

* Restriction sites are added to 5' ends of each tyrosinase primer.

Figure 1. The sequences of primer pair 3′ and 5′ used in this study.

after incubation (**Fig 4*a***). These levels were significantly increased by about 1.6- and 1.4-fold at day 1 and about 1.8- and 2.4-fold, respectively, at day 2 (**Fig 4*b***). The increased levels of these transcripts were very similar when the same experiments were repeated.

Effects of UVB Irradiation on ET-1 and Tyrosinase Gene Transcript in Human Epidermis To clarify the *in vivo* effect of UVB exposure upon human skin, we studied changes in gene expression of ET-1, IL-1α, and tyrosinase using RT-PCR in human epidermis exposed to UVB. Biopsy specimens were obtained from back skin before and 2 or 5 d after UVB irradiation. In the skin exposed to two or three MED of UVB, an erythematous reaction markedly occurred at day 2 and pigmentation began to appear at day 5. The epidermal sheet was separated by heating at 60°C. Total cellular RNA was extracted and amplified by reverse transcription polymerase chain reaction (RT-PCR) with the specific primers. As shown in **Fig 5**, whereas in the non-exposed site ET-1, IL-1α, and tyrosinase mRNA signals were scarcely detected, UVB irradiation with a dose of twice the MED caused a significant increase in the expression of the three genes 5 d later.

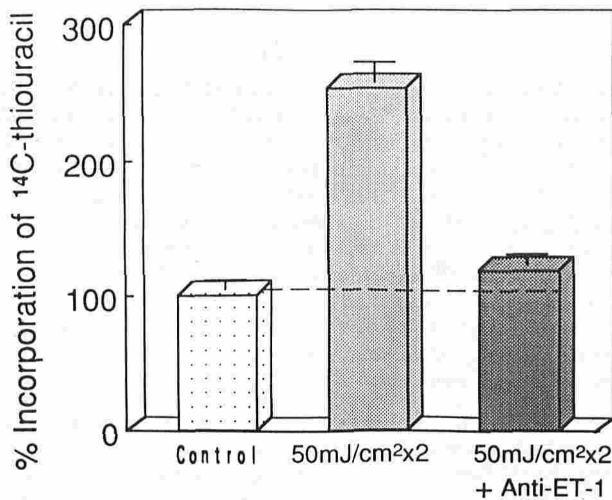

Figure 2. Stimulatory effect of the conditioned medium from UVB-exposed human keratinocytes on the melanin synthesis of cultured human melanocytes and its suppression by anti–ET-1 antibody. The conditioned media were concentrated tenfold and added in a 1:2 dilution to cultured melanocytes. To determine the ET-1 antibody effect, 5-10 μg/ml of anti-human ET-1 IgG was added to the culture medium. Three hours later, the cells were labeled for 21 h with 0.5 μCi/ml [^{14}C] thiouracil. The values are means ± SD derived from three experiments.

DISCUSSION

We recently demonstrated that human melanocytes are upregulated by potent vasoconstrictive peptides, ETs, which stimulate melanocyte proliferation *via* a receptor-mediated signal-transduction pathway [1]. The conditioned medium of normal cultured human keratinocytes exposed to UVB light was found to contain factors that stimulate DNA synthesis in human melanocytes [2]. This suggested that the epidermal cells, which surround melanocytes in a 30:1 ratio, play an essential role in regulating melanocyte proliferation by secreting various growth factors in response to stimuli. In a parallel study, we found that human keratinocytes can produce and secrete the vasoconstrictive peptides, endothelins [2]. Most importantly, this biologic process was considerably accentuated by exposing human keratinocytes to UVB light, implying that ETs are an intrinsic mitogen for human melanocytes in UVB-induced pigmentation [2]. Direct evidence for the involvement of ET-1 in UVB melanogenesis was that the stimulatory effect on DNA synthesis in cultured human melanocytes by conditioned medium of keratinocytes exposed to UVB was completely neutralized by anti–ET-1 antibody [2].

Unlike the action of ET-1 on melanocyte proliferation, little is yet known about the mechanisms by which melanocytes are activated to produce more melanin polymer after exposure to ultraviolet light, because melanogenic factors produced by UVB-exposed keratinocytes have not been identified. It is unlikely that the accentuated melanin synthesis within melanocytes always occurs subsequently to an effect on proliferation under cytokine-stimulated conditions. The evidence that bFGF, a known mitogen of human melanocytes, has no potential to stimulate melanin synthesis* [11] supports the notion that there are distinctly different stimulation mechanisms between proliferation and melanization within melanocytes.

The incorporation of ^{14}C-thiouracil into pigment cells occurs in parallel with the hydroxylation of tyrosine and the activity of tyrosine hydroxylase in living cells [2,15–17] and is a convenient indicator of melanin synthesis due to its selective incorporation into melanin-synthesizing cells [14,15]. The quantification of melanin synthesis by ^{14}C-thiouracil incorporation demonstrated that the conditioned medium from human keratinocytes exposed to UVB light contained stimulatory factors for melanin synthesis in human melanocytes. This effect was abolished by anti–ET-1 to the level of the conditioned medium of normal keratinocytes, indicating that melanogenic factors in the UVB-conditioned medium are also ascribed to ET-1. The notion that ET-1 is involved in the biologic process leading to the stimulation of melanin synthesis was directly confirmed by the fact that the exogenous addition of ET-1 effec-

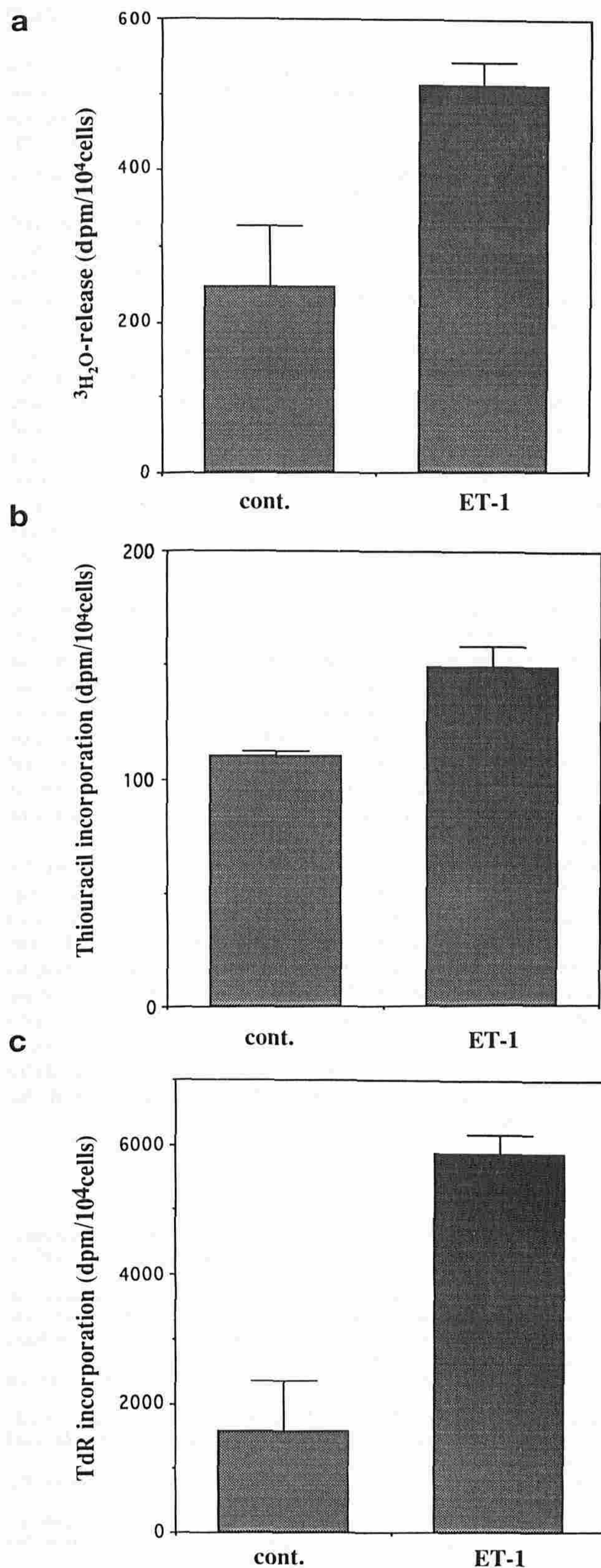

Figure 3. Stimulatory effects of ET-1 on human melanocytes. *a*, Effects of ET-1 on tyrosinase activity. The ET-1 was added at a concentration of 10 nM to human melanocytes culture. Three hours later, tyrosinase activity was evaluated by measuring release of ^3H_2O for 21 h. *b*, Effects of ET-1 on melanin synthesis. Melanin synthesis was evaluated by measuring ^{14}C-thiouracil incorporation for 21 h after 3 h incubation with 10 nM ET-1. *c*, Effects of ET-1 on DNA synthesis. DNA synthesis was evaluated by measuring [^{3}H]thymidine incorporation for 4 h after 20 h incubation with 10 nM ET-1. The values are mean ± standard deviation derived from three experiments. Cont, control.

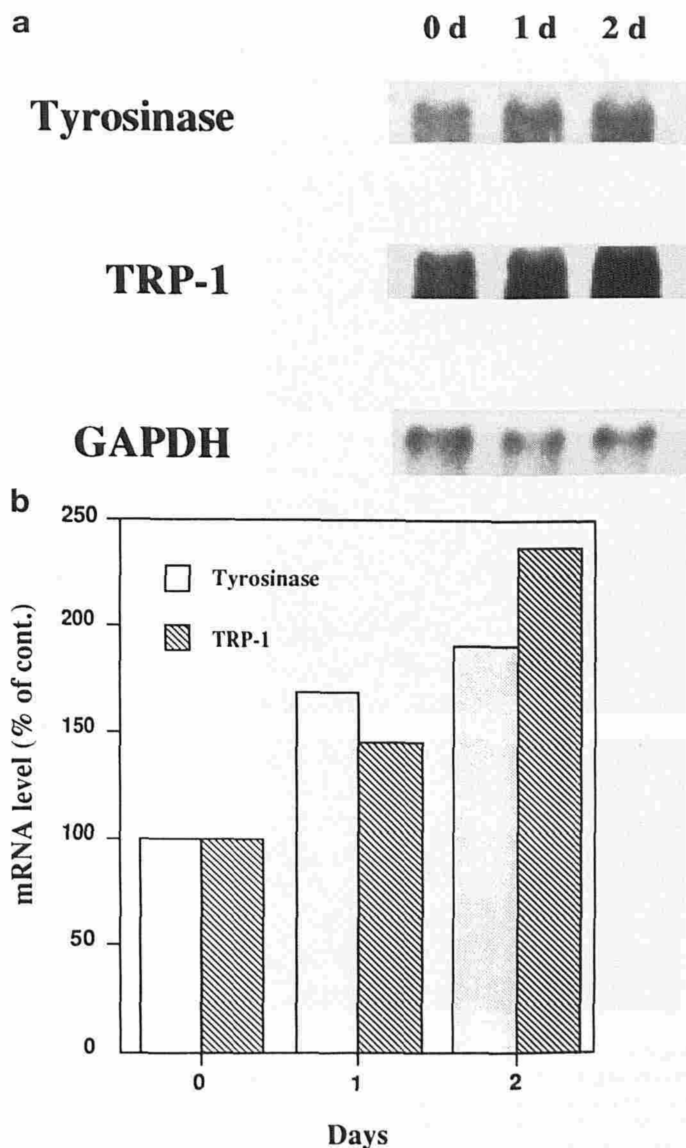

Figure 4. Northern blot analysis of tyrosinase and TRP-1 mRNAs. *a*, Northern blots of poly(A)+ RNA (1 μg/lane) isolated from human melanocytes after hybridization with ^{32}P-labeled tyrosinase and TRP-1 cDNA probe. The extraction of poly(A)+ RNA from human melanocytes was carried out before and 1 and 2 d after treatment with 10 nM ET. Portions of the poly(A)+ RNA were blotted onto another filter and hybridized to the ^{32}P-labeled human beta-actin probe. *b*, Quantitative analysis of Northern blots. The total radioactivity of hybridized bands was quantified using a Fujix Bio-Image analyzer BAS 2000.

tively stimulated tyrosinase activity and melanin synthesis in cultured human melanocytes. This melanogenic stimulation was associated with increased tyrosinase and TRP-1 mRNA gene expression, suggesting the role of ET-1 in the accentuated production of tyrosinase. These findings are consistent with the biologic events in the skin after UVB-exposure, where the stimulation of melanin synthesis concurs with the increased proliferation of melanocytes [8–10]. We therefore conclude that ET-1 is probably a major UVB-associated melanogenic stimulator in UVB-induced pigmentation.

The sequence of subcellular events following cytokine exposure that leads melanocytes to differentiate into melanin-producing and/or highly proliferative cells is unknown. Several investigators have reported that the protein-kinase C activation pathway plays a critical role during the stimulation of melanin synthesis. Park *et al* [18] have found that the activation of PKC by PMA increases

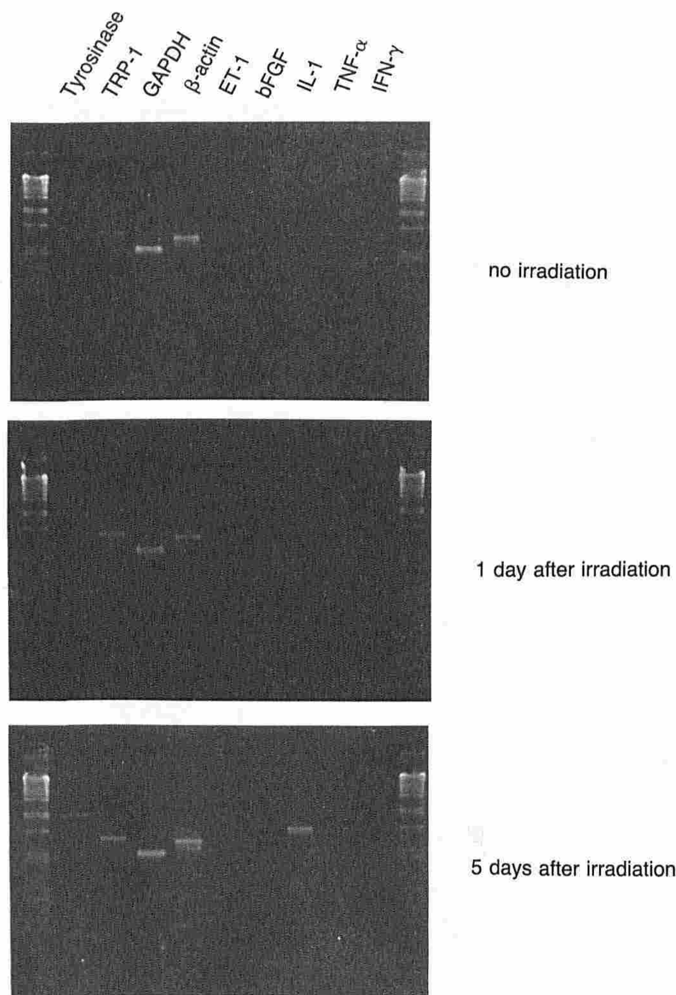

Figure 5. RT-PCR analysis of RNA isolated from human epidermis following UVB exposure. Biopsy specimens were obtained from human back skin before and 2 or 5 days after UVB irradiation with a dose of twice the MED. Total RNA extracted from the biopsied epidermal sheet was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase using oligo(dT)16 as a primer. The cDNA synthesized was PCR amplified with the ET-1, tyrosinase, TRP-1, IL-1α, γ-IFN, TNFα, and bFGF specific primer sets or β-actin and GAPDH primer set as the positive controls.

tyrosinase activity in human melanocytes, whereas PKC depletion by phorbol-12,13-dibutyrate (PDBu) rapidly reduces tyrosinase activity, and the recovery of PKC activity after withdrawal of PDBu conversely leads to normalization of tyrosinase activity. Human melanocytes cultured in the presence of 1-oleoyl-2-acetylglycerol, an active analog of diacyglycerol, had a higher level of melanogenesis [19]. Furthermore, the α-melanocyte stimulating hormone (MSH)–stimulated melanization in murine melanoma cells can also be abolished through the depletion of PKC by PDBu.† In addition, we showed in another study that the tyrosinase activity and melanin synthesis in guinea pig melanocytes stimulated by allergic reaction-derived soluble factors that are associated with the enhanced formation of intracellular inositol 1,4,5-trisphosphate, is completely blocked by the PKC inhibitor, H-7, or its downregulatory agent, PDBu [16]. With regard to the melanogenic stimulation in melanocytes induced by cytokines or chemicals, there is most likely a close relationship between the increased melanogenesis and PKC activation.

Based upon our preliminary study,* which showed that ET-1–induced PKC activation in human melanocytes is accompanied by protein kinase A activation as shown by the elevated level of cyclic AMP, the major issue to be resolved is the mechanism by which ET is able to cross talk among diverse signal-transduction pathways. Further studies are required to address this issue.

The physiologic significance of ETs in UVB-induced epidermal pigmentation has remained unclear in the absence of *in vivo* evidence of increased ET production by surrounding keratinocytes in the epidermis. No intrinsic melanogenic factor associated with UVB-induced epidermal pigmentation has so far been characterized *in vivo*, specifically with regard to the expression of melanogenesis-related gene transcripts. In this study, RT-PCR of the RNA isolated from the epidermis of UVB-exposed human skin revealed that, whereas in non-exposed sites, ET-1, IL-1α, and tyrosinase mRNA signals were scarcely detectable, there was a definite increase in the expression of the IL-1α, ET-1, and tyrosinase genes 5 d after irradiation with UVB. This shows that the ET-1 gene is expressed in human epidermis and that UVB light increases it, as well as those of tyrosinase and IL-1α. The concurrent gene expression of IL-1α and ET-1 in UVB-exposed epidermis is consistent with the previous evidence [2] that IL-1α is an essential cytokine for the production of ET-1 in cultured human keratinocytes in an autocrine fashion. Therefore, together with the evidence that ET-1 is a powerful melanogen, these findings suggest that UVB light stimulates the secretion of IL-1α by keratinocytes to increase ET-1 gene expression in an autocrine fashion. The up-regulated ET-1 secretion in keratinocytes results in the increased expression of the tyrosinase gene, together with the expression of growth-related genes in melanocytes, which lead to cutaneous pigmentation *in vivo*.

In conclusion, this study provides a new insight into the mechanisms underlying UVB-associated pigmentation. UVB light triggers human keratinocytes in an autocrine fashion to produce and secrete a completely different species of growth factors. Human melanocytes undergo proliferation and melanization in response to the same growth factor secreted from human keratinocytes. Most importantly, the present findings suggest that ET-1 makes a considerable impact upon the expression of the melanogenesis-stimulation cascade. This will facilitate fundamental understanding of the melanization mechanism underlying UVB melanogenesis and the homeostatic maintenance of skin color.

† Park HY, Russakovsky V, Gilchrest BA: Depletion of protein kinase C blocks α-melanocyte stimulating hormone-induced melanogenesis (abstr). *J Invest Dermatol* 98:645, 1992.

REFERENCES

1. Imokawa G, Yada Y, Miyagishi M: Endothelins secreted from human keratinocytes are intrinsic mitogens for human melanocytes. *J Biol Chem* 267:24675–24680, 1992
2. Yada Y, Higuchi K, Imokawa G: Effects of endothelins on signal transduction and proliferation in human melanocytes. *J Biol Chem* 266:18352–18357, 1991
3. Hearing VJ, Ekel TM: A comparison of tyrosine hydroxylation and melanin formation. *Biochem J* 157:549–557, 1976
4. Pawelek JM, Korner AM: The biosynthesis of mammalian melanin. *Am Sci* 70:136–145, 1987
5. Imokawa G, Mishima Y: Isolation and biochemical characterization of tyrosinase-rich GERL and coated vesicle in melanin synthesizing cells. *Br J Dermatol* 104:169–178, 1981
6. Lerner AB, Fitzpatrick TB: Biochemistry of melanin formation. *Physiol Rev* 30:91–126, 1950
7. Imokawa G, Mishima Y: Loss of melanogenic properties in tyrosinases induced by glycosylation inhibitors within malignant melanoma cells. *Cancer Res* 42:1994–2002, 1982
8. Imokawa G, Kawai M, Mishima Y, Motegi I: Differential analysis of experimental hypermelanosis induced by UVB, PUVA and allergic contact dermatitis using brownish guinea pig model. *Arch Dermatol Res* 278:352–362, 1986
9. Blog FB, Szabo G: The effects of psoralen and UVA (PUVA) on epidermal melanocytes of the tail in C57 BL mice. *J Invest Dermatol* 73:533–537, 1979
10. Jimbow K, Uesugi T: New melanogenesis and photobiological process in activation and proliferation of precursor melanocytes after UV exposure: ultrastructural differentiation of precursor melanocytes from Langerhans cells. *J Invest Dermatol* 78:108–115, 1982
11. Yaar M, Gilchrest BA: Human melanocyte growth and differentiation: a decade of new data. *J Invest Dermatol* 97:611–617, 1991

12. Isseroff RR, Fusenig NE, Ridkin DB: Plasminogen activator in differentiating mouse keratinocytes. *J Invest Dermatol* 80:217–222, 1983
13. Oikawa A, Nakayasu M, Nohara M, Tchen T: Fate of L-[3,5-^{3}H] tyrosine in cell-free extracts and tissue cultures of melanoma cells: a new assay method for tyrosinase in living cells. *Arch Biochem Biophys* 148:548–557, 1972
14. Farishian RA, Whittaker JR: Phenylalanin lowers melanin synthesis in mammalian melanocytes by reducing tyrosine uptake: implications for pigment reduction in phenylketonuria. *J Invest Dermatol* 74:85–89, 1980
15. Whittaker JR: Biosynthesis of a thiouracil pheomelanin in embryonic pigment cells exposed to thiouracil. *J Biol Chem* 246:6217–6226, 1971
16. Imokawa G, Yada Y, Okuda M: Allergic contact dermatitis release soluble factors that stimulate melanogenesis through activation of protein kinase C-related signal transduction pathway. *J Invest Dermatol* 99:482–488, 1992
17. Imokawa G, Motegi I: Skin organ culture model for examining epidermal melanization. *J Invest Dermatol* 100:47–54, 1993
18. Park HY, Russakovsky V, Ohno S, Gilchrest BA: The beta isoform of protein kinase C stimulates human melanogenesis by activating tyrosinase in pigment cells. *J Biol Chem* 268:11742–11749, 1993
19. Gordon PR, Gilchrest BA: Human melanogenesis is stimulated by diacylglycerol. *J Invest Dermatol* 93:700–702, 1989