Endothelin-1 Is a Paracrine Growth Factor That Modulates Melanogenesis of Human Melanocytes and Participates in Their Responses to Ultraviolet Radiation[1]

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Abstract

Endothelin (ET)-1, α-melanocyte stimulating hormone (α-melanotropin; α-MSH), and basic fibroblast growth factor (bFGF) are keratinocyte-derived factors that interact synergistically to stimulate human melanocyte proliferation. ET-1 has a dose-dependent mitogenic effect on human melanocytes and a biphasic effect on melanogenesis: a stimulatory effect at subnanomolar concentrations, and an inhibitory effect at concentrations equal to or higher than 1 nM. Human melanocytes express ET B receptors. Brief treatment of melanocytes with ET-1 caused up-regulation of α-MSH receptor mRNA but did not alter ET B receptor mRNA level. ET-1 modulates the response of human melanocytes to UV rays (UVRs). Treatment of melanocytes with 10 nM ET-1 immediately after exposure to UVRs enabled them to overcome the G_1 growth arrest. However, ET-1 did not inhibit p53 accumulation or $p21^{Waf\text{-}1/SDI\text{-}1/Cip\text{-}1}$ overexpression, nor did it reverse the hypophosphorylated state of pRb or the reduction in Bcl2 level in irradiated melanocytes. These results substantiate the role of ET-1 as a paracrine regulator that modulates the response of human melanocytes to UVRs.

Introduction

ET[4]-1 was first described as an endothelium-derived factor with potent vasoconstrictive effects (1). However, it is now known to be synthesized by numerous cell types and to act, for the most part, as a paracrine regulator of many target cells (Ref. 1; reviewed in Ref. 2). Human epidermal keratinocytes in culture were found to synthesize ET-1, particularly after treatment with UVRs or interleukin-1 (3, 4). Moreover, ET-1 was found to be mitogenic for cultured human epidermal melanocytes in the presence of a cAMP inducer such as cholera toxin or α-MSH (5, 6). The effects of ET-1 were mediated by binding to specific receptors expressed by human melanocytes (5). These results suggest a paracrine role for ET-1 in the regulation of human pigmentation, particularly in the response of melanocytes to sun exposure or inflammation.

There are three forms of ETs, ET-1, ET-2, and ET-3, each of which is coded for by a distinct gene (7). ETs bind to two types of receptors, ET AR, which has highest affinity for ET-1, and ET BR, which binds all three ETs with a similar affinity (8–10). ETs elicit their biological effects by a complex signaling pathway (2). Many reports showed that ET-1 stimulates 1,4,5-inositol-triphosphate formation and intracellular calcium mobilization (5, 11, 12) as well as activates protein kinase C and nonreceptor tyrosine kinase(s) (5, 13–18).

We have reported previously that ET-1 interacts synergistically with α-MSH and bFGF to stimulate human melanocyte proliferation (6). α-Melanotropin is classically known as a pituitary derived factor that increases integumental pigmentation of many vertebrate species (19, 20). It is now recognized that α-MSH as well as adrenocorticotropic hormone are synthesized by human keratinocytes and melanocytes and are mitogenic and melanogenic for human melanocytes (21–25). Both peptides elicit their effects on human melanocytes by binding to the melanocortin 1 receptor (MC1R), a G protein-coupled receptor, resulting in activation of the cAMP pathway (26–28). bFGF is a keratinocyte-derived mitogen that activates a receptor tyrosine kinase (29, 30). The cooperation of these three epidermal factors strongly suggests the existence of a cutaneous paracrine network that regulates the proliferation and melanization of human melanocytes.

The results hereby presented indicate that human melanocytes express ET BR and respond to both ET-1 and ET-3 with a dose-dependent increase in proliferation and a decrease or an increase in melanogenesis. One level of cooperation between ET-1 and α-MSH is exemplified by the up-regulation of the MC1R mRNA level by brief ET-1 treatment. Moreover, we demonstrate that ET-1 modulates the re-

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[4] The abbreviations used are: ET, endothelin; cAMP, cyclic AMP; α-MSH, α-melanocyte stimulating hormone; UVR, UV ray; AR and BR, A receptor and B receptor, respectively; MC1R, melanocortin-1 receptor; bFGF, basic fibroblast growth factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; BPE, bovine pituitary extract; TRP-1, tyrosinase-related protein-1; PI, propidium iodide.

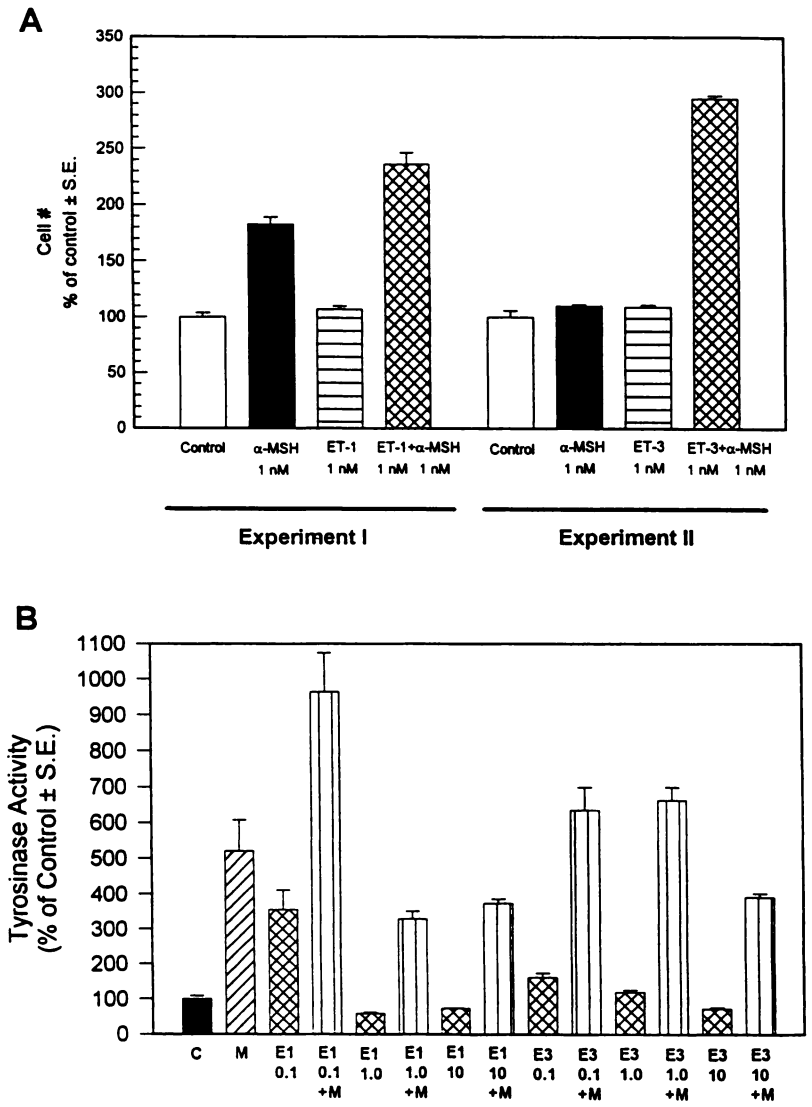

Fig. 1. Dose-dependent effects of ET-1 and ET-3, in the presence or absence of α-MSH, on human melanocyte proliferation and tyrosinase activity. In *A*, human melanocytes were treated with 1 nM ET-1 or ET-3, in the absence or presence of 1 nM α-MSH, for a total of 6 days as described in "Materials and Methods." The cell number in each flask (triplicate flasks/experimental group) was determined at the end of the experiment using a Coulter counter (model ZM). Two different melanocyte lines were used in experiments I and II. These experiments were repeated four times with similar findings. *Bars*, SE. In *B*, tyrosinase activity was determined after treatment of melanocytes with 0 (*C*), 0.1, 1, or 10 nM ET-1 (*E1*) or ET-3 (*E3*), in the presence or absence of 1 nM α-MSH (*M*) as described in "Materials and Methods." The data represent results of an experiment that was repeated four times using different cell lines with similar findings. *Bars*, SE.

sponse of human melanocytes to UVR, an effect that might have implications on the UV-induced photocarcinogenesis.

Results

Regulation of Human Melanocyte Proliferation and Melanogenesis by ETs. Previously, we reported that in the presence of a low concentration of bFGF (0.6 μg/ml) in the growth medium, ET-1 and α-MSH interacted synergistically to stimulate human melanocyte proliferation (6). In this study, we noted that the mitogenic effect of ET-1 was mimicked by ET-3 (Fig. 1*A*). To further investigate the mitogenic effect of ET-1, we examined the effects of 0.1 or 10 nM ET-1 in the presence or absence of α-MSH on the cell cycle (Table 1). As expected, control melanocytes that were deprived of TPA and BPE were quiescent, and 97% of the cells were in G_1 phase. The addition of 0.1 nM ET-1 did not induce the entry into S phase, and the cell cycle profile of this experimental group was almost identical to that of the control untreated group. Treatment with 10 nM ET-1 for 48 h resulted in a 7% decrease in the number of cells in G_1, a 3% increase in the number of cells in S, and a 4% increase in the number of cells in G_2-M. Similarly, treatment of melanocytes with 10 nM α-MSH for 48 h reduced the number of cells in G_1 by 9% and increased the number of cells in S and G_2-M by 2 and 7%, respectively. The percentage of cells in S continued to increase after 72 h of treatment with either 10 nM ET-1 or α-MSH. The most profound change in the cell cycle profile was observed following combined treatment of melanocytes with 10 nM ET-1 and 10 nM α-MSH. In comparison to control melanocytes that were maintained in TPA- and BPE-free medium, melanocytes treated with 10 nM ET-1 and α-MSH for 48 h demonstrated a 17% reduction in the number of cells in G_1 and a 4 and a 13% increase in S and G_2-M, respectively. After 72 h of such treatment, there was a 20% reduction in the number of melanocytes in G_1, accompanied by a 21% increase in the number of melanocytes in S.

Table 1 Dose-dependent effects of ET-1 in the absence or presence of α-MSH on the cell cycle profile of human melanocytes

Melanocytes in TPA- and BPE-free medium were treated 48 h after plating with 10 nM α-MSH, 0.1 or 10 nM ET-1, 0.1 nM ET-1 + 10 nM α-MSH, or 10 nM ET-1 + 10 nM α-MSH. The medium was replaced, and the appropriate dose of hormone(s) was added every other day. Flow cytometric analysis of the cell cycle was carried out 24, 48, and 72 h after treatment, as described in "Materials and Methods." Data represent the percentage of the total population of melanocytes in G_1, S, or G_2-M. Similar results were obtained in two independent experiments.

	% in G_1	% in S	% in G_2-M
24 h			
Control	96.7	0.6	2.7
10 nM α-MSH	96.4	0.9	2.7
0.1 nM ET-1	97.2	0.1	2.7
0.1 nM ET-1 + 10 nM α-MSH	96.9	0.4	2.7
10 nM ET-1	96.8	0.5	2.8
10 nM ET-1 + 10 nM α-MSH	96.6	0.8	2.6
48 h			
Control	97.1	0.6	2.3
10 nM α-MSH	88.3	2.7	8.9
0.1 nM ET-1	97.3	0.1	2.6
0.1 nM ET-1 + 10 nM α-MSH	91.5	1.3	7.2
10 nM ET-1	90.4	3.1	6.5
10 nM ET-1 + 10 nM α-MSH	79.8	5.3	14.9
72 h			
Control	97.1	0.9	2.0
10 nM α-MSH	90.0	8.2	1.8
0.1 nM ET-1	96.9	1.2	1.9
0.1 nM ET-1 + 10 nM α-MSH	90.0	8.4	1.6
10 nM ET-1	91.7	6.5	1.8
10 nM ET-1 + 10 nM α-MSH	76.7	22.4	1.0

Tyrosinase is the rate-limiting enzyme in the melanin synthetic pathway (31). We found that ET-1 at 1 or 10 nM significantly reduced tyrosinase activity by 30–40%. More extensive dose-response experiments revealed that ET-1 at 0.1 nM increased tyrosinase activity up to 350% of control (Fig. 1*B*). Also, like ET-1, ET-3 had a biphasic effect on tyrosinase activity, a stimulatory effect at subnanomolar concentrations, and an inhibitory effect at concentrations higher than 1 nM. Treatment with α-MSH in combination with either 0.1 nM ET-1 or ET-3 had an additive effect on tyrosinase activity (Fig. 1*B*). The inhibitory effect of 10 nM ET-1 on tyrosinase activity was accompanied by a marked reduction in the amount of tyrosinase protein, whereas the stimulatory effect of 0.1 nM ET-1 was accompanied by a profound increase in the amount of tyrosinase expressed in melanocytes, as determined by Western blot analysis (Fig. 2). Consistent with our results reported previously, α-MSH increased the protein level of tyrosinase (25). Combined treatment with 0.1 nM ET-1 and α-MSH for 6 days resulted in a higher level of tyrosinase than that observed after treatment with either hormone alone (Fig. 2). In these experiments, we observed similar but less remarkable changes in the level of TRP-1, which is thought to act as a DOPA-oxidase in the melanin synthetic pathway (32), after ET-1 treatment (Fig. 2).

Identification of the ET Receptor and the Regulation of ET BR and MC1R by ET-1 and α-MSH. The similarities in the mitogenic and melanogenic responses to ET-1 and ET-3 suggested that human melanocytes express the ET B receptor. Northern blot analysis revealed that melanocytes expressed the mRNA for ET BR (Fig. 3) but not ET AR (data not shown). In these experiments, we examined the possible regulation of ET BR mRNA expression by treatment with 10 nM ET-1 and/or 10 nM α-MSH. We found that the level of ET BR mRNA was not altered by treatment with either hormone. We also investigated the regulation of MC1R by ET-1 and/or α-MSH (Fig. 3). We found that 10 nM ET-1 resulted in a significant increase in the level of MC1R mRNA after 6 h of treatment. However, after 5 days of treatment, this effect of ET-1 was no more evident. In these experiments, brief treatment with α-MSH induced an increase in MC1R mRNA transcript, as we have reported previously (28), and combined treatment with α-MSH and ET-1 caused a greater increase in MC1R mRNA level than treatment with either hormone alone.

To confirm further the expression of ET BR on human melanocytes, we conducted receptor binding assays to compare the ability of ET-1 and ET-3 to displace ^{125}I-labeled ET-1 from its binding sites (Fig. 4). We found that the displacement curves of ET-1 and ET-3 were almost identical, with IC_{50}s of 1.479 ± 0.149 nM and 1.757 ± 0.202 nM for ET-1 and ET-3, respectively, suggesting that both have the same affinity for the receptor.

Mitogenic and Melanogenic Effects of ET-1 on UV-irradiated Melanocytes. Because ET-1 has been shown to be important in the melanogenic response of human melanocytes to UVRs (33), we investigated its dose-dependent effects on the UV-induced growth arrest and melanogenesis. We found that treatment of UV-irradiated melanocytes with ET-1 did not reduce the lethal effects of UVRs. Irradiation of melanocytes with a single dose of 28 mJ/cm^2 UVRs resulted in the killing of ~40% of the total population within 48 h, an effect that was not altered by ET-1 treatment (data not shown). In comparison to the control group, the number of UV-irradiated melanocytes was lower by 30 and 60% at 24 and 48 h after irradiation, respectively (Fig. 5*A*). This decrease was due to cell death and growth arrest. Beginning 4 days after UV treatment, irradiated melanocytes resumed proliferation but at a much slower rate than the control unirradiated group. In comparison, 6 days after UV treatment (9 days after plating), irradiated melanocytes that were treated with 0.1 nM ET-1 resumed proliferation at the same rate as control melanocytes. Treatment of UV-irradiated melanocytes with 10 nM ET-1 for 6 days stimulated their proliferation at a markedly higher rate than control untreated melanocytes. The increase in number of melanocytes from day 4 to day 6 after UV and/or ET-1 treatment was 40% in the control group, 12% in the UV-treated group, 44% in the UV-irradiated group that received treatment with 0.1 nM ET-1, and 364% in the UV-irradiated group that was treated with 10 nM ET-1. These results clearly illustrate the dose-dependent mitogenic effect of ET-1 on UV-irradiated melanocytes and is consistent with the profound mitogenic effect of 10 nM ET-1.

Comparison of the cell cycle profile of melanocytes exposed only to 28 mJ/cm^2 UVRs to that of melanocytes irradiated with this dose of UVRs and treated with ET-1, revealed the following results (Table 2). In comparison with control melanocytes, irradiated melanocytes were arrested in G_1, as evident in the data obtained 24 or 48 h after UV exposure. Treatment of UV-irradiated melanocytes with 0.1

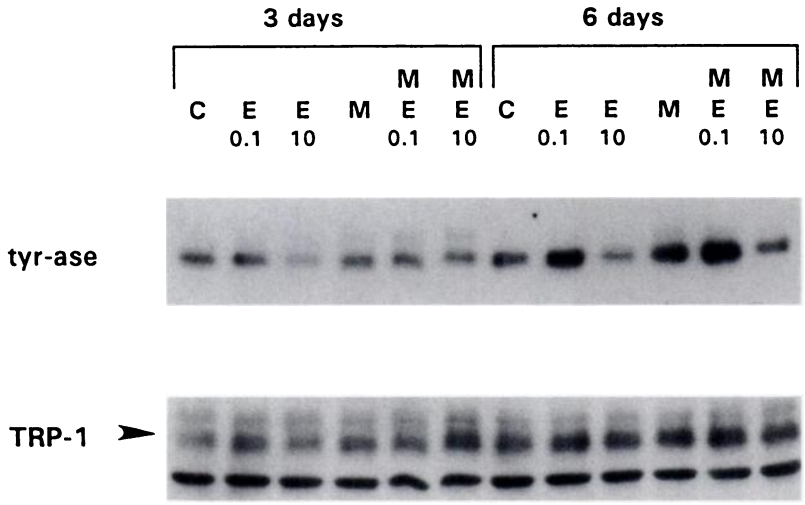

Fig. 2. Western blot analysis of tyrosinase and TRP-1 after treatment with different concentrations of ET-1 in the presence or absence of α-MSH. Cell extracts were prepared from melanocytes that were treated with 0.1 or 10 nM ET-1 in the presence or absence of 10 nM α-MSH for 3 or 6 days (*C*, control; *E*, ET-1; *M*, α-MSH). Western blot analysis for tyrosinase (*tyr-ase*) and TRP-1 was carried out as described in "Materials and Methods." This experiment was repeated three times with similar results.

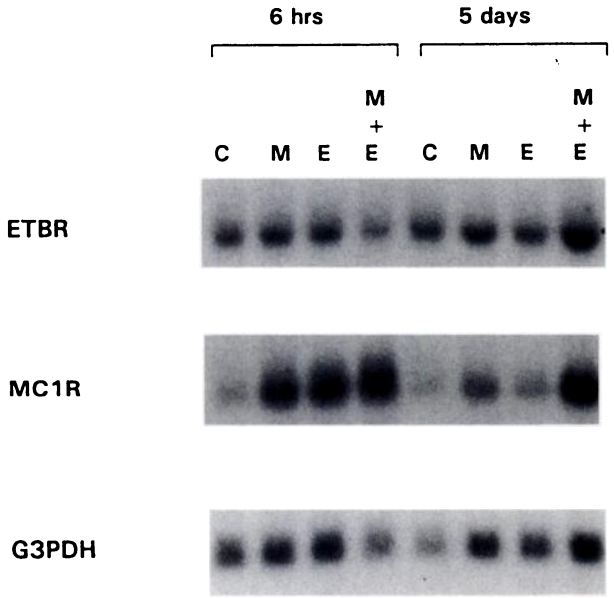

Fig. 3. Northern blot analysis of ET BR and MC1R after treatment with ET-1 and/or α-MSH. Melanocytes were treated for 6 h or 5 days with 0 (*C*), 10 nM ET-1 (*E*), 10 nM α-MSH (*M*), or concomitantly with ET-1 and α-MSH. Northern blot analysis of total RNA was carried out as described in "Materials and Methods" using a cDNA probe for human ET BR mRNA, a cDNA probe for human MC1R mRNA, or a cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) to control for loading. Similar results were obtained in two independent experiments.

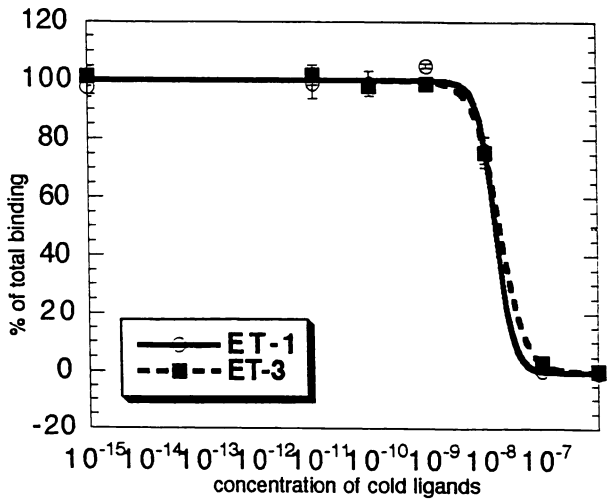

Fig. 4. Competition of ^{125}I-ET-1 binding by ET-1 or ET-3. The displacement of ^{125}I ET-1 binding by increasing concentrations of ET-1 or ET-3 (10^{-12}-10^{-6} M) was determined as described in "Materials and Methods." Total binding represents binding of ^{125}I ET-1 in the absence of any cold ligand. *Bars*, SE.

nM ET-1 did not significantly alter the UV-induced G_1 arrest and resulted in a delayed and modest increase in the percentage of cells in S phase. This was consistent with our finding that this dose of ET-1 had a minimal mitogenic effect on UV-irradiated melanocytes. However, treatment of UV-irradiated melanocytes with 10 nM ET-1 for 48 h increased the percentage of cells in S phase from 3 to 8.6% and the percentage of cells in G_2-M from 4.4 to 17.5%. These effects were accompanied by a 22% decrease in the percentage of cells in G_1. The same trend continued after 72 h of treatment and was consistent with the profound mitogenic effect of 10 nM ET-1.

As for the effects of 0.1 or 10 nM ET-1 on the UV-induced melanogenesis we found that the former markedly potentiated the stimulatory effect of UVRs on tyrosinase activity, whereas the latter had an inhibitory effect (Fig. 5*B*). These results are consistent with the biphasic effects of ET-1 on melanogenesis of unirradiated human melanocytes (Fig. 1*B*). On days 4 and 6 after UVRs and/or ET-1 treatment, unirradiated melanocytes responded to 0.1 nM ET-1 with 31 and 37% increase in tyrosinase activity, respectively. In contrast, melanocytes treated with 10 nM ET-1 had a 48 and 42% reduction in tyrosinase activity, respectively. Melanocytes irradiated with UVR had a 30% reduction in tyrosinase activity on day 4, and a 12% increase above control activity on day 6 after irradiation. Treatment of UV-irradiated melano-

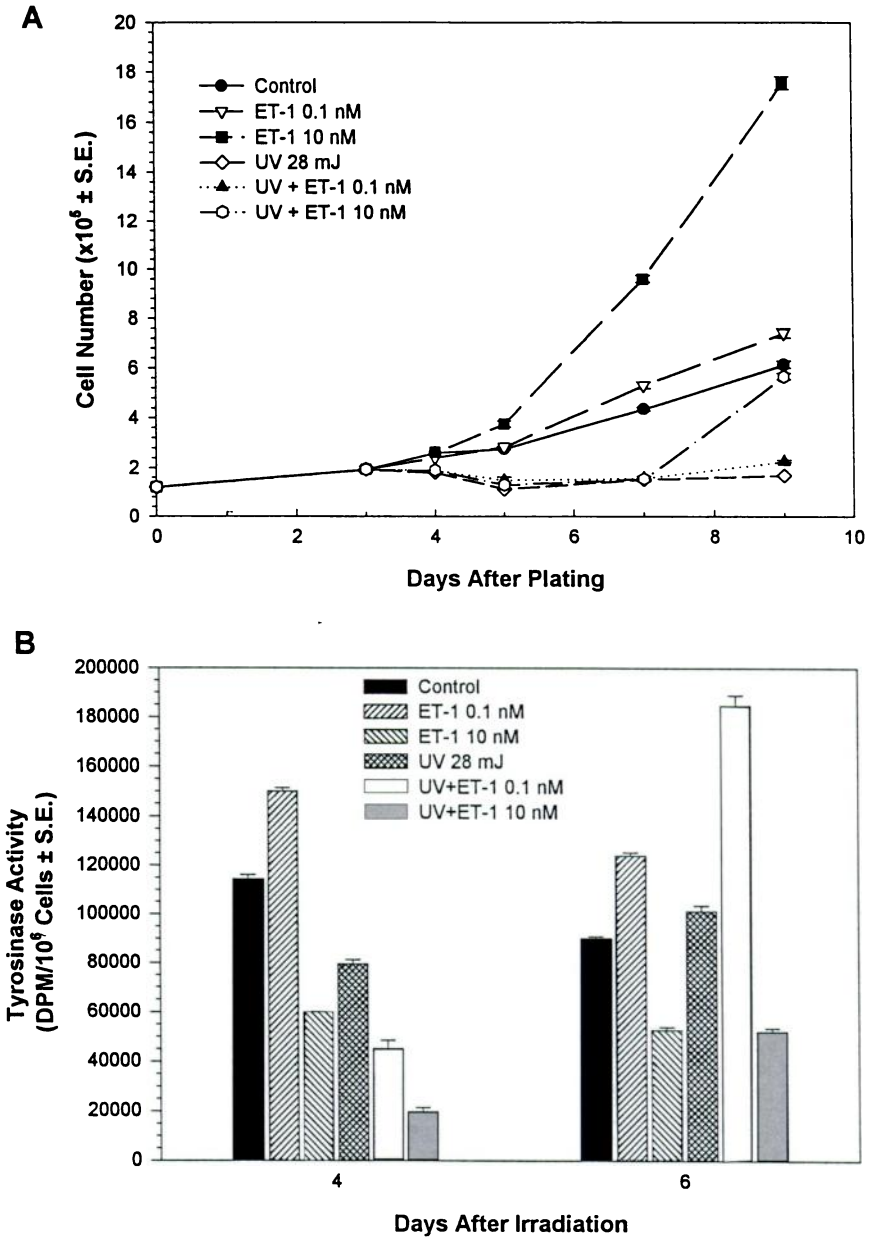

Fig. 5. Effect of ET-1 on the proliferation and tyrosinase activity of human melanocytes after irradiation with 28 mJ/cm^2 UVRs. Melanocytes were maintained in TPA-free medium for 2–3 days prior to, and for the duration of, the experiment. Cells were plated at 1 × 10^5 cells/60-mm dish, and triplicate dishes were included in each of the control and experimental groups. On day 3 after plating, melanocytes were irradiated with 28 mJ/cm^2 and/or treated with 0.1 or 10 nM ET-1. Fresh growth medium with or without the appropriate concentration of ET-1 was added to each dish every other day for a total of 6 days after irradiation. Cell number was determined 1, 2, 4, and 6 days after UVR and/or ET-1 treatment. In *A*, each data point represents the mean cell number of triplicate dishes; *bars*, SE. In the same experiment, tyrosine hydroxylase activity was assayed on days 4 and 6 after UVRs and/or ET-1 treatment. On days 3 or 5 after treatment, each dish was incubated in medium containing [^{3}H]tyrosine (0.7 μCi/ml) for 24 h. The conditioned medium from each dish was then collected and assayed for tyrosine hydroxylase activity. In *B*, tyrosine hydroxylase activity is expressed as DPM/10^6 cells. Each data point represents the mean of six determinations; *bars*, SE. This experiment was repeated three times with similar findings.

cytes with 0.1 nM ET-1 had a delayed stimulatory effect on the activity of tyrosinase, evident as a 2-fold increase in enzyme activity after 6 days of treatment. Compared with control, the response of UV-irradiated melanocytes to 10 nM ET-1 was a striking 83 and 42% inhibition of tyrosinase activity after 4 and 6 days of treatment, respectively.

Effects of ET-1 on the UV-induced Expression of p53, p21, pRb, and Bcl2. We have shown previously that UVRs resulted in the accumulation of p53, increased expression of p21, reduced expression of Bcl2, and maintenance of pRb in a hypophosphorylated form (34–36). We found that treatment of melanocytes that were irradiated with 28 mJ/cm^2 UVRs with either 0.1 or 10 nM ET-1 did not alter the above UV-induced effects (Fig. 6). In the absence as well as in the presence of ET-1, p53 accumulated over time in UV-irradiated melanocytes, reached a peak at 48 h, and remained highly elevated 72 h after irradiation. In these cells, the increase in p21 expression was time dependent and was highest 48 h after irradiation. An increase in p21 level was also observed in unirradiated melanocytes that were treated with 10 nM ET-1. This mitogen-dependent increase occurred earlier (at 7 h), continued to increase with time, but remained less pronounced than the increase induced by UVR. In unirradiated melanocytes, treatment with 10 nM ET-1 induced pRb phosphorylation, an effect that is consistent with the mitogenic effect of ET-1 and its ability to recruit melanocytes to the cell cycle. The phosphorylated form of pRb was detectable 24 h after the addition of 10 nM ET-1 and became

Table 2 Dose-dependent effects of ET-1 on the cell cycle profile of UV-irradiated human melanocytes

Melanocytes in TPA-free medium were irradiated once with 28 mJ/cm^2 UVRs 72 h after plating and/or treated with 0.1 or 10 nM ET-1. The growth medium was replaced with fresh medium, and the appropriate concentration of ET-1 was added every other day. Flow cytometric analysis of the cell cycle was determined as described in "Materials and Methods." Data represent the percentage of melanocytes in G_1, S, or G_2-M. This experiment was carried out twice with similar results.

	% in G_1	% in S	% in G_2-M
24 h			
Control	90.6	5.3	4.1
28 mJ/cm^2 UVRs	94.6	1.2	4.1
0.1 nM ET-1	89.8	6.1	4.0
28 mJ/cm^2 UVRs + 0.1 nM ET-1	93.6	1.7	4.7
10 nM ET-1	89.3	7.7	3.0
28 mJ/cm^2 UVRs + 10 nM ET-1	94.0	1.1	5.0
48 h			
Control	85.8	4.4	9.8
28 mJ/cm^2 UVRs	92.5	3.1	4.4
0.1 nM ET-1	81.3	7.9	10.8
28 mJ/cm^2 UVRs + 0.1 nM ET-1	92.4	3.5	4.2
10 nM ET-1	70.4	12.1	17.5
28 mJ/cm^2 UVRs + 10 nM ET-1	85.9	8.6	5.5
72 h			
Control	82.9	12.7	4.4
28 mJ/cm^2 UVRs	75.0	15.8	9.2
0.1 nM ET-1	80.6	14.1	5.3
28 mJ/cm^2 UVRs + 0.1 nM ET-1	70.0	20.4	9.6
10 nM ET-1	57.8	33.5	8.7
28 mJ/cm^2 UVRs + 10 nM ET-1	60.2	22.5	17.3

clearly obvious 72 after treatment. However, despite the mitogenic effect of 10 nM ET-1 on UV-treated melanocytes, phosphorylation of pRb was not evident after ET-1 treatment. The reduction in Bcl2 level was observed in UV-irradiated melanocytes whether or not they were subsequently treated with ET-1. Exposure of melanocytes to UVRs resulted in a significant decrease in Bcl2 level after 24 h of treatment. This effect persisted for at least 72 h and was not altered in the presence of ET-1. The observation that ET-1 did not reverse the UV-induced reduction in Bcl2 level is consistent with our finding that ET-1 did not increase the survival of melanocytes after UV irradiation.

Discussion

There is considerable evidence that a paracrine regulatory network exists in the epidermis and affects the proliferation and/or melanogenesis of human melanocytes. Members of this network include ET-1, α-MSH, and bFGF, which support the long-term proliferation of human melanocytes *in vitro* (3, 4, 21, 22, 37). The cross-talk of the signaling pathways activated by these mitogens is important for the regulation of melanocyte proliferation and melanogenesis (6, 17, 25, 29, 37, 38).

By Northern blot analysis, we found that human melanocytes express ET BR but not ET AR (Fig. 3). This was confirmed by the results of receptor binding assays that showed almost identical affinities of ET-1 and ET-3 for the receptor and by the results of dose-response experiments in which ET-1 and ET-3 were equally potent in stimulating the proliferation and modulating tyrosinase activity of human melanocytes (Figs. 1 and 4). Our results differ from those of Imokawa *et al.* (17), who reported that human melanocytes express ET AR, based on the use of the ET AR antagonist BQ123. Additional results from our experiments revealed that the mRNA levels of ET BR was not modulated by either brief or prolonged treatment with 10 nM ET-1 and/or 10 nM α-MSH (Fig. 3).

The melanotropic hormone α-MSH binds the G protein-coupled MC1R on human melanocytes, resulting in increased cAMP accumulation (26–28). We have reported recently that the level of the MC1R mRNA in human melanocytes is up-regulated by brief treatment with α-MSH (28). Here, we found that brief treatment of melanocytes with ET-1 increased the level of MC1R mRNA (Fig. 3). Others have shown that UVR increases the synthesis of ET-1 by epidermal keratinocytes and of α-MSH by epidermal keratinocytes and melanocytes (4, 22). Based on these findings, it is conceivable that up-regulation of MC1R mRNA level by ET-1 represents a mechanism by which ET-1 increases the responsiveness of melanocytes to α-MSH, particularly after UV exposure.

Additionally, we showed that in the presence of bFGF, α-MSH and ET-1 interacted in a synergistic manner to stimulate melanocyte proliferation (6). An intriguing finding is that ET-1 and ET-3 had a biphasic effect on tyrosinase activity, a stimulatory effect at subnanomolar concentrations, and an inhibitory effect at 10 nM concentration (Fig. 1*B*). However, the concomitant presence of ET-1 and α-MSH had an additive effect on the activity and amount of tyrosinase, ultimately resulting in increased melanogenesis (Figs. 1*B* and 2). Our results differ from those of Imokawa *et al.* (33), who observed a dose-dependent stimulation of melanogenesis by ET-1 in the presence of a cAMP inducer in the growth medium. It is important to note that we observed a biphasic effect of ET-1 in the absence as well as in the presence of cAMP inducers in the melanocyte growth medium (Fig. 1*B* and Fig. 5*B*).

We speculate that the biphasic effect of ET-1 on the activity and the amount of tyrosinase is related to the dose-dependent stimulation of Ca^{+2} mobilization from intracellular stores. In preliminary experiments, we observed that ET-1, at concentrations equal to or higher than 1 nM, induced intracellular Ca^{+2} mobilization in a dose-dependent manner (data not shown). A significant increase in Ca^{+2} mobilization after treatment with 10 nM ET-1 was associated with inhibition of tyrosinase (Figs. 1*B* and 5*B*). A role for Ca^{+2} in regulating melanogenesis has been described by other investigators. Treatment with the calcium channel blockers verapamil or TMB8 increased basal as well as α-MSH-stimulated tyrosinase activity, whereas treatment with the calcium ionophore A23187 was inhibitory for basal as well as cAMP-induced tyrosinase activity of murine melanoma cells (39, 40). Also, A23187 was reported to inhibit tyrosinase activity and TRP-1 expression in normal human melanocytes (41). These results suggest an inhibitory effect of high intracellular calcium concentrations on melanogenesis.

In a previous study, ET-1 was shown to be crucial for UV-induced melanogenesis (33). Recently, we demonstrated that stimulation of the cAMP pathway by physiological factors, such as α-MSH, is absolutely required for the melano-

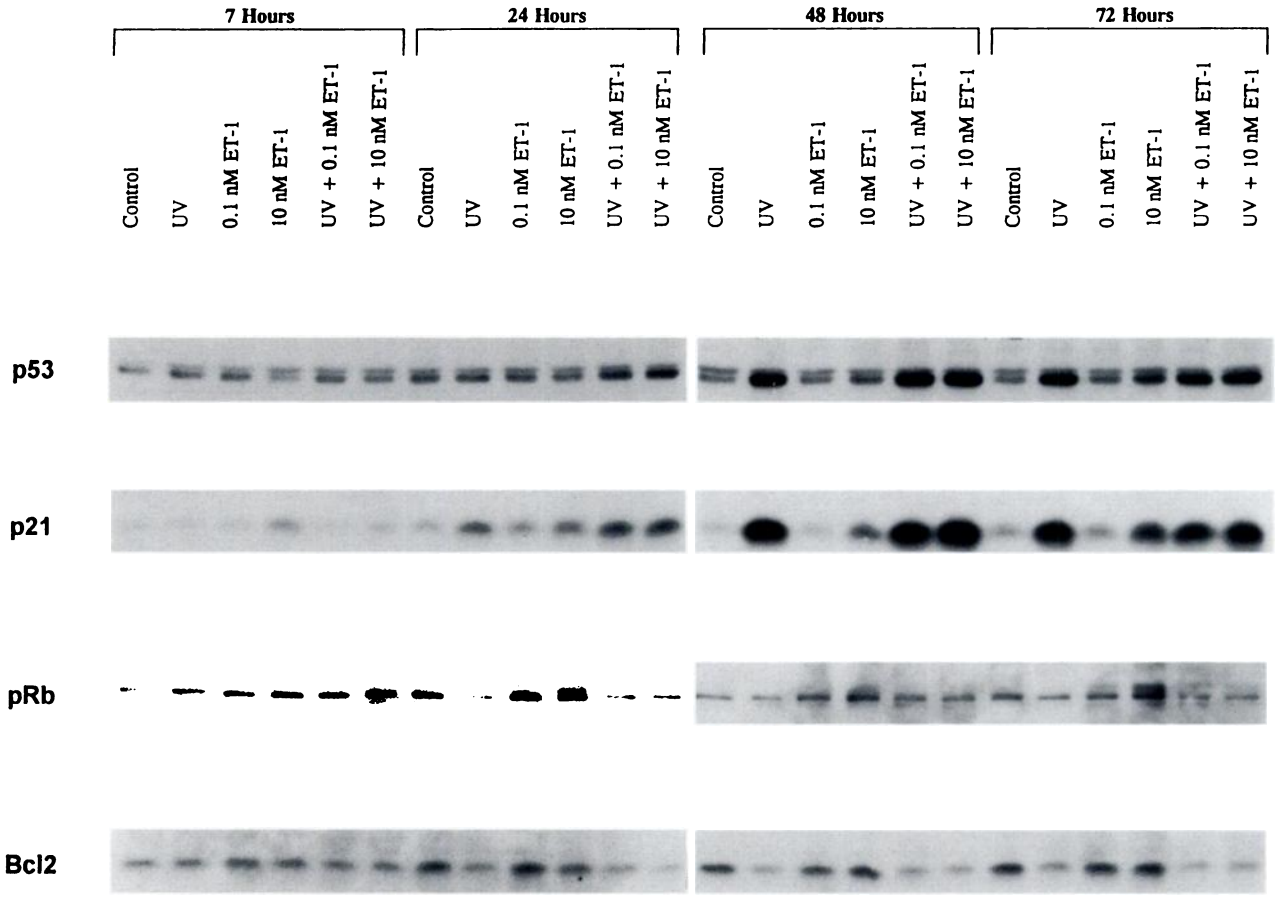

Fig. 6. Effect of ET-1 on the UVR-induced expression of p53, p21, pRb, and Bcl2. Melanocytes in TPA-free medium were plated at a density of 5–7 × 10^5 cells/60-mm dish. Seventy-two h later, cells were irradiated with 28 mJ/cm^2 UVRs and/or treated with 10 nM ET-1. Cell lysates were prepared 7, 24, 48, or 72 h after UVRs and/or ET-1 treatment. Western blot analysis for p53, p21, pRb, and Bcl2 was carried out as described in "Materials and Methods."

Table 3 Summary of the effects of 0.1 or 10 nM ET-1

	0.1 nM ET-1	10 nM ET-1
Proliferation of nonirradiated melanocytes	+	++
Proliferation of UV-irradiated melanocytes	±	+
Tyrosinase activity of nonirradiated and UV-irradiated melanocytes	+	−
Tyrosinase level in nonirradiated and UV-irradiated melanocytes	+	−
MC1R mRNA level	ND[a]	+
ET BR mRNA level	ND	No effect
UV-induced p53 and p21 levels	No effect	No effect
UV-induced pRb hypophosphorylation	No effect	No effect
UV-induced reduction in Bcl2 level	No effect	No effect

[a] ND, not done.

genic effect of UVRs on human melanocytes (36). In the experiments hereby presented, we found that after exposure to 28 mJ/cm^2 UVR, irradiated melanocytes that were treated with 0.1 nM ET-1 demonstrated a significant potentiation of their melanogenic response (Fig. 5*B*). The melanogenic response of melanocytes to UVR in the presence or absence of 0.1 nM ET-1 was delayed and took longer than 4 days to be detected. However, the response of irradiated melanocytes to 10 nM ET-1 was similar to that of unirradiated melanocytes that demonstrated profound inhibition of tyrosinase activity. Treatment with 10 nM ET-1 stimulated the proliferation of UV-irradiated melanocytes and enhanced their entry into S and G_2-M (Table 2). In contrast, 0.1 nM ET-1 had a minimal effect and could not rescue melanocytes from the UV-induced growth arrest. Similar responses to ET-1 were observed in experiments whereby melanocytes were irradiated once with 21 mJ/cm^2 UVR, a dose that is significantly less lethal than the dose of 28 mJ/cm^2 that we used in the above-described experiments (data not shown).

We have shown previously that a single exposure to UVRs arrests human melanocytes in G_1 (34, 36). This effect is thought to result from accumulation of p53 that acts as a cell cycle check point and induces the expression of the cyclin-cdk inhibitor p21 (35, 42–44). This in turn inhibits the activation of pRb via phosphorylation by cyclin-cdk complexes and maintains pRb in association with E2F proteins, thus suppressing their transcriptional activity (45, 46). Analysis of the effects of ET-1 on this UV-induced pathway revealed that neither 0.1 nor 10 nM ET-1 inhibited p53 accumulation or overexpression of p21 (Fig. 6). In the same experiments, we observed an early and modest increase in p21 after 7–24 h of

treatment of unirradiated melanocytes with 10 nM ET-1. This increase is known to be mitogen induced and p53 independent. In other experiments, we observed a similar early and transient increase in p21 after the addition of fresh growth medium to melanocytes and keratinocytes as well as fibroblasts (data not shown). This can be explained by the newly described role of p21 and the related cyclin-cdk inhibitors $p27^{KIP}$ and $p57^{KIP2}$ as adapter proteins that assemble cdk4 and cyclin D and target the cyclin/cdk complexes to the nucleus (47). Although 10 nM ET-1 induced pRb phosphorylation in unirradiated melanocytes, it failed to do so in UV-irradiated melanocytes, despite its mitogenic effect on these cells. These results are similar to our previous findings that α-MSH enhances the proliferation of UV-irradiated melanocytes without reversing the UV-induced p53 accumulation, p21 overexpression, or the dephosphorylation of pRb (36). Also, similar to α-MSH, ET-1 did not rescue melanocytes from the lethal effects of UVRs (36). The percentage of dead cells remained virtually the same in irradiated melanocytes that were treated or untreated with 10 nM ET-1 (data not shown). This suggests that ET-1, like α-MSH, acts as a mitogen, not as a survival factor for melanocytes (36). The inability of ET-1 or α-MSH to alter the above genotoxic effects of UVRs raises questions about the role of the p53-mediated pathway in UV-induced growth arrest and suggests that this pathway is mainly involved in UV-induced cell death. Furthermore, our results suggest that alternative pathways are activated by mitogens that enable UV-irradiated melanocytes to overcome the G_1 arrest.

In summary, we have found that ET-1, a potential paracrine factor for human melanocytes, stimulates the proliferation and modulates melanogenesis of these cells by binding to the ET BR (Table 3). ET-1 potentially might enhance the responsiveness of melanocytes to melanotropic hormones by up-regulating the expression of the MC1R. As we reported recently (36), ET-1 is similar to α-MSH in that it acts as a mitogen, rather than a survival factor for melanocytes after UV irradiation. Our results underscore the significance of paracrine factors in the modulation of melanocyte proliferation and melanogenesis, particularly in response to UV exposure.

Materials and Methods

Melanocyte Culture Conditions. Normal human melanocytes were derived from neonatal foreskins as described previously (6) and maintained in a growth medium consisting of: MCDB 153, 4% heat-inactivated FCS, 13 μg/ml BPE (Clonetics, San Diego, CA), 8 nM TPA, 0.6 ng/ml human recombinant bFGF, 5 μg/ml insulin, 1 μg transferrin, 1 μg/ml α-tocopherol, and 1% penicillin-streptomycin (10,000 units/ml and 10,000 μg/ml, respectively), as described by Medrano and Nordlund (48). All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. The cultures were maintained in a humidified incubator with 5% CO_2 at 37°C. For all experiments, early passage (ranging from passages 2 to 6) cultures were used. The doubling time of melanocytes in culture varies among donors and is on the average of 48–72 h.

For experiments aimed at describing the combined effects of ET-1 and α-MSH, melanocytes were maintained in culture medium lacking TPA and BPE for 2–3 days prior to beginning, and for the entire duration of, the experiments. For experiments in which the effects of ET-1 on UV-irradiated melanocytes were investigated, melanocytes were maintained in growth medium devoid of TPA only, as described above. Removal of TPA was reported to be important for an optimal response to ETs, because TPA down-regulates the expression of the ET Rs (49). Also, as we reported earlier, removal of BPE was essential for an optimal response to melanotropins, because it contains high concentrations of α-MSH (25).

Determination of Melanocyte Proliferation and Tyrosinase Activity after Treatment of UV-irradiated or Unirradiated Melanocytes with ETs. To investigate the effects of ETs and α-MSH on melanocyte proliferation and tyrosinase activity, cells were plated at a density of 1×10^5 cells/12.5 cm^2 flask. The culture medium was replenished, and fresh treatment (0.1–10 nM ET-1 or ET-3 and/or 1 nM α-MSH) was added every other day for a total of 6 days. At the end of each experiment, melanocytes from each individual flask were harvested and counted using a Coulter counter (Coulter Electronics, Hialeah, FL). During the final 24 h of the experiment, [3,5-^{3}H]L-tyrosine (specific activity, 52 mCi/mmol; Dupont NEN, Boston, MA) at a concentration of 0.7 μCi/ml (total of 2.1 μCi/3 ml/flask) was added to the culture medium, and the conditioned medium from each flask was saved to be assayed for the activity of tyrosinase, the rate-limiting enzyme in the melanogenic pathway. The tyrosine hydroxylase activity was measured *in situ*, as described previously (50, 51).

To investigate how ET-1 treatment might alter the effects of UVRs on melanocyte proliferation and tyrosinase activity, cells were plated at a density of 1×10^5 cells/60-mm dish and maintained in growth medium lacking TPA. On day 3 after plating, melanocytes were irradiated once with 28 mJ/cm^2 UVRs, as described previously (36). The UV source we are using has 75% emission in the UVB range (290–320 nm wavelength) and 25% emission in the UVA range (320–400-nm wavelength). Peak emission of the light source is at 313-nm wavelength. One set of UV-irradiated culture dishes was treated with 0.1 nM, and another was treated with 10 nM ET-1. Fresh medium and the appropriate concentration of ET-1 were added every other day for a total of 6 days. Cell number was determined on days 1, 2, 4, and 6 after UV and/or ET-1 treatment. Tyrosinase activity was determined on days 4 and 6 after UV exposure and/or ET-1 treatment.

Western Blot Analysis of Tyrosinase and TRP-1, p53, p21, Bcl2, and pRB. For Western blot analysis of tyrosinase and TRP-1, cells were plated at a density of 5×10^5 cells/60-mm dish and treated 48 h later with 0.1 nM ET-1, 10 nM ET-1, and/or 10 nM α-MSH for a total of 3 or 6 days. Fresh medium and appropriate treatment were added every other day. Cell lysates were prepared using RIPA buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) containing the phosphatase inhibitor Na_3VO_4 (10 mM) and the protease inhibitors phenylmethylsulfonyl fluoride (200 mM), aprotinin (10 μg/ml), and leupeptin (10 μg/ml). Equal amounts of protein (5–8 μg) were loaded on each lane and separated by electrophoresis on a 7.5% polyacrylamide gel. After transblotting onto nitrocellulose membranes, the membranes were reacted with αhPEP-7 (1:1500), a polyclonal antibody raised against the COOH terminus of the human tyrosinase (a gift from Richard King and William Oetting, University of Minnesota, Minneapolis, MN), or with TA99 (1:1500), a mouse monoclonal antibody raised against the human TRP-1 (kindly provided by Setaluri Vijayasaradhi, Wake Forest University, Winston-Salem, NC). The membranes were then reacted with horseradish peroxidase conjugated anti-rabbit IgG (Amersham; 1:3000 dilution after incubation with αhPEP-7) or anti-mouse IgG (Amersham; 1:15,000 dilution after incubation with TA99). The immunoreactive bands were detected by chemiluminescence, using the Renaissance kit (Dupont NEN, Boston, MA).

For detection of p53, p21, pRb, and Bcl2, melanocytes maintained in medium devoid of TPA were irradiated with 28 mJ/cm^2 UVRs, as described before (36). One group of irradiated melanocytes was treated with 0.1 nM, and another was treated with 10 nM ET-1 for 7, 24, 48, or 72 h. Cell lysates were prepared as described above, and 15–20 μg were loaded per lane on a minigel apparatus. Gel (7.5%) was used for p53 and pRb, and 12% gel was used for p21 and Bcl2 detection. After electrophoresis and transblotting, the membranes were reacted with p53 monoclonal antibody DO1 (Santa Cruz Biotechnology, Santa Cruz, CA), and the same membrane was used for the detection of pRb using If8 monoclonal antibody (Santa Cruz Biotechnology), as described previously in detail (36). The other membranes were reacted with C-19 polyclonal antibody against p21, then with N-19 polyclonal antibody against Bcl2 (both antibodies were obtained from Santa Cruz Biotechnology). Western blot analysis was carried out as described in detail previously (36), and the immunoreactive bands were visualized by enhanced chemiluminescence.

Cell Cycle Analysis of Unirradiated and UV-irradiated Melanocytes after Treatment with ET-1. The effects of 0.1 or 10 nM ET-1, alone or in combination with 10 nM α-MSH, on the progression of melanocytes through the cell cycle were determined as follows. Melanocytes maintained in medium lacking TPA and BPE were plated at a density of 7.5×10^5 cells/100-mm dish and were treated 48 h later with 0.1 or 10 nM ET-1 and/or 10 nM α-MSH. Fresh medium and hormone(s) were added every other day. After 24, 48, or 72 h of treatment, melanocytes were harvested, and bare nuclei were prepared and stained with PI, as described previously in detail (36). To determine the effects of ET-1 on the UV-induced G_1 arrest, melanocytes were maintained in medium lacking TPA and were plated into 100-mm dishes at a density of 7.5×10^5 cells/dish. Three days later, melanocytes were irradiated a single time with 28 mJ/cm^2 UVR and/or treated with 0.1 or 10 nM ET-1. Fresh medium and ET-1 were added every other day. Twenty-four, 48, or 72 h after treatment, melanocytes were harvested, and their DNA was stained with PI. Flow cytometric analysis of the cell cycle was performed using a Coulter EPICS XL flow cytometer (Coulter Cytometry, Coulter Corp., Miami, FL). The PI signals were collected and analyzed using System II software (Coulter Corp.), and the DNA histograms were analyzed using Multicycle (Phoenix Software, San Diego, CA).

Northern Blot Analysis of ET BR, ET AR, and MC1R. Melanocytes in BPE- and TPA-free medium were plated onto 100-mm dishes at a density of 7×10^5 cells/dish. Forty-eight hours later, melanocytes were treated with 10 nM ET-1 and/or 10 nM α-MSH for 6 h or 5 days and subsequently lysed using phenol and guanidine thiocyanate (Molecular Research Center, Cincinnati, OH). Total RNA was purified by phase separation, followed by isopropanol precipitation, and the concentration of RNA was determined spectrophotometrically. Fifteen to 20 μg of total RNA were separated by electrophoresis through agarose-formaldehyde gel. After electrophoresis, the RNA was blotted onto nylon membrane filters (Hybond-N$^+$; Amersham) and the amount and quality of RNA were detected using methylene blue staining. Filters were hybridized with ^{32}P-labeled pMEhET$_A$ or pMEhET$_B$ for detection of ET AR or ET BR mRNA, respectively (both probes were a gift from Masashi Yanagisawa, University of Texas Southwestern Medical Center, Dallas, TX), or with 9-1 neo hMSH-R expression vector for the detection of MCIR mRNA (a gift from Roger Cone, Vollum Institute, Portland, OR). After hybridization, filters were washed three times in a solution of 2× SSC, 0.1% SDS at room temperature for a total of 45 min, then exposed to X-ray film for 20 h with intensifying screen. Filters were rehybridized with glyceraldehyde-3-phosphate dehydrogenase cDNA probe (Clontech Laboratory, Inc., Palo Alto, CA) to normalize for the amount of RNA.

Receptor Binding Assays: Competition of ^{125}I-labeled ET-1 Binding by ET-1 or ET-3. Melanocytes were inoculated into 24-well plates at a concentration of 4×10^5 cells/well and allowed to attach for 2 days. Cells were then washed twice with 1 ml of MCDB medium containing 0.5% BSA (fraction V; Sigma Chemical Co.) and incubated in 0.5 ml of MCDB medium containing 0.5% BSA, 0.3 mM 1,10-phenanthroline (proteinase inhibitor; Sigma Chemical Co.), and 100,000–150,000 cpm of 3-[^{125}I] iodotyrosyl ET-1 (Amersham; specific activity, 2000 Ci/mmol) and various concentrations of cold ET-1 or ET-3 (10^{-12}-10^{-6} M) at room temperature for 2 h. After incubation, melanocytes were washed three times with 1 ml of ice-cold MCDB medium containing 0.5% BSA and lysed with 1 ml of 0.5 N NaOH, 0.4% deoxycholate. Radioactivity was counted in a gamma counter, and the data were analyzed by linear regression using Kaleidagraph software package.

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