Increase of Pro-opiomelanocortin mRNA Prior to Tyrosinase, Tyrosinase-Related Protein 1, Dopachrome Tautomerase, Pmel-17/gp100, and P-Protein mRNA in Human Skin After Ultraviolet B Irradiation

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In ultraviolet-induced tanning, the protein levels of various gene products critical for pigmentation (including tyrosinase and tyrosinase-related protein-1) are increased in response to ultraviolet B irradiation, but changes in mRNA levels of these factors have not been investigated *in vivo*. We have established an *in situ* hybridization technique to investigate mRNA levels of pro-opiomelanocortin, tyrosinase, tyrosinase-related protein-1, dopachrome tautomerase, P-protein, Pmel-17/gp100, and microphthalmia-associated transcription factor, and have analyzed the changes in mRNA levels in the ultraviolet B-exposed skin *in vivo*. The right or left forearm of each volunteer was irradiated with ultraviolet B, and skin biopsies were obtained at 2 and 5 d postirradiation. mRNA level of pro-opiomelanocortin was increased 2 d after ultraviolet B irradiation, and returned to a near-basal level after 5 d, whereas the mRNA levels of tyrosinase, tyrosinase-related protein-1, dopachrome tautomerase, P-protein, and Pmel-17/gp100 showed some or no increase at 2 d, but were significantly increased 5 d after ultraviolet B irradiation. Microphthalmia-associated transcription factor mRNA was slightly increased on days 2 and 5 after ultraviolet B irradiation. Our results suggest that the mechanism of the tanning response of human skin may involve the transcriptional regulation of certain pigmentary genes, and that pro-opiomelanocortin-derived melanocortins such as α-melanocyte-stimulating hormone and adrenocorticotropic hormone may play a part in regulating these genes *in vivo*. *Key words: in situ hybridization/melanocyte/pro-opiomelanocortin/tanning. J Invest Dermatol 118:73–78, 2002*

It is well known that skin darkening due to melanin deposition, with an increase of tyrosinase activity, appears 3 or 4 d after ultraviolet (UV) B irradiation (Fitzpatrick, 1965; Fitzpatrick *et al*, 1967; Quevedo *et al*, 1975; Gilchrest *et al*, 1979; Pathak *et al*, 1980; Nordlund *et al*, 1981). With the development of culture techniques for normal human melanocytes and the recent progress of molecular biology, it has been shown that α-melanocyte-stimulating hormone (α-MSH) may play a part in UVB-induced pigmentation in human skin (Chakraborty *et al*, 1995). Im *et al* (1998) reported that UVB can directly stimulate melanization in cultured normal human melanocytes only in the presence of α-MSH or other cyclic adenosine monophosphate inducers in the growth medium. Furthermore, Valverde *et al* (1995) reported that loss-of-function mutations in melanocortin receptor-1 (MC1R) in humans are associated with poor tanning ability, further emphasizing the importance of melanocortins in UVB-induced pigmentation. Melanocortins [(α-MSH, β-MSH, γ-MSH, and adrenocorticotropic hormone (ACTH)] are a group of hormones that originate from a common precursor, pro-opiomelanocortin (POMC) (Rubinstein *et al*, 1978; Liotta *et al*, 1980). We showed that ACTH, α-MSH, and β-MSH, but not γ-MSH, increase melanogenesis and proliferation of normal human melanocytes by binding to the common receptor MC1R (Suzuki *et al*, 1996). α-MSH and ACTH have the highest affinity for MC1R and the greatest effect on melanogenesis and proliferation. This, and the observation that both melanocortins are expressed in the skin *in vivo* (Wakamatsu *et al*, 1997), suggest that they play a part as paracrine regulators of human melanocytes. In cultured normal human melanocytes, α-MSH and ACTH greatly increase tyrosinase activity *in situ*, yet cause only small increases in the protein levels of tyrosinase, tyrosinase-related protein (TYRP) 1, and dopachrome tautomerase (DCT), with no change in the mRNA levels of these melanogenic enzymes (Abdel-Malek *et al*, 1995). In human skin, the protein levels of tyrosinase and TYRP1 are increased following UVB irradiation (Virador *et al* 2001); however, it is not known whether or not this is due to the increase of tyrosinase and TYRP1 mRNA.

In this study, we established an *in situ* hybridization technique for mRNA of various gene products critical for pigmentation and then determined the mRNA levels of POMC as well as tyrosinase,

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Abbreviations: POMC, pro-opiomelanocortin; TYRP, tyrosinase-related protein; DCT, dopachrome tautomerase; Pmel-17, Pmel-17/gp100; MSH, melanocyte-stimulating hormone; MC1R, melanocortin receptor-1; MITF, microphthalmia-associated transcription factor.

TYRP1, DCT, Pmel-17/gp100 (Pmel-17), P-protein, and microphthalmia-associated transcription factor (MITF) by *in situ* hybridization in control and UVB-irradiated human skin.

MATERIALS AND METHODS

Skin biopsies Eight healthy male volunteers of oriental origin (age range, 25–38 y) were recruited for this study. The right or left forearm (area of 1.5 cm × 1.5 cm) of each volunteer was irradiated with UVB (equivalent to 2 minimum erythema dose; 101.2–193.2 mJ per cm^2) (FL 20 SE30 lamp, Toshiba, Tokyo, Japan) 2 and 5 d prior to the skin biopsy. Skin punch biopsies (6 mm) were obtained from the boundary of the UVB-irradiated region and unirradiated region so that each cross-sectional sample contained both control (unirradiated) and irradiated regions (two biopsies from each volunteer). The samples were fixed in 10% formalin neutral buffer solution, pH 7.4 (062-01661 WAKO, Osaka, Japan) for one night and embedded in paraffin. All procedures involving human subjects received prior approval from both the University of Nagoya Ethical Committee and the POLA Chemical Industries Ethical Committee, and all subjects provided written informed consent.

Probes Probes that were used in this study were tyrosinase (374-2254, M38297), TYRP1 (63-2065, X51420), DCT (365-2133, D17547), Pmel-17 (168-1953, M77348), P-protein (1151-2832, M99564), MITF (60-1717, Z29678), and POMC (170-843, M38297) (parentheses show the cDNA fragments that were used as probes and the accession numbers of the sequences). Complementary DNA fragments of these genes were amplified from total RNA isolated from normal human melanocytes (tyrosinase, TYRP1, DCT, Pmel-17, and P-protein) or from a human pituitary cDNA library (POMC) (9510, Takara, Tokyo, Japan) by reverse transcription–polymerase chain reaction. Amplified fragments were inserted into PCR-II vector (Invitrogen, CA, USA). Digoxigenin-labeled cRNA probes for *in situ* hybridization were produced using a digoxigenin RNA labeling kit (1175 025, Roche Diagnostics, Mannheim, Germany). Produced cRNA probes were shortened to about 130 bp by alkaline treatment.

***In situ* hybridization** *In situ* hybridization was carried out according to a protocol provided by WAKO (Tokyo, Japan) with minor modifications. Most of the reagents used were included in *In situ* Hybridization Reagents (316-01951, WAKO, Tokyo, Japan). The modified protocol is described briefly below.

Cross-sectional samples (thickness of 4 μm) were cut from paraffin-embedded skin samples. The samples were washed three times in xylene for 5 min, and three times with 100% ethanol for 5 min, then washed in 90, 80, 70, and 50% ethanol (5 min each), and left in phosphate-buffered saline for 10 min. They were treated with proteinase K (5 μg per ml in phosphate-buffered saline) for 10–30 min at 37°C, and washed in glycine solution (2 mg per ml glycine in phosphate-buffered saline, WAKO, Osaka, Japan) for 10 min, then twice in phosphate-buffered saline for 3 min. Next they were each placed in 200 ml of acetylation buffer (315-02761, WAKO, Osaka, Japan), and 500 μl of acetic anhydride was slowly added. The mixture was incubated for 15 min with continuous agitation. They were then washed twice in 4 × sodium citrate/chloride buffer (319-02781, WAKO, Osaka, Japan) for 10 min, and incubated in prewarmed prehybridization solution (2 × sodium citrate/chloride buffer, 50% deionized formamide, at 43°C) for more than 30 min. Excess prehybridization solution was wiped off, hybridization solution (316-02791, WAKO, Osaka, Japan) containing labeled cRNA probes (1 ng per μl) was placed on the samples, and incubation was conducted overnight at 43°C. The samples were washed in prewarmed prehybridization solution three times for 20 min at 43°C. After the incubation in prewarmed NTE buffer (316-02811, WAKO, Osaka, Japan) for 5 min at 37°C, the samples were treated with prewarmed RNase A solution (20 μg per ml in NTE buffer) for 30 min at 37°C, then returned to NTE buffer, and incubated for 3 min at 37°C. They were washed in prewarmed 0.1 × sodium citrate/chloride buffer three times for 20 min at 43°C, and then washed once in Buffer 1 (0.1 M Tris–HCl pH 7.5, 0.15 M NaCl) for 1 min. Blocking solution [5% blocking agent (1175 041, Roche) in Buffer 1] was placed on the samples, and incubation was continued for about 30 min. The samples were washed with Buffer 1 for 1 min, the samples were treated with anti-digoxigenin–alkaline phosphatase conjugate (1175 041, Roche) (diluted to 1/500 in Buffer 1 with 1% blocking agent and 0.075% Brij 35) for 1 h. They were washed with Buffer 1 with 0.075% Brij35 three times for 10 min and incubated in Buffer 1 for 5 min. Then they were washed with Buffer 3 (0.1 M Tris–HCl pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$) for 3 min, substrate solution [1/20 diluted solution of NBT/BCIP stock solution (1175 041, Roche) in Buffer 3] was placed on the samples and incubation was conducted overnight at room temperature. They were then washed with TE buffer (10 mM Tris–HCl pH 8.0, 1 mM ethylenediamine tetraacetic acid) to stop the reaction. For less abundant mRNA (P-protein and MITF) the GenPoint System (K0620, DAKO, CA, USA) was used for the detection step according to the manufacturer's instructions.

In both methods (WAKO, Osaka, Japan and DAKO, CA, USA), RNA stained violet and could be easily distinguished from brownish melanin.

RESULTS

***In situ* hybridization of UVB-irradiated skin** Anti-sense RNA probes for tyrosinase, TYRP1, DCT, Pmel-17, MITF, and P-protein reacted with cells in the basal layer of the epidermis (**Figs 1** and **2**), corresponding to the distribution of melanocytes, whereas the sense RNA probe for tyrosinase gave no signal (data not shown). The anti-sense RNA probe for POMC reacted with epidermal keratinocytes as well as dermal cells (**Figs 1** and **2**). This signal disappeared when the section was coincubated with an excess of cold anti-sense POMC RNA, but not with an excess of cold anti-sense Pmel-17 RNA (**Fig 1**).

Messenger RNA levels of tyrosinase, TYRP1, DCT, P-protein, and Pmel-17 were significantly increased 5 d after UVB irradiation (**Fig 2**), but the increases were not as dramatic at 2 d after UVB irradiation (**Fig 1**). On the other hand, POMC mRNA level was significantly increased 2 d after UVB irradiation (**Fig 1*A***), and returned to near normal by 5 d (**Fig 2*A***). MITF mRNA was slightly increased both 2 and 5 d after UVB irradiation (**Figs 1*G*** and **2*G***).

The results of preliminary statistical analyses of the numbers of positive cells per unit length of basal layer were consistent with the visual observation (**Fig 3**). It is important to note that an increase in the number of positive cells does not necessarily represent proliferation of melanocytes. It may mean that there are melanocytes that produce enough mRNA to be detected by our method only after UV irradiation. It should also be mentioned that, by visual observation, both the number of positive cells per unit length of basal layer and the intensity per cell are increased.

DISCUSSION

In this study of *in situ* hybridization of mRNA, we demonstrated for the first time that mRNA levels of POMC, tyrosinase, TYRP1, DCT, P-protein, Pmel-17, and MITF increase in the human epidermis in response to UVB exposure. Our data suggest that transcriptional regulation plays an important part in UVB-induced melanogenesis.

As cultured human keratinocytes express POMC, and its production increases in response to UVB irradiation (Schauer *et al*, 1994; Chakraborty *et al*, 1996; Wintzen *et al*, 1996), it is suggested that keratinocytes are the source of melanocortins influencing human skin. Here, we have demonstrated for the first time that keratinocytes express POMC mRNA *in vivo*, and the expression increases in response to UVB radiation (**Fig 1*A***). This is consistent with the findings of Wakamatsu *et al* (1997).

The increase in POMC mRNA level after UVB irradiation preceded the peak increases of mRNA of other genes except MITF mRNA, which was slightly increased at 2 d and remained at almost the same level at 5 d after UVB irradiation (**Figs 1–3**). This may suggest that increased POMC levels regulate mRNA levels of the melanogenesis-associated genes. As there were no detectable changes in tyrosinase, TYRP1, or DCT mRNA in cultured normal human melanocytes following α-MSH treatment (Abdel-Malek *et al*, 1995), the increase in tyrosinase, TYRP1 and DCT mRNA in UVB-irradiated skin may be due to the production of other melanogenic stimulators in the skin in response to UVB (Abdel-Malek, 1998); however, as cotransfection experiments showed that MITF has the ability to activate promoters for

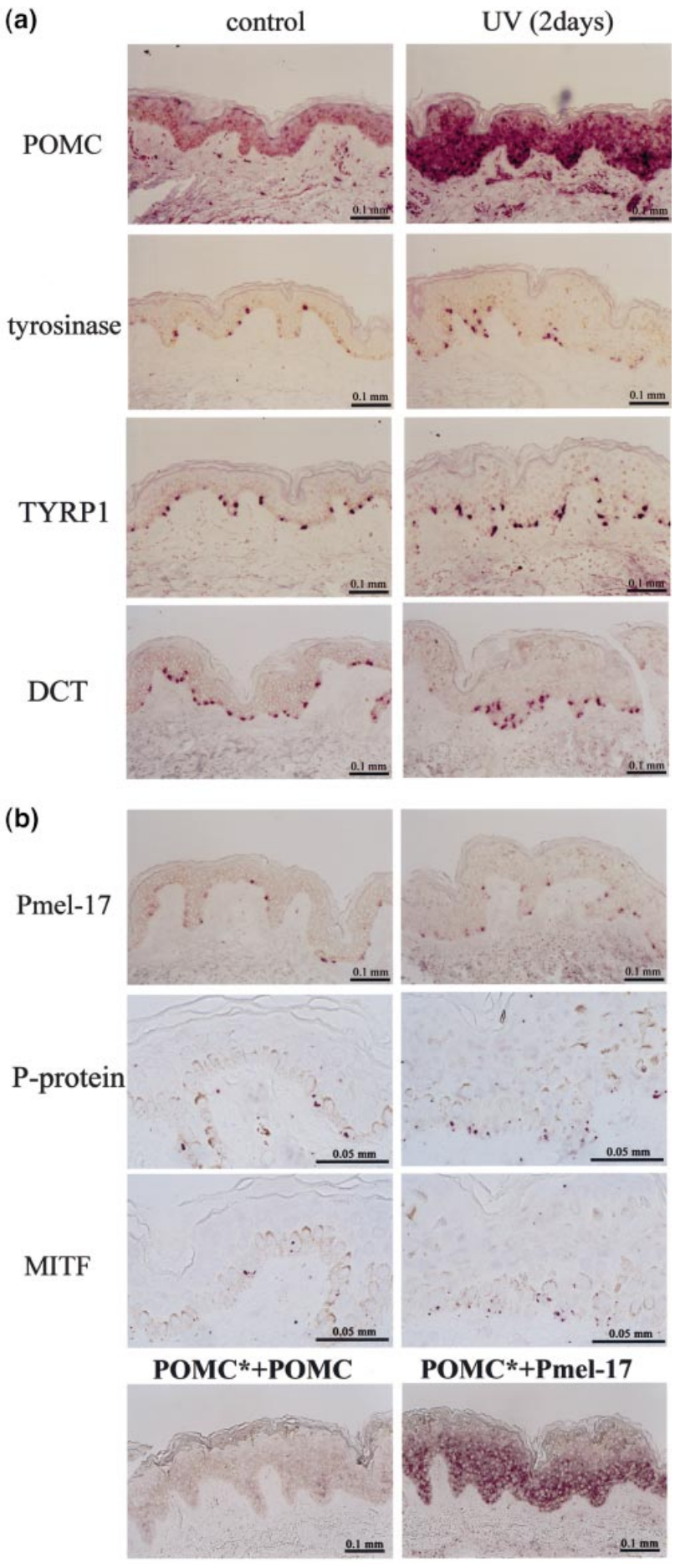

Figure 1. Comparison of the mRNA levels of a panel of melanogenesis-related genes in human skin, as detected by *in situ* hybridization 2 d after UVB irradiation. Left and right panels represent unirradiated controls and UVB-irradiated samples, respectively. Skin sections were hybridized with specific probes as indicated. POMC*+ POMC represents a sample stained with labeled anti-sense POMC probe and cold POMC probe. POMC*+ Pmel-17 represents a sample stained with labeled anti-sense POMC and cold Pmel-17 probe.

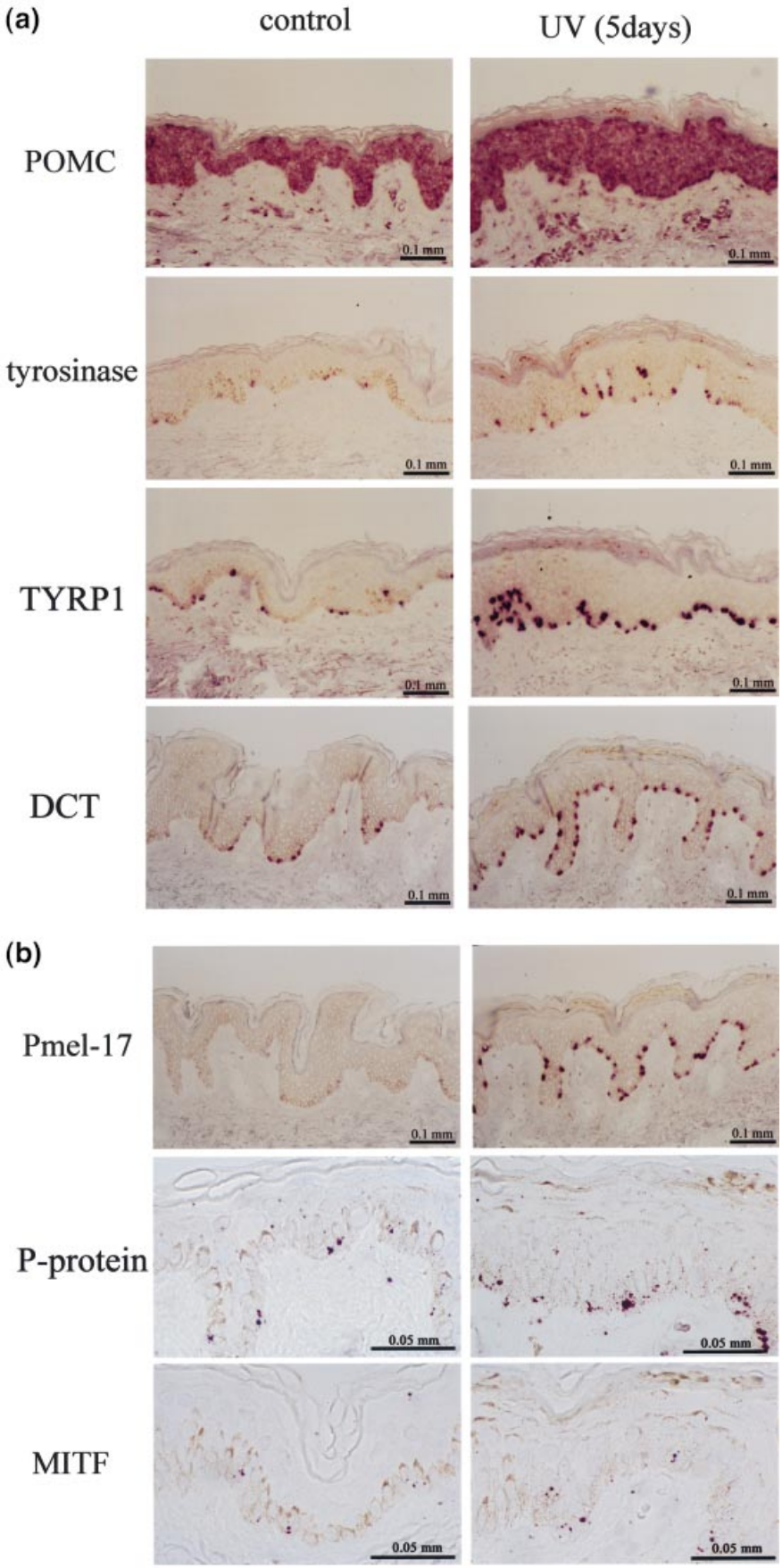

Figure 2. Comparison of the mRNA levels of a panel of melanogenesis-related genes in human skin, as detected by *in situ* hybridization 5 d after UVB irradiation. Left and right panels represent unirradiated controls and UVB-irradiated samples, respectively. Skin sections were hybridized with specific probes as indicated.

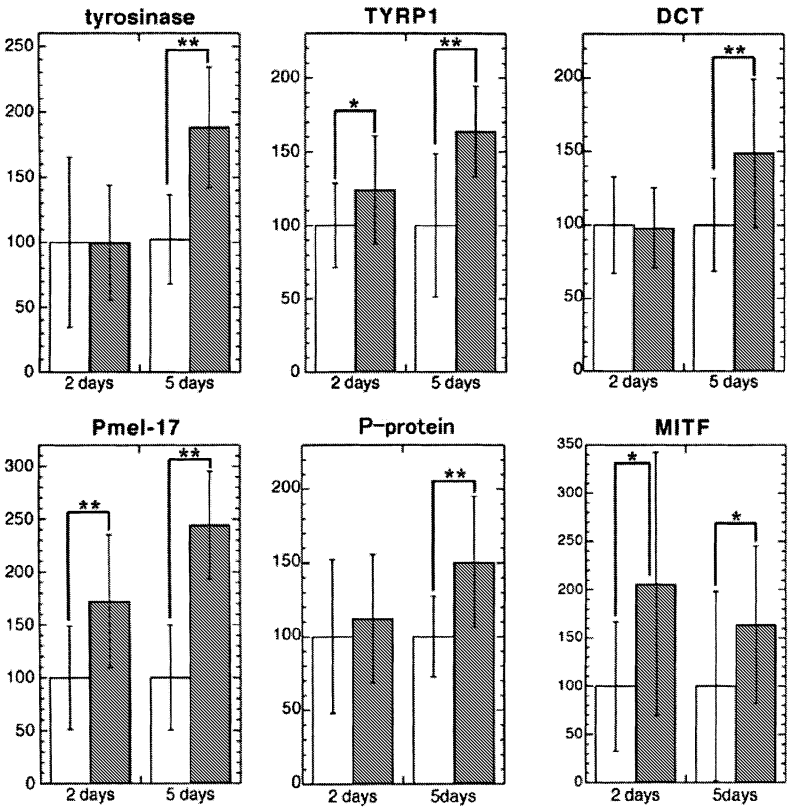

Figure 3. Comparison of the numbers of positive cells per unit length of basal layer. The numbers of positive cells were counted and divided by the length of the basal layer, and are presented as percentage of control (mean ± standard deviation, n = 8). Open bars represent control regions and black bars represent UVB-irradiated regions. Statistical analysis was performed using paired t test. $^{**}p < 0.01$, $^{*}p < 0.05$.

tyrosinase and TYRP1 (Yasumoto *et al*, 1997), and MITF mRNA levels are increased by α-MSH in cultured normal human melanocytes, it is possible that the mRNA levels of those enzymes are under the control of α-MSH *in vivo*. Our results, together with the association of loss-of-function mutations in MC1R with poor tanning ability, suggest that α-MSH plays a pivotal role in UVB-induced pigmentation. The regulatory role of α-MSH in UVB-induced pigmentation could be further clarified by conducting similar experiments on individuals with nonfunctional MC1R.

The results of preliminary statistical analyses of the numbers of positive cells per unit length of the basal layer were consistent with the visual observation (**Fig 3**); however, it is not clear if the number of melanocytes is increased by these doses of UVB irradiation, as *in situ* hybridization only detects cells that produce more than a certain amount of target mRNA. By visual observation, both the numbers of positive cells and the intensity were increased. Considering that UVB increases POMC expression in keratinocytes and that melanocortins have the ability to stimulate cellular proliferation of cultured normal human melanocytes, it is not unreasonable to think that UVB stimulates both cellular proliferation of melanocytes and transcription of melanogenic genes. Furthermore, if technical problems could be solved, it would be interesting to perform immunohistochemistry/*in situ* hybridization double staining using an antibody specific for melanocytes to see if there are melanocytes that are negative for mRNA of certain melanogenic genes.

In conclusion, our results suggest that the tanning response of human skin is regulated at the mRNA level, and that POMC-derived melanocortins, such as α-MSH and ACTH, may be key factors in the transcriptional regulation of these genes *in vivo*.

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REFERENCES

Abdel-Malek Z. Regulation of human pigmentation by ultraviolet light and by endocrine, paracrine, and autocrine hormones. In: Nordlund JJ, Boissy RE, Hearing VJ, King RA, Ortonne J-P (eds). *The Pigmentary System*. Oxford University Press, Oxford, 1998: pp 115–122

Abdel-Malek Z, Swope VB, Suzuki I, *et al:* Mitogenic and melanogenic stimulation of normal human melanocytes by melanotropic peptides. *Proc Natl Acad Sci USA* 92:1789–1793, 1995

Chakraborty A, Slominski A, Ermak G, Hwang J, Pawelek J: Ultraviolet B and melanocyte-stimulating hormone (MSH) stimulate mRNA production for alpha MSH receptors and proopiomelanocortin-derived peptides in mouse melanoma cells and transformed keratinocytes. *J Invest Dermatol* 105:655–659, 1995

Chakraborty AK, Funasaka Y, Slominski A, Ermak G, Hwang J, Pawelek JM, Ichihashi M: Production and release of proopiomelanocortin (POMC) derived peptides by human melanocytes and keratinocytes in culture: regulation by ultraviolet B. *Biochim Biophys Acta* 1313:130–138, 1996

Fitzpatrick TB: Mammalian melanin biosynthesis. *Trans St Johns Hosp Dermatol Soc* 51:1–26, 1965

Fitzpatrick TB, Miyamoto M, Ishikawa K: The evolution of concepts of melanin biology. *Arch Dermatol* 96:305–323, 1967

Gilchrest BA, Blog FB, Szabo G: Effects of aging and chronic sun exposure on melanocytes in human skin. *J Invest Dermatol* 73:141–143, 1979

Im S, Moro O, Peng F, *et al:* Activation of the cyclic AMP pathway by alpha-melanotropin mediates the response of human melanocytes to ultraviolet B radiation. *Cancer Res* 58:47–54, 1998

Liotta AS, Loudes C, McKelvy JF, Krieger DT: Biosynthesis of precursor corticotropin/endorphin-, corticotropin-, alpha-melanotropin-, beta-lipotropin-, and beta-endorphin-like material by cultured neonatal rat hypothalamic neurons. *Proc Natl Acad Sci USA* 77:1880–1884, 1980

Nordlund JJ, Ackles AE, Traynor FF: The proliferative and toxic effects of ultraviolet light and inflammation on epidermal pigment cells. *J Invest Dermatol* 77:361–368, 1981

Pathak MA, Jimbow K, Fitzpatrick TB. Photobiology of pigment cell. In: Seiji M (ed.). *Phenotypic Expression in Pigment Cells*. Tokyo: University of Tokyo Press, 1980: pp 655–670

Quevedo WC, Fitzpatrick TB, Pathak MA, Jimbow K: Role of light in human skin color variation. *Am J Phys Anthropol* 43:393–408, 1975

Rubinstein M, Stein S, Udenfriend S: Characterization of pro-opiocortin, a precursor to opioid peptides and corticotropin. *Proc Natl Acad Sci USA* 75:669–671, 1978

Schauer E, Trautinger F, Kock A, *et al:* Proopiomelanocortin-derived peptides are

synthesized and released by human keratinocytes. *J Clin Invest* 93:2258–2262, 1994

Suzuki I, Cone RD, Im S, Nordlund J, Abdel-Malek ZA: Binding of melanotropic hormones to the melanocortin receptor MC1R on human melanocytes stimulates proliferation and melanogenesis. *Endocrinology* 137:1627–1633, 1996

Valverde P, Healy E, Jackson I, Rees JL, Thody AJ: Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans. *Nat Genet* 11:328–330, 1995

Virador V, Matsunaga N, Matsunaga J, *et al*: Production of melanocyte-specific antibodies to human melanosomal proteins: expression patterns in normal human skin and in cutaneous pigmented lesions. *Pigment Cell Res* 14:289–297, 2001

Wakamatsu K, Graham A, Cook D, Thody AJ: Characterisation of ACTH peptides in human skin and their activation of the melanocortin-1 receptor. *Pigment Cell Res* 10:288–297, 1997

Wintzen M, Yaar M, Burbach JP, Gilchrest BA: Proopiomelanocortin gene product regulation in keratinocytes. *J Invest Dermatol* 106:673–678, 1996

Yasumoto K, Yokoyama K, Takahashi K, Tomita Y, Shibahara S: Functional analysis of microphthalmia-associated transcription factor in pigment cell-specific transcription of the human tyrosinase family genes. *J Biol Chem* 272:503–509, 1997