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BQ-788 (ENDOTHELIN-B RECEPTOR ANTAGONIST) BLOCKS KERATINOCYTE-INDUCED DENDRICITY OF CULTURED MELANOCYTES

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Summary

Facial hyperpigmentation in women, which is considered to be a serious cosmetic disability and a cause of mental distress, requires proper management. Melanocyte dendricity is a crucial factor affecting epidermal pigmentation. We found that BQ-788, the endothelin-B (ETB) receptor antagonist, blocks the formation of multi-dendricity which is induced by cocultured keratinocytes.

Melanocytes in vivo show numerous dendrites which are in close contact with multiple keratinocytes, forming the epidermal-melanin unit. While melanocytes transfer their melanosomes into the neighboring keratinocytes via dendrites, keratinocytes secrete many growth factors and cytokines that influence viability, morphology, and melanin formation of melanocytes. Endothelin-1 (ET-1), prostaglandin E2(PGE2), and leukotriene-C4 (LT-C4) have been suggested as the candidates for increasing dendricity. Other reports suggested that ET-1 has stimulatory effects on proliferation and melanin formation of melanocytes in vitro.

In the present study, using type-specific ET receptor antagonists, we observed how the morphology of melanocytes could be modulated in a coculture system. In addition, the roles of ET-1 for morphology and proliferation on melanocytes were evaluated in different culture media.

We suggest that ET-1 increases dendricity and proliferation of melanocytes, and that its dendrite-inducing effect and mitogenic effect are regulated independently.

Introduction

The morphology of melanocytes in vivo is characterized by dendrite formation and ramification. Through dendritic processes, melanocytes transfer melanosomes into neighboring keratinocytes [1,2]. Therefore, melanocyte dendricity is a crucial factor affecting epidermal pigmentation [3,4]. Although it is not clearly understood how this dendritic appearance of melanocytes is controlled, the role of growth factors secreted by keratinocytes has been suggested by many investigators [5,6,7,8,9,10,11,12]. Recently, it was found that keratinocytes synthesize and secrete endothelin-1(ET-1), which was originally isolated from endothelial cells [13,8]. It was further reported that ET-1 has stimulatory effects on proliferation, melanin formation, and dendricity of melanocytes in vitro[7,4]. However, little is known about the action mechanisms of ET-1 on melanocytes.

To determine which type of ET-1 receptor is involved in the biologic functions of melanocytes, we compared the effects ETA receptor antagonist (BQ-123) and ETB receptor antagonist (BQ-788) in a keratinocyte-melanocyte coculture system. We also evaluated the biologic effects of ET-1 on melanocytes under various culture conditions.

Materials & Methods

Human Melanocyte and Keratinocyte Culture

Neonatal foreskins obtained from circumcisions were used to culture human melanocytes and keratinocytes, as described previously. [14,15]. Briefly, the epidermis was separated from the dermis after overnight incubation in 0.25% trypsin at 4 °C.

Melanocyte cultures were then established in MCDB 153 supplemented with 5% fetal bovine serum(FBS) (Gibco), 16nM 12-O-tetradecanoylphorbol-13-acetate(TPA) (Sigma), 1ng/ml basic fibroblast growth factor (b FGF)(Calbiochem, San Diego, CA), 1ug/ml vitamin E acetate(Sigma), 1ug/ml transferrin(Sigma), 5ug/ml insulin(Sigma), and antibiotics. In some experiments, melanocytes were cultured in the absence of TPA. Keratinocyte cultures were established in keratinocyte growth medium (Gibco BRL, Grand Island, NY) supplemented with 10ng/ml epidermal growth factor (Gibco BRL). In the coculture system, keratinocytes were seeded and allowed to attach for 3-4h. Then, melanocytes were seeded directly over the keratinocytes (1:10 ratio). They were maintained in MCDB 153 media supplemented with 5% fetal bovine serum, 10ng/ml epidermal growth factor, 1ng/ml b FGF, 1ug/ml vitamin E acetate, 1ug/ml transferrin, 5ug/ml insulin, and antibiotics. Medium changes were performed twice a week.

Effects of ET-1 on Morphology of Melanocyte

Pure-cultured melanocytes at second and third passage were treated with various concentrations of ET-1(0.1-200 nM) in the presence or absence of TPA. The morphologic changes of melanocytes were compared and photographed under a phase-contrast microscope (Nikon SMZ-V).

Effect of BQ-788 and BQ-123 on the melanocyte morphology

First passaged cells were treated with BQ-123(10uM) or BQ-788 (10 uM) for 5 days in the keratinocyte-melanocyte coculture system, and thier morphologic changes were observed under the phase-contrast microscope.

Image Analysis

Cultured melanocytes were photographed under the phase-contrast and bright field microscope(Nikon SMZ-V) at ramdom microscopic fields (at least 5 fields), and their images were scanned using Nikon coolscan II system with Nikon control 2.1 software. The cell length, perimeter, area, number of dendrites per cell, length of dendrites were analysed with a Macintosh LC475 computer using the NIH Image program.

Effects of ET-1 on Proliferation of Melanocyte

The proliferation of cultured melanocytes was determined by MTT assay, as described [16]. Briefly melanocytes were treated with various concentrations of ET-1 (0.1-200 nM) in the presence or absence of TPA. After 2 and 5 days of culture, 15 ul of MTT solution (5mg/ml in PBS) was added to the medium and cells were incubated for 3h at 37°C. The medium was gently removed from each well and 150 ul of DMSO (dimethyl sulfoxide) was added. After 30 minutes, the plates were read on a microelisa reader, using a test wavelength of 540 nm, with a reference wave length of 650 nm.

RESULTS

ET-1 increased dendricity of melanocytes

The morphology of melanocytes was dependent on culture media, which included or excluded TPA. Melanocytes cultured in a medium without TPA showed a short, thick, fibroblast-like appearance(Fig. 1-A), while those cultured in a media containing TPA showed a long, slender bipolar appearance(Fig. 1-C). Stimulation with ET-1 induced a dendritic appearance of melanocytes, and this dendrite-inducing effect was not affected by the presence of TPA, although it was a little more prominent in the TPA-deprived culture medium (Fig. 1-B, D).

Image analysis also showed that, compared to control, ET-1(200 nM) induced statistically significant increases in the length of dendrites (71.2 18.9 vs 33.4 9.5 um, $p < 0.01$), cell length (179.1 35.0 vs 94.1 16.2 um, $p < 0.01$), area (850.0 594.6 vs 523.2 333.1 um2, $p < 0.05$), perimeter (438.3 176.5 vs 213.6 53.4 um, $p < 0.01$), and the number of dendrites per cell (2.9 1.1 vs 2.2 0.5, $p < 0.01$) (Fig.2). Compared to ET-1 alone, combination of ET-1 and TPA did not induce any statistically significant changes in the morphology of melanocytes.

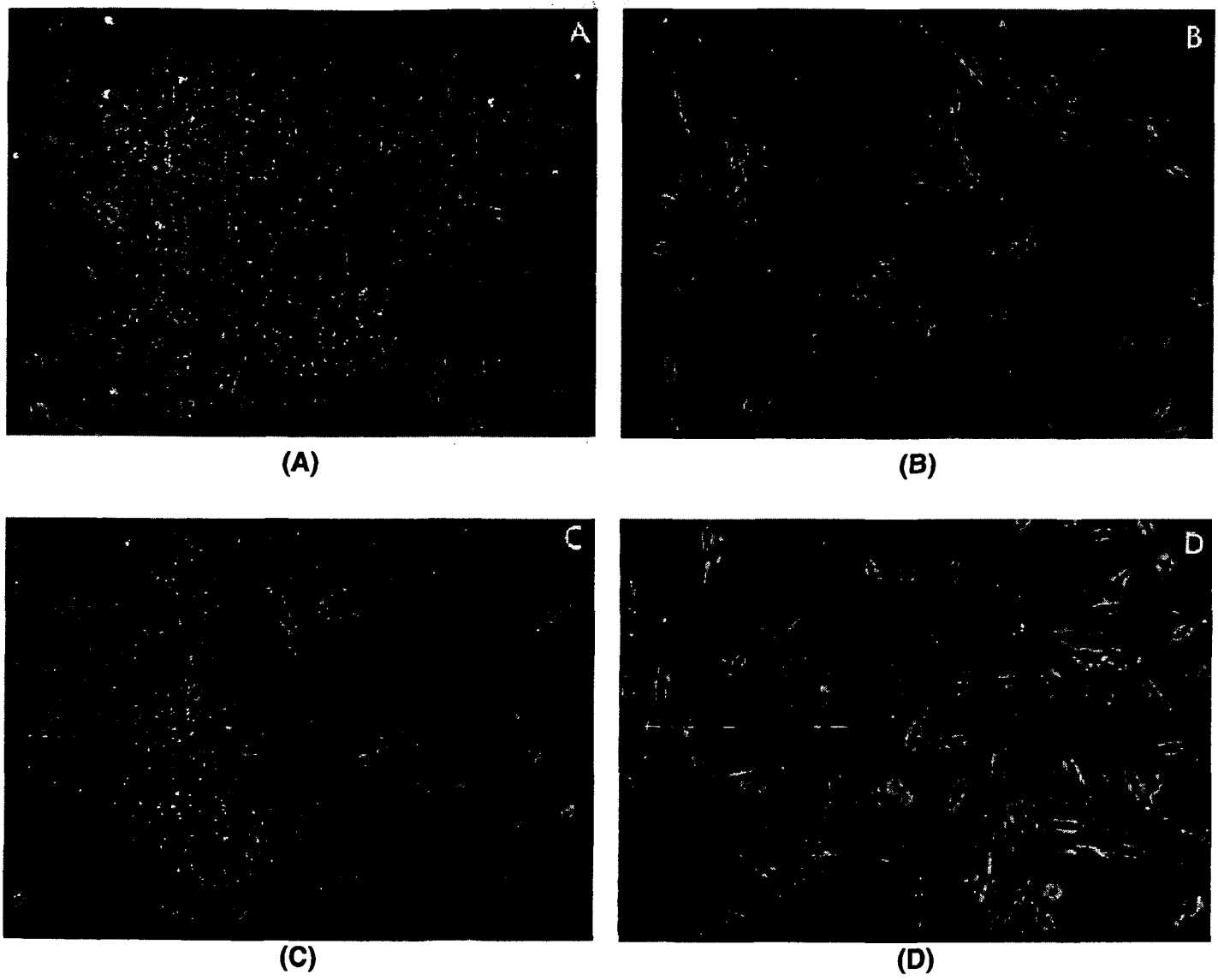

Fig.1. Effect of ET-1 on the morphology of cultured melanocytes

Melanocytes were isolated and cultured as described in Materials & Method. The morphology of melanocytes was dependent on culture media, which included or sexcluded TPA. Melanocytes cultured in a medium without TPA showed a short, thick, fibroblast-like appearance(A), while those cultured in a media containing TPA showed a long, slender bipolar appearance(C). Stimulation with ET-1 induced a dendritic appearance of melanocytes, and this dendrite-inducing effect was not affected by the presence of TPA, although it was a little more prominent in the TPA-deprived culture medium (B, D). All the melanocytes used were second passaged pure melanocytes. Seven days after seeding. Concentration of ET-1 : 200 nM. Original magnification : x 200.

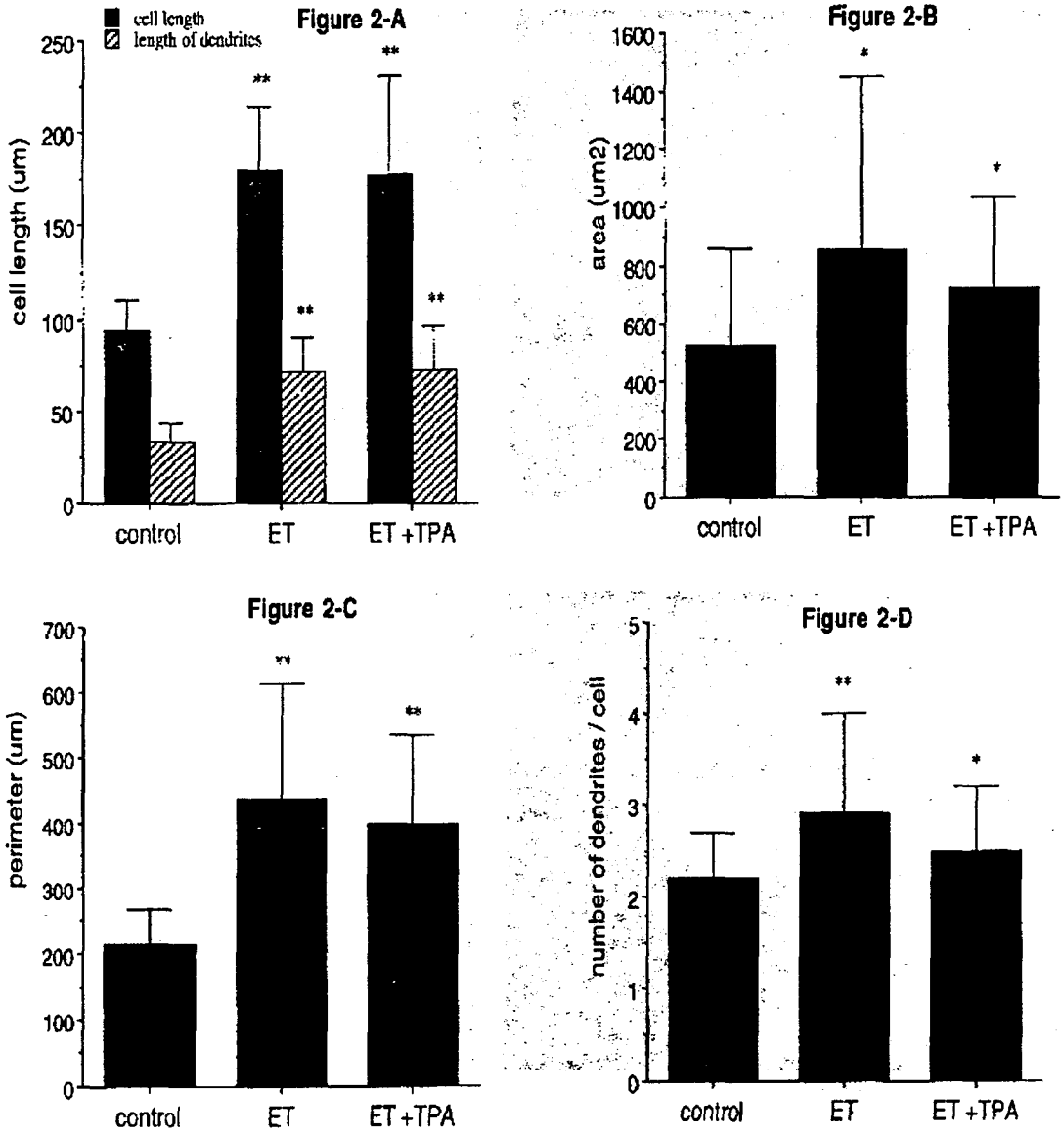

Fig.2. ET-1 induced morphologic changes measured by image analysis.

*The cell length, perimeter, area, number of dendrites per cell, length of dendrites were analysed with the NIH Image program as described. Image analysis also showed that, compared to control, ET-1(200 nM) induced statistically significant increases in the length of dendrites (A), cell length (A), area (B), perimeter (C), and the number of dendrites per cell (D). Compared to ET-1 alone, combination of ET-1 and TPA did not induce any statistically significant changes in the morphology of melanocytes. Second passaged melanocytes were measured on the 7th day of culture(n=30). ET-1 : 200 nM, TPA : 16 nM, mean S.D., n=30, * $p < 0.05$ compared to control, ** $p < 0.01$ compared to control.*

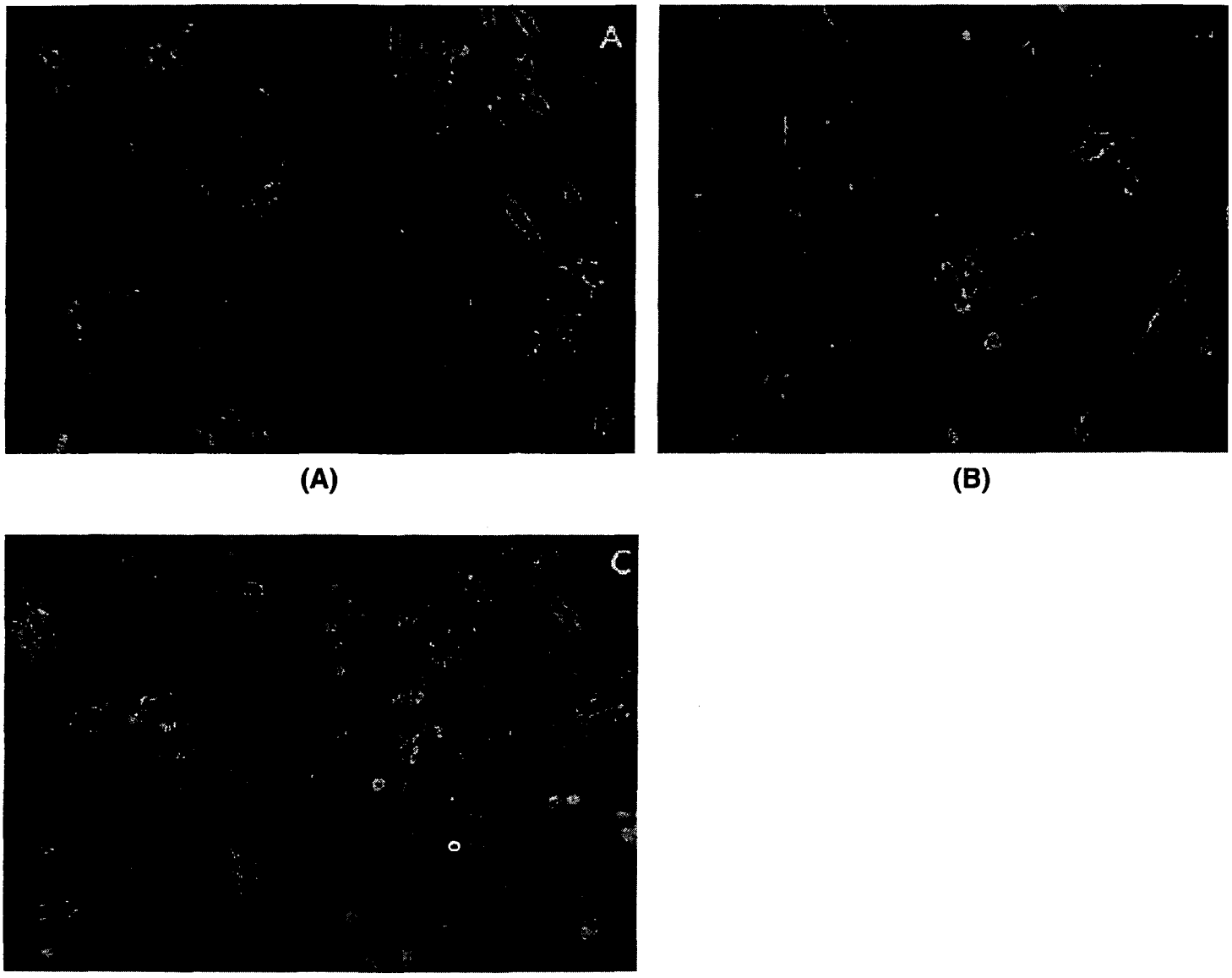

Fig. 3. Effects of ET-1 receptor antagonists on melanocyte morphology

Melanocytes cocultured with keratinocytes appeared as dendritic cells with highly developed ramifications (A). Through their dendrites, melanocytes were in contact with each other and/or with keratinocytes. Addition of BQ-788, the ETB receptor antagonist, induced significant morphologic changes resulting in decreased dendricity of melanocytes (B). Most melanocytes altered their dendritic appearance into bipolar or tripolar cells, although some remained dendritic. However, addition of BQ-123, the ETA receptor antagonist, to these cocultured cells had no effect on the morphology of melanocytes (C). First passaged cells were treated with BQ-123(10uM) or BQ-788 (10 uM) for 5 days. Original magnification x 200.

BQ-788 Inhibited Dendricity of Melanocyte in the Coculture System

Melanocytes cocultured with keratinocytes appeared as dendritic cells with highly developed ramifications (Fig.3-A). Through their dendrites, melanocytes were in contact with each other and/or with keratinocytes. Addition of BQ-788, the ETB receptor antagonist, induced significant morphologic changes resulting in decreased dendricity of melanocytes (Fig.3-B). Most melanocytes altered their dendritic appearance into bipolar or tripolar cells, although some remained dendritic. However, addition of BQ-123, the ETA receptor antagonist, to these cocultured cells had no effect on the morphology of melanocytes (Fig.3-C). Image analysis showed that BQ-788 decreased cell length (167.7 37.0 vs 200.9 50.7 um, $p < 0.05$)(Fig.4-A) and surface area (2069.9 580.5 vs 3549.9 948.2um2, $p < 0.05$)(Fig.4-B) as well as dendricity of melanocytes (3.4 0.9 vs 5.5 1.9 dendrites / cell, $p < 0.05$)(Fig.4-C). In melanocytes treated with BQ-123, no significant changes in length, area, and dendricity were found.

ET-1 stimulated the proliferation of melanocytes

The effect of ET-1 on the proliferation of melanocytes was dependent on TPA. In the absence of TPA, ET-1 increased the proliferation of melanocytes in a concentration-dependent manner. Statistically significant increase in proliferation of melanocytes was found at the concentration of 200 nM on the second day of culture (0.511 0.057 vs 0.346 0.072 O.D., $p < 0.05$)(Fig. 5-A), and at the concentration of 2 nM on the fifth day of culture (0.429 0.047 vs 0.347 0.035 O.D., $p < 0.05$) (Fig. 5-B). In the presence of TPA, however, this mitogenic effect of ET-1 on the proliferation of melanocytes was not found at any of concentrations tested.

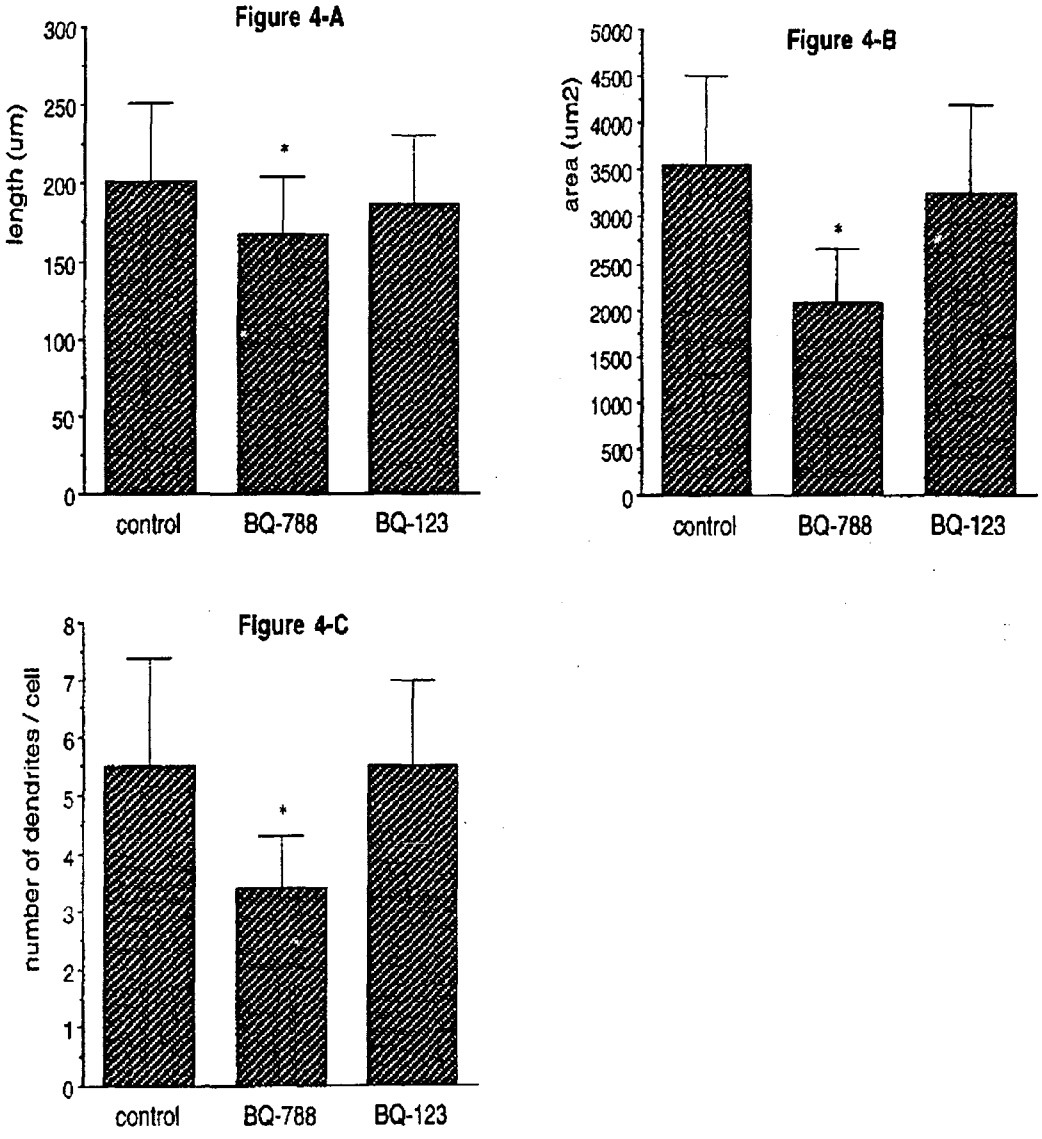

Fig. 4. Effects of ET-1 receptor antagonists measured by image analysis

*Image anaiysis showed that BQ-788 decreased cell length (A) and surface area (B) as well as dendricity of melanocytes (C). In melanocytes treated with BQ-123, no significant changes in length, area, and dendricity were found. First passaged cells were treated with BQ-123(10um) or BQ-788 (10 uM) for 5 days. mean ± S.D., n=20, * p < 0.05 compared to control, ** p < 0.01 compared to control.*

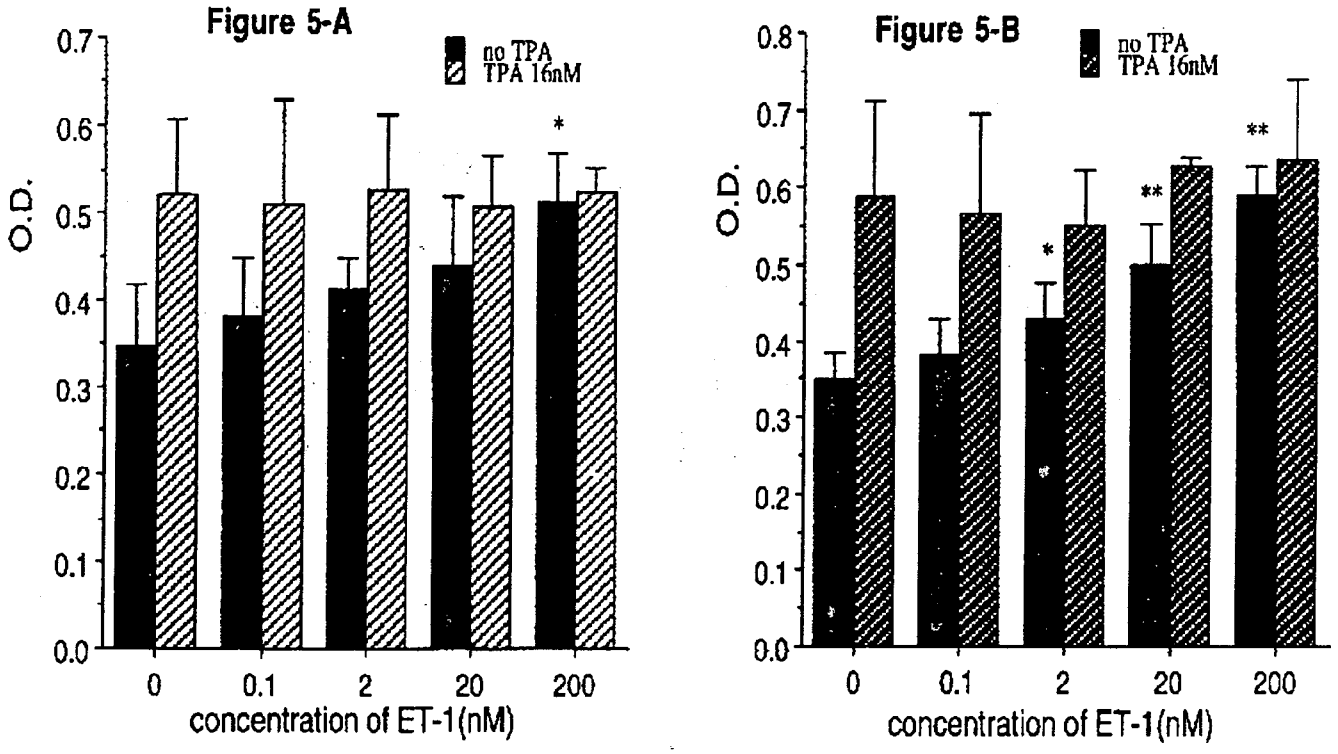

Fig. 5. Effects of ET-1 on proliferation of cultured melanocytes

*The effect of ET-1 on the proliferation of melanocytes was dependent on TPA. In the absence of TPA, ET-1 increased the proliferation of melanocytes in a concentration-dependent manner. Statistically significant increase in proliferation of melanocytes was found at the concentration of 200 nM on the second day of culture (A), and at the concentration of 2 nM on the fifth day of culture (B). In the presence of TPA, however, this mitogenic effect of ET-1 on the proliferation of melanocytes was not found at any of concentrations tested. mean S.D., n=3, * $p < 0.05$ compared to control, ** $p < 0.01$ compared to control.*

Discussion

In the present study, we found that ET-1 induces morphologic changes (increase in dendricity, cell length, perimeter, and area) of melanocytes. Our results correlate well with those of Hara et al[4], who demonstrated that ET-1 increases dendricity of melanocytes. However, their culture medium was supplemented with cAMP inducers such as cholera toxin and isobutylmethylxanthine(IBMX), which are known to increase the dendricity of melanocytes [14,2]. Thus, the possibility that ET-1 affects melanocyte dendricity only in the presence of increased intracellular cAMP can not be excluded. We investigated the effect of ET-1 on melanocyte dendricity in the absence of cAMP inducers and demonstrated that ET-1 has its own dendrite-inducing effect. Moreover, we also found that TPA, a potent protein kinase C (PKC) activator, did not affect the ET-1-induced morphologic changes. These results suggest that ET-1 induces dendricity of melanocytes, which is independent of activation of cAMP and PKC signal pathway.

Recently, Yada et al[7] demonstrated that ET-1 stimulates melanocyte proliferation. We also demonstrated that ET-1 stimulates the proliferation of melanocytes in a concentration dependent manner and that high concentration of ET-1(200 nM) was comparable to TPA(16nM) in its effect on melanocyte proliferation. However, stimulation with ET-1 in the presence of TPA did not induce any synergistic or additive mitogenic effect on proliferation of melanocytes. These results indicate that TPA inhibits mitogenic effect of ET-1 on melanocytes. TPA is not a physiologic stimulator, therefore, its regulating effect does not seem to occur in vivo, but any biological events inducing PKC activation might regulate the effects of ET-1 on melanocytes. Although it has been previously proposed that stimulatory effect of ET-1 on DNA synthesis of fibroblasts is markedly reduced by TPA [17], further studies are needed to determine the regulatory role of TPA on ET-1-induced proliferation of melanocytes.

It is generally agreed that keratinocytes produce a variety of cytokines, which regulate melanocyte growth and differentiation [5,18]. Recent evidence suggests that ET-1 produced by keratinocytes also plays an important part in the biology of melanocytes [7,4]. However, little is known about the signal-transduction mechanism of ET-1 on melanocytes. In our coculture system, dendricity of melanocytes was significantly reduced by the ETB receptor antagonist (BQ-788), but not by the ETA receptor antagonist (BQ-123). Moreover, in the pure-culture system of melanocytes, dendrite-inducing effect of ET-1 was blocked by BQ-788 (data not shown). These findings strongly suggest that ET-1 is secreted from cultured keratinocytes, and its dendrite-inducing effect is mediated by the ETB receptor of melanocytes. However, dendricity of melanocytes was not completely inhibited by BQ-788, indicating that dendrite formation of melanocytes is also mediated by other undefined factors as well as ET-1. Dendricity of melanocytes seems to be maintained in a complex manner, and further studies are needed to clarify the regulating mechanisms.

We suggest that ET-1 increases dendricity of melanocytes through the ETB receptor, which is independent of activation of cAMP and TPA, and that ET-1 stimulates proliferation of melanocytes,

which is blocked by TPA. In conclusion, the dendrite-inducing effect and mitogenic effect of ET-1 seem to be regulated independently.

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