Journal of Enzyme Inhibition and Medicinal Chemistry, August 2008; 23(4): 526–534

informa
healthcare

Effects of hydroxybenzyl alcohols on melanogenesis in melanocyte-keratinocyte co-culture and monolayer culture of melanocytes

SZU-HSIU LIU[1,2], I-MING CHU[1], & I-HORNG PAN[2]

[1]*Department of Chemical Engineering, National Tsing Hua University, Hsinchu, Taiwan, 30013, R.O.C., and* [2]*Biomedical Engineering Research Laboratories, Industrial Technology Research Institute, Hsinchu, Taiwan, R.O.C.*

(Received 28 June 2007; in final form 24 July 2007)

Abstract
In mammalian skin, melanocyte proliferation and melanogenesis can be stimulated by keratinocytes, fibroblasts and other regulatory factors. To determine whether hydroxybenzyl alcohols (HBAs) show more inhibitory in melanocytes cultured alone or in melanocytes co-cultured with keratinocytes, we developed a murine melanocyte–keratinocyte co-culture model to investigate the pigmentation regulators in company with other melanogenic inhibitors and stimulators. It was found that the effects of HBAs and melanogenic factors were more evident in melanocytes co-cultured with keratinocytes. Keratinocytes may play a synergistic role in melanocyte melanogenesis and influence the pigment production. The tests in the co-culture model also imply that the inhibitory effects of HBAs on melanogenesis are due to the direct inhibition of melanosomal tyrosinase activity. HBAs showed a low cytotoxicity. The eventual results proved that HBAs are promising and safe agents for skin whitening in melanocyte alone and in co-culture systems. The co-culture model provides a more physiologically realistic condition to study the interaction between melanocytes and keratinocytes, which enables a reliable screening system for depigmenting compounds.

Keywords: *Hydroxybenzyl alcohol, melanocytes, keratinocytes, co-culture, melanogenesis*

Introduction

Melanin is polyphenol-like biopolymer with a complex structure and colors varying from yellow to black, [1] which was formed through a series of oxidative reactions involving the amino acid tyrosine in the presence of tyrosinase. [2] Tyrosinase (monophenol monooxygenase EC 1.14.18.1) is a copper-containing enzyme, which is widely found in plants and animals. [3] It is known to be a key enzyme for melanin biosynthesis in plants, microorganisms and mammalian cells. Many tyrosinase inhibitors have been tested as cosmetics and pharmaceuticals for reducing melanin in epidermal layers. [4–6] In our previous study, we showed that *p*-hydroxybenzyl alcohol (4HBA) is an irreversible inhibitor of tyrosinase. Its effect on melanogenesis in B16 melanoma cells was demonstrated. [7] Here, we attempted to develop a melanocyte–keratinocyte co-culture model for further characterizing the effects of 4HBA on the pigmentation of skin.

A typical epidermal melanin unit in human skin is composed of one melanocyte in contact with approximately 35 to 40 neighboring keratinocytes. In the vertebrate epidermis, melanocytes are associated with surrounding keratinocytes via dendritic processes. This close contact allows keratinocytes to support the growth of melanocytes and conduct their primary function of producing and delivering melanin to keratinocytes. [8,9] Previous studies have showed that melanocytes adhere to keratinocytes through E-cadherin, [10] resulting in the formation of gap-junction mediated channels between the two cell types. It is probably that keratinocytes control the growth, shape and expression of cell-surface adhesion receptors as well as their cytoplasmic associated

Correspondence: I-Ming Chu, Department of Chemical Engineering, National Tsing Hua University, 101, Section 2 Kuang Fu Road, Hsinchu, Taiwan 30013, R.O.C. E-mail: imchu@che.nthu.edu.tw

ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.1080/14756360701654894

protein in melanocyte via these tight connections. [11–13] Keratinocytes produce and secrete basic fibroblast growth factor (bFGF), α-melanocyte stimulating hormone (α-MSH), endothelin-1 and other factors that can stimulate the proliferation, melanogenesis and dendritogenesis of melanocytes in vivo. [14–16] The expressions of the melanogenic enzyme, such as tyrosinase, TRP1 and TRP2, were differently modulated when melanocytes cultured with keratinocytes, and thus it suggests that keratinocytes derived signals were involved in the coordinated expression of these three enzymes. [17] Therefore, various skin equivalent models have been reconstituted to provide realistic models for skin physiology.

The aim of our study is to establish a co-culture system using immortalized murine melanocytes and murine keratinocytes to supply a screening model for depigmenting compounds. The effects of HBAs and melanogenesis stimulators, such as α-melanocyte-stimulating hormone (α-MSH) and L-tyrosine, were investigated in melanocytes that were cultured alone or cultured in the presence of keratinocytes.

Materials and methods

Chemicals

Mushroom tyrosinase (EC 1.14.18.1), L-tyrosine, 2-hydroxybenzyl alcohol (2HBA), 3-hydroxybenzyl alcohol (3HBA), 4-hydroxybenzyl alcohol (4HBA), hydroquinone (HQ), dimethyl sulfoxide, trypsin/EDTA, α-melanocyte-stimulating hormone (α-MSH), mitomycin C, Phosphate buffered saline (PBS) and arbutin were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (New York, NY, USA). Other chemicals were purchased from Aldrich.

Sources of tyrosinases

Mushroom tyrosinase was purchased from Sigma. Murine tyrosinase was prepared from murine melanocytes (B16 cells) as previously described. [18] B16 cells (2 $X10^6$) were in PBS buffer. The cell pellet was lysed with 0.5 mL of 50 mM sodium phosphate buffer (pH 6.8) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5% Triton X-100. The cell were sonicated six times for 30 s each with 1-min intervals and then incubated at 4°C for 1 h. After centrifugation at 10,000 rpm for 5 min at 4°C, supernatant was used as a source of murine tyrosinase.

Enzyme assay

The enzyme activity was monitored by dopachrome formation at 475 nm for an appropriate period (not exceeding 10 min, unless otherwise noted). The assay was performed as previously described. [19] First, L-tyrosine aqueous solution was mixed with phosphate buffer (pH6.8) and incubated at 25°C for 10 min. Then, the solution and mushroom tyrosinase were added in this order to the mixture. This assay solution (1 ml: contains 0.8 mM L-tyrosine, 0.9 M phosphate sodium buffer at pH 6.8 and 40.0 units/ml mushroom tyrosinase) was immediately monitored for the formation of dopachrome by measuring the linear increase in optical density at 475 nm.

Cell culture

Murine melanocytes (B16) and murine keratinocytes (XB-2) were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). B16 melanocytes were cultured in DMEM with 10% FBS. XB-2 murine keratinocytes were cultured as previously described. [20] Briefly, XB-2 keratinocytes were cultured in DMEM with 20% FBS together with a 3T3 feeder layer (treated with mitomycin C for 4hours) at the outset of culture, after which they were grown without a feeder. Mitomycin C-treated XB2 feeder cells were used in establishment and thawing out of frozen stocks of this cell line.

All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. After three days culture, the cells were harvested after trypsinization and then enumerated by tryptan blue inclusion under microscopy.

Co-culture

XB-2 keratinocytes and B16 melanocytes were harvested after trypsinization and then resuspended in DMEM with 20% FBS. The cell count was carried out using a hemocytometer, and the trypan blue exclusion was used to assess cell viability. Melanocytes (3×10^4 cells/well) and keratinocytes (3×10^5 cells/well) were seeded together in 6-well plates and maintained in DMEM containing 20% FBS. The initial seeding ratio of keratinocytes to melanocytes was thus 10: 1. Three days later, culture medium was replaced with fresh medium (DMEM contain 10% FBS medium) containing different compounds (melanogenic inhibitors or stimulators) in each well. After four days, cells were photographed and were then harvested with trypsin/EDTA.

Cell viability

The cells were harvested after trypsinization. Counted by tryptan blue inclusion under microscopy and assayed for cell viability.

Melanin assay

The melanin content was determined by the published protocol with minor modifications. [21] The cells were washed with PBS and lysed with 1 mL of 1N NaOH. The relative melanin content was determined by optical density at 490 nm and the melanin content was calculated as $\mu g/10^6$ cells.

Assay of cellular tyrosinase activity

The cellular tyrosinase activity using L-DOPA as the substrate was assayed based on the method of Tomita et al. [22] The cells were washed with PBS and lysed with 1 ml 0.5% Triton-X/PBS. The lysated sample was mixed by vibration, and 0.8 mM L-DOPA was added to each sample. After incubating at 37°C for 3 h, the absorbance at 475 nm was measured to calculate the activity.

Cyclic AMP determination

Cells were seeded into 12-well plates and incubated with different compounds for 4 days. The cells were treated with 0.1M NaOH for 10 min. The lysated cells were centrifuged at 2000rpm for 10 min. The supernatant were assayed directly in Direct cAMP assay (Assay Designs, USA). The protein content of the cells was determined after dissolving samples in 1mL 1N NaOH and measured with the Bio-Rad protein assay reagent (Bio-Rad, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using TRIZOL-reagent (Invitrogen) according to the manufacturer's protocol. RNA samples (2 μg/reaction) were reverse-transcribed with Superscript in the presence of oligo-dT. The RT reaction was used for amplification with Taq polymerase. The resulting cDNA was amplified using tyrosinase, melanocortin-1 receptor (MC1-R) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific primers. The PCR products were separated by electrophoresis on 1.0% agarose gel.

Statistics

Significant differences between results were determined by Student's *t*-test. p Values < 0.05 were taken to be significant.

Results

Kinetic study of HBAs and cytotoxicity assay

Inhibitory effects of hydroxybenzyl alcohol compounds (see Figure 1) on mushroom and murine tyrosinase activity were evaluated (see Table I). In our previous study, we verified that *p*-hydroxybenzyl alcohol (4HBA) reduced melanogenesis through the inhibition of tyrosinase rather than suppression of tyrosinase gene. [7] The 4HBA has inhibitory concentration (IC_{50}) of 350 μM when L-tyrosine is used as the substrate, and it is an irreversible inhibitor of mushroom tyrosinase. Interestingly, it was found that both of 2HBA and 3HBA, which have similar structures to 4HBA, can also effectively decrease the tyrosinase activity and showed a dose-dependent manner of inhibitions. According to the kinetic analysis (see Table I), 2HBA is a competitive inhibitor and the IC_{50} value was 894.3 μM. 3HBA showed the irreversible inhibitory that is similar to 4HBA, and its IC_{50} value was 402.5 μM. For comparison, the IC_{50} values of HQ and arbutin were also determined to be 87.6 and 295.7 μM respectively. The tyrosinase activity in crude murine tyrosinase solution was inhibited by all of these agents in a concentration-dependent manner. However, inhibitory effects of compounds on murine tyrosinase were weaker than mushroom tyrosinase activity.

The B16 cells were treated with different concentrations of HBAs, and the numbers of the cultured cells were compared for determining whether these compounds have cytotoxic effects. The results showed that no significant decreases in cell viability were observed, when the concentrations of HBAs were below 1.0 mM (see Figure 2).

Effects of HBAs in co-culture

To study the effects of HBAs on melanogenesis in co-culture, we used melanogenic inhibitors (HQ and arbutin) and stimulators (α-melanocyte stimulating hormone and L-tyrosine) in company with HBAs to investigate the pigmentation regulators. Melanocytes retained normal morphology and grew very well when

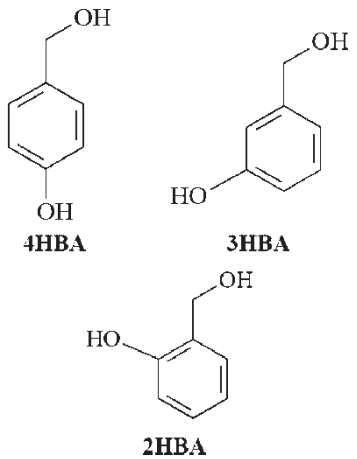

Figure 1. Chemical structures of 2-hydroxybenzyl alcohol (2HBA), 3-hydroxybenzyl alcohol (3HBA) and 4-hydroxybenzyl alcohol (4HBA).

Table I. Inhibitory effects on mushroom and murine tyrosinase by different compounds.

		Mushroom			Murine	
Compound	Concentration μM	Inhibition[a] %	IC 50[b] μM	Model of inhibition	Inhibition[a] %	IC 50[b] μM
2HBA	400	26.5 ± 1.1	894.28	Competitive	7.9 ± 2.3	2393
	800	47.1 ± 0.5			15.3 ± 1.2	
3HBA	400	49.8 ± 0.6	402.47	Irreversible	22.3 ± 1.8	952.3
	800	82.2 ± 1.2			42.9 ± 1.0	
4HBA	400	55.3 ± 0.8	349.95	Irreversible	33.6 ± 1.7	649.9
	800	91.5 ± 2.1			59.6 ± 2.1	
HQ	100	56.4 ± 1.1	87.57	Competitive	30.1 ± 2.2	188.2
	200	98.4 ± 0.8			53.2 ± 1.8	
Arbutin	400	60.4 ± 1.4	295.7	Competitive	35.2 ± 0.5	624.6
	800	93.6 ± 1.5			62.0 ± 1.3	

[a] 1 mL solution contains 0.9 M phosphate sodium buffer at pH 6.8, different concentrations of compounds and tyrosinase. The assay solution was pre-incubated at 25°C for 5 min. Then, 0.8 mM L-tyrosine was added, and the absorbance was read at 475 nm. Each value represents the mean ± S.E. of three experiments; [b] 50% inhibitory concentration. HQ = hydroquinone.

cultured with keratinocytes XB-2 cells (see Figure 3). Melanocytes showed more pigment and dendritic in morphology when grown in co-culture treated with α-MSH. Under phase contrast microscopes, the L-tyrosine-treated melanocytes were found evidently increasing in pigment compared with the untreated control. By contrast, melanocytes became lighter in the presence of the melanogenic inhibitors HQ, arbutin and 4HBA.The aberrant morphology was observed in the case that melanocytes were treated with HQ. 4HBA showed the depigmenting ability and had no influence on cells morphology.

Effects of HBAs on melanin synthesis and cellular tyrosinase activity

The melanin synthesis and tyrosinase activity were measured to evaluate the effects of HBAs, melanogenic inhibitors and stimulators in melanocytes cultured alone and in melanocytes co-cultured with keratinocytes. As shown in Figure 4 and Figure 5, the melanin content and tyrosinase activity were stimulated by α-MSH. α-MSH led to a 50% increase on tyrosinase activity in melanocytes cultured alone, and the effects were significantly enhanced when melanocytes were co-cultured with keratinocytes. The melanin production also showed significant enhancement in co-cultured melanocytes when treated with α-MSH. Similarly, L-tyrosine also elicited increase in melanin production, and the effects of L-tyrosine were observed higher in melanocytes co-cultured with keratinocytes. However, L-tyrosine in melanocytes did not affect the tyrosinase activity in both of cultured alone and co-culture models.

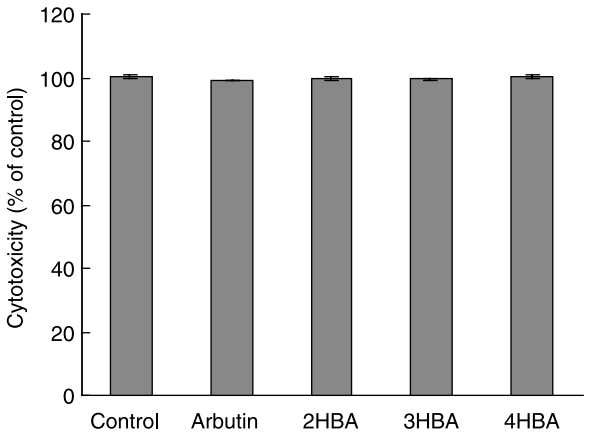

Figure 2. Effect of hydroxybenzyl alcohols on B16 cell viability. B16 cells were cultured in 6-well plates and incubated with different agents for 4 days. Bars represent means ± S.E. of at least three independent experiments. Significant differences were determined by Student's *t*-test; *P < 0.05 compared to control.

In Figure 4 and Figure 5, the data indicate that HBAs reduced melanin content and inhibited the tyrosinase activity in a concentration-dependent manner. Obviously, 4HBA had the strongest inhibitory effects, compared with other HBAs, in melanocytes that cultured alone and co-cultured with keratinocytes. 2HBA and 3HBA also have significant inhibition ability on tyrosinase at 1.0 mM, at which the melanin content was reduced to 55.9%, 53.7% and 55.3%, 49.4% respectively in melanocytes alone and in the co-culture. Arbutin also decreased tyrosinase activity and melanin content in melanocytes alone and had evident effects in co-culture models.

To investigate whether HBAs can affect pigment production with the presence of stimulating factors, melanocytes were co-treated with HBAs and α-MSH in cultured alone and co-culture models. The tyrosinase activity and melanin content could be greatly enhanced by α-MSH in both cases, as described ahead. However, both of melanin synthesis and tyrosinase activity in α-MSH-treated melanocytes were significantly inhibited by HBAs. (right three columns shown in Figure 4 and Figure 5) Moreover,

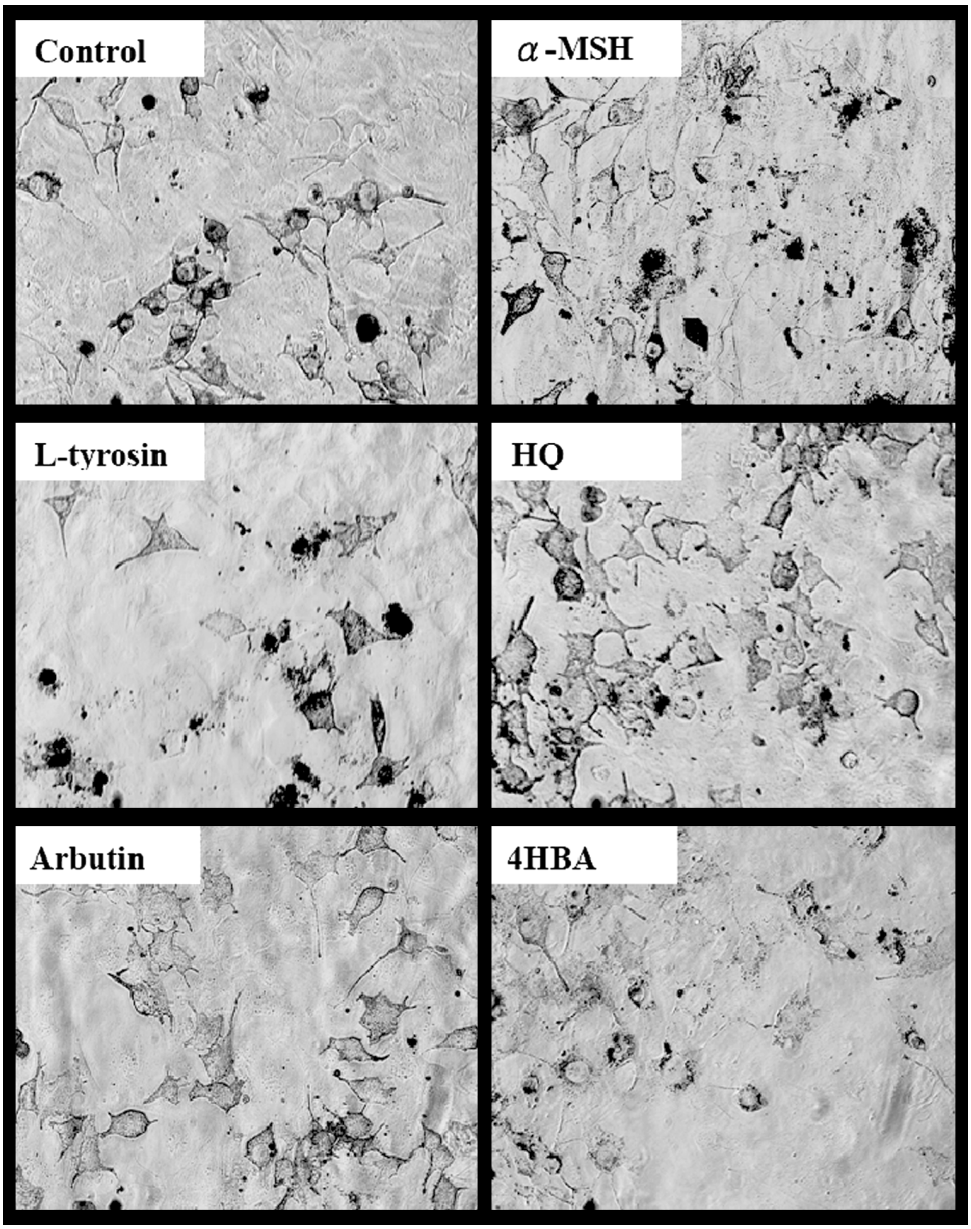

Figure 3. Bright-field images of B16 cells (co-cultured with keratinocytes) with 4 days exposure to 100 nM α-MSH, 1.0 mM L-tyrosine, 0.5 mM HQ, 1.0 mM arbutin, and 1.0 mM 4HBA (× 200). Co-cultures were described in Materials and Methods. HQ, hydroquinone.

the results showed that HBAs inhibited melanogenesis more effectively in co-culture model.

Effects of HBAs on cyclic AMP

α-MSH induced melanogenesis is coupled with increase level of cyclic AMP (cAMP). We measured intracellular cAMP levels in the presence or absence of HBAs. As shown in Figure 6, α-MSH led to threefold increase on cAMP levels over untreated controls, but the effect was significantly stronger when the melanocytes were co-cultured with keratinocytes. (about 13 fold) The data also show that HBAs had no effect on cAMP levels in melanocytes that culture alone or co-cultured with keratinocytes.

Effects of HBAs on the expression of the tyrosinase gene

The cellular RNA was extracted from cells incubated in the presence or absence of α-MSH and HBAs. The mRNA levels of MC1-R and tyrosinase showed no difference between HBAs-treated and non-treated cells in both melanocytes alone and co-culture cases. (see Figure 7). Treatment with α-MSH increased mRNA levels of MC1-R and tyrosinase in melanocytes compared with untreated control. Cells exposing to α-MSH and HBAs together produced similar patterns to that of α-MSH treated cells. The results indicated that α-MSH up-regulates the expression levels of MC1-R and tyrosinase mRNA, but HBAs has no effect on the expression of these mRNA in both melanocytes and co-cultured cells.

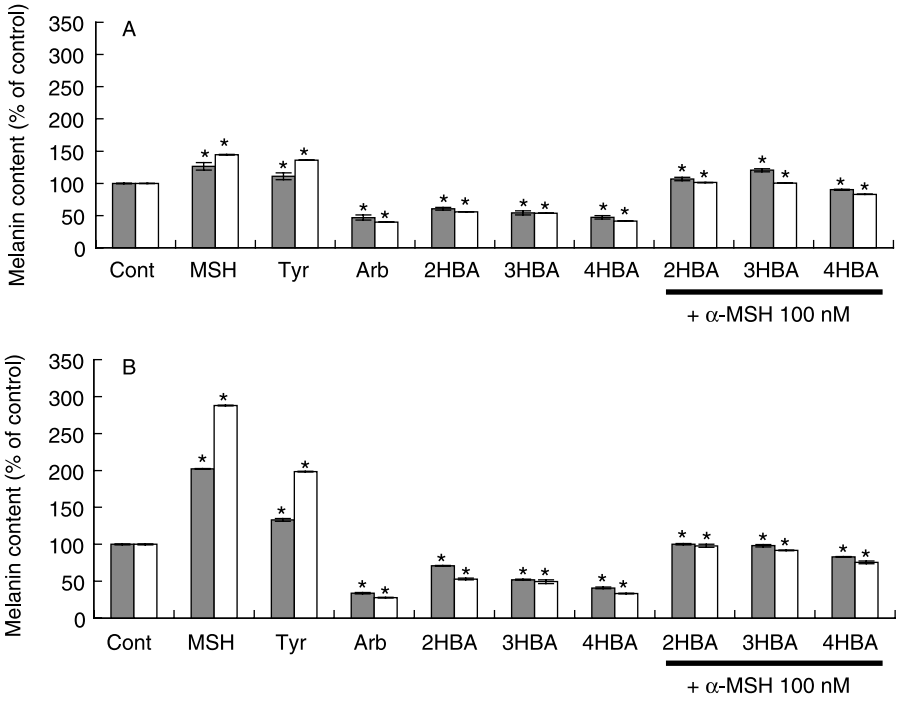

Figure 4. Melanin content in cultures of melanocytes with and without keratinocytes. B16 melanocytes alone (A) and co-cultured with Xb-2 keratinocytes (B) were treated for 4 days with the 0.5 mM (grey bars) or 1.0 mM (open bars) indicated compounds (L-tyrosine, arbutin, and HBAs). α-MSH was used as 50 (grey bars) and 100 nM (open bars). Cellular melanin content was measured as described in Materials and Methods. Bars represent means ± S.E. of at least three independent experiments. Significant differences were determined by Student's *t*-test; $\star P < 0.05$ compared to control. Cont, control; Tyr, L-tyrosine; Arb, arbutin.

Discussion

The aim of this study was to develop a melanocyte–keratinocyte co-culture model that can be induced to differentiate and investigate the effects of pigmentation regulators. Earlier studies have shown that the melanin production in melanocytes is influenced by keratinocytes and fibroblasts. [23,24] Primary human melanocytes and keratinocytes have some drawbacks such as the limited proliferate capacity and the donor variability. Thus the use of immortalized cell lines would be more suitable for investigating the effects of pigmentation regulators. B16 melanocytes have been used widely in the pigmentation research model for normal melanocytes behavior. Until now, there is no report on the interaction between B16 cells and murine keratinocytes in their co-culture model. The murine keratinocytes XB-2 was used in this study because it expressed similar stem cell factor (SCF) as human keratinocytes, which can associated with hyperfunction of melanocytes and is necessary for normal melanocyte function in the epidermis. [25–27]

In preliminary experiments, we found that an initial seeding ratio of 1:10 (melanocytes to keratinocytes) in DMEM (with 10% FBS) is ideal for maintaining the co-culture in 6 days or longer. When keratinocytes and melanocytes were co-seeded at the same time, the keratinocytes and melanocytes survived well and retained their normal morphology. We used melanogenic stimulators (α-MSH and L-tyrosine) and inhibitors (HQ, arbutin, and HBAs) to investigate their effects on pigmentation. The tyrosinase activity and melanin content increased when melanocytes were treated with α-MSH, and the effect was greater when the melanocytes were co-cultured with keratinocytes. Meanwhile, melanocytes treated with L-tyrosine also elicited the increase on melanin production, but tyrosinase activity was not enhanced. Earlier studies have indicated that α-MSH receptor (MC1-R) is expressed on melanocytes, keratinocytes and melanoma cells, [28] which cause keratinocytes synergistic effect on the regulation of melanogenesis by α-MSH. The findings in our work agree with their reports on α-MSH effect in the co-culture model. Recent studies further demonstrated that keratinocytes produce and secrete basic fibroblast growth factor (bFGF), endothelin-1, and other factors that can regulate morphology, proliferation and melanogenesis of melanocytes *in vivo*. [14,15,29–32]

Besides, tyrosinase is known to be a key enzyme for melanin biosynthesis in mammalian cells, and most of skin lightings show significant inhibitory activity to tyrosinase. To evaluate the inhibitory effects of HBAs on melanogenesis in the presence or absence of co-cultured keratinocytes, we compared the inhibitory effects of HBAs and arbutin on melanin formation and

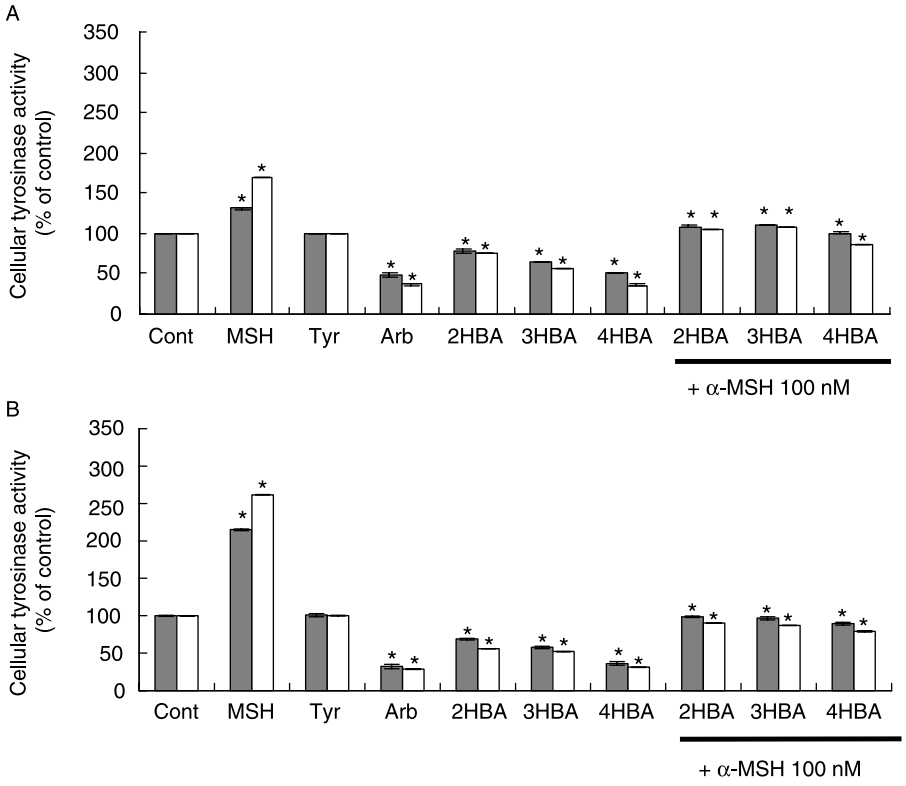

Figure 5. Tyrosinase activities in cultures of melanocytes with and without keratinocytes. B16 melanocytes alone (A) and co-cultured with Xb-2 keratinocytes (B) were treated for 4 days with the 0.5 mM (grey bars) or 1.0 mM (open bars) indicated compounds (L-tyrosine, arbutin, and HBAs). α-MSH was used as 50 (grey bars) and 100 nM (open bars). Cellular melanin content was measured as described in Materials and Methods. Bars represent means ± S.E. of at least three independent experiments. Significant differences were determined by Student's *t*-test; $\star P < 0.05$ compared to control. Cont, control; Tyr, L-tyrosine; Arb, arbutin.

tyrosinase activity in B16 melanocytes. In our study, HBAs showed a dose-dependent inhibitory effect on melanogenesis in melanocytes alone. 4HBA and arbutin showed stronger inhibitory effects on melanin content than did 2HBA and 3HBA. It should be mentioned that the inhibitory effects in melanocytes co-cultured with keratinocytes were more conspicuous. The higher inhibitory effects observed in the co-culture model disclosed that keratinocytes indeed relate with modulating melanocytes function and pigment production, no matter *in vivo* or *in vitro*.

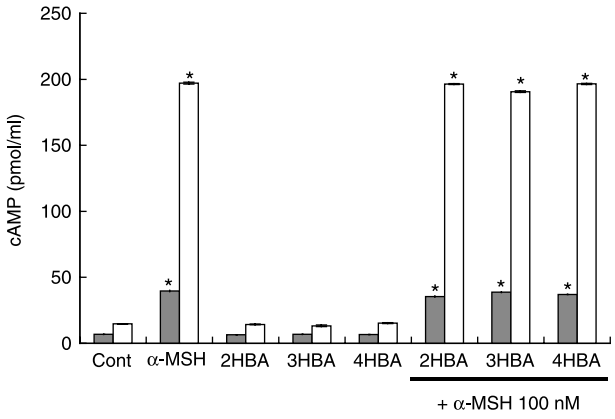

Figure 6. Cyclic AMP production of B16 cells after stimulating with α-MSH or HBAs. B16 melanocytes alone (grey bars) and co-cultured with Xb-2 keratinocytes (open bars) were treated with 1.0 mM HBAs in the presence or absence of α-MSH (100nM) for 4 days. Cyclic AMP concentration was measured as described in Materials and Methods. Bars represent means ± S.E. of at least three independent experiments. Significant differences were determined by Student's *t*-test; $\star P < 0.05$ compared to control.

The cytokines released by keratinocytes might regulate the melanogenesis. To test this hypothesis, we used α-MSH to investigate the pigmentation regulator. α-MSH is known to be an important cytokine involved in regulating the function of various mammalian cells. In the human skin, α-MSH is synthesized in keratinocytes and can increase the expression of MC1-R in melanocytes. Stimulation of MC1-R activates adenylate cyclase (results in increased levels of cAMP content), an accumulation of tyrosinase mRNA, and stimulation of tyrosinase activity. [33–35] Therefore, if we want to investigate how HBAs modulate pigment production in melanocytes, we can trace cAMP and MC1-R expression in culture and co-culture models with the presence of both HBAs and α-MSH. Our results indicated that tyrosinase activity, melanin content, and cAMP greatly stimulated by α-MSH in melanocytes alone and co-culture model. HBAs significantly inhibited

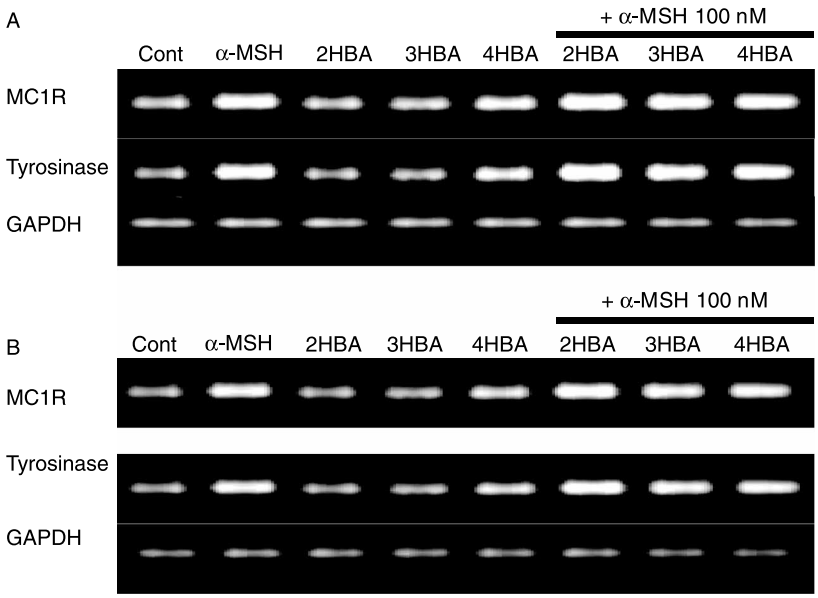

Figure 7. Effect of HBAs on MC1-R (melanocortin 1 receptor) and tyrosinase expression in B16 melanoma cells. B16 melanocytes alone (A) and co-cultured with Xb-2 keratinocytes (B) were treated with 1.0 mM HBAs in the presence or absence of α-MSH (100nM) for 4 days. Message RNA levels were measured by a reverse transcription polymerase chain reaction (RT-PCR) assay, as described in Materials and Methods. GAPDH = glyceraldehydes-3-phosphate dehydrogenase.

pigment production by α-MSH but had no effect on cAMP levels, expression level of MC1-R, and tyrosinase mRNA in melanocytes (alone and co-cultured with keratinocytes). Therefore, the suppression of melanin synthesis by HBAs was thus attributed to its behavior in inhibiting cellular tyrosinase activity rather than in the interference in gene expression and pigmentation regulator.

In this study, we demonstrated that HBAs possess an inhibitory effect on melanogenesis in murine melanocyte at noncytotoxic concentrations. The effects were significantly stronger when melanocytes were co-cultured with keratinocytes. To investigate the effect of pigmentation regulator in co-culture system, we used α-MSH to treat melanocytes. The melanin synthesis, cAMP, the expression level of MC1-R, and tyrosinase mRNA were markedly increased when treated with α-MSH in co-culture. These findings point to the better behavior of co-cultured murine cells, and the usefulness of this system for evaluating the regulation of pigmentation.

In summary, this study showed that keratinocytes may play a synergistic role in melanogenesis and may influence the pigment production. Co-culture model displays a more physiologically realistic condition to study the interaction between melanocytes and keratinocytes, which enables a reliable screening system for depigmenting compounds. We also proved that HBAs are effective agents for skin whitening in melanocytes alone and co-culture system, and may be capable of using in cosmetic products in the future.

References

[1] Prota G. Progress in the chemistry of melanias and related metabolites. Med Res Rev 1988;8:525–556.
[2] Pawelek JM, Korner AM. The biosynthesis of mammalian melanin. Am Sci 1982;70:136–145.
[3] Whitaker JR. Polyphenoloxidase. In: Wong DWS, editor. Food Enzymes. Structure and Mechanism. New York: Chapman & Hall; 1995.
[4] Funayama M, Arakawa H, Yamamoto R, Nishino Shin T, Murao S. Effects of a- and b-arbutin on activity of. tyrosinases from mushroom, and mouse melanoma. Biosci Biotechnol Biochem 1995;59:143–144.
[5] Giuseppe P. Melanins and Melongenesis. Cosmetics and Toiletries 1996;111:43–51.
[6] Kim YJ, Uyama H. Tyrosinase inhibitors from natural and synthetic sources: Structure, inhibition mechanism and perspective for the future. Cell Mol Life Sci 2005;62:1707–1723.
[7] Liu SH, Pan IH, Chu IM. Inhibitory effect of *p*-hydroxybenzyl alcohol on tyrosinase activity and melanogenesis. Bio Phar Bull 2007;30:1135–1139.
[8] Jimbow K, Quevedo WC, Prota G. Biology of melanocytes. In: Freedberg IM, Eisen AZ, Wolff K, editors. Fitzpatrick's dermatology in general medicine. 5th ed. New York: McGraw-Hill; 1999.
[9] Jimbow K. Current update and trends in melanin pigmentation and melanin biology. Keio J Med 1995;44:9–18.
[10] Hsu M, Andl T, Li G, Meinkoth JL, Herlyn M. Cadherin repertoire determines partner-specific gap junctional communication during melanoma progression. J Cell Sci 2000;113:1535–1542.
[11] Herlyn M, Shih IM. Interactions of melanocytes and melanoma cells with the microenvironment. Pigment Cell Res 1994;7:81–88.
[12] Kippenberger S, Bernd A, Bereiter-Hahn J, Ramirez-Bosca A, Kaufmann R. The mechanism of melanocyte dendrite formation: The impact of differentiating keratinocytes. Pigment Cell Res 1994;11:34–37.

[13] Jouneau A, Yu YQ, Pasdar M, Larue L. Plasticity of cadherin-catenin expression in the melanocyte lineage. Pigment Cell Res 2000;13:260–272.

[14] Gordon PR, Mansur CP, Gilchrest BA: Regulation of human melanocyte growth, dendricity and melanization by keratinocyte derived factors. J Invest Dermatol 1989;92:565–572.

[15] Imokawa G, Kobayashi T, Miyagishi M, Higashi K, Yada Y. The role of endothelin-1 in epidermal hyperpigmentation and signaling mechanisms of mitogenesis and melanogenesis. Pigment Cell Res 1997;10:218–228.

[16] Virador VM, Muller J, Wu X, Abdel-Malek ZA, Yu ZX, Ferrans VJ, Kobayashi N, Wakamatsu K, Ito S, Hammer JA, Hearing VJ. Influence of alpha-melanocyte-stimulating hormone and ultraviolet radiation on the transfer of melanosomes to keratinocytes. FASEB J 2002;16:105–107.

[17] Kippenberger S, Bernd A, Bereiter-Hahn J, Ramirez-Bosca A, Kaufmann R, Holzmann H. Transcription of melanogenesis enzymes in melanocytes: Dependence upon culture conditions and co-cultivation with keratinocytes. Pigment Cell Res 1996;9:179–184.

[18] Kim YM, Yun J, Lee CK, Lee H, Min KR, Kim Y. Oxyresveratrol and hydroxystilbene compounds. Inhibitory effect on tyrosinase and mechanism of action. J Biol Chem 2002;3:16340–16344.

[19] Kubo I, Kinst HI. Tyrosine inhibitors from cumin. J Agric Food Chem 1998;46:5338–5341.

[20] Rheinwald JG, Green H. Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. Cell J 6 1975. p 6317–6330

[21] Curto EV, Kwong C, Hermersdorfer H, Glatt H, Santis C, Virador V, Hearing VJ, Dooley TP. Inhibitors of mammalian melanocyte tyrosinase: *In vitro* comparisons of alkyl esters of gentisic acid with other putative inhibitors. Biochem Pharmacol 1999;57:663–672.

[22] Tomita Y, Maeda K, Tagami H. Melanocyte-stimulating properties of arachidonic acid metabolites: Possible role in postinflammatory pigmentation. Pigment Cell Res 1992;5:357–361.

[23] Hedley SJ, Layton C, Heaton M, Chakrabarty KH, Dawson RA, Gawkrodger DJ, MacNeil S. Fibroblasts play a regulatory role in the control of pigmentation in reconstructed human skin from skin types I and II. Pigment Cell Res 2002;15:49–56.

[24] Lei TC, Virador VM, Vieeira WD, Hearing VJ. A melanocyte-keratinocyte coculture model to assess regulators of pigmentation in vitro. Anal Biochem 2002;305:260–268.

[25] Kunisada T, Lu SZ, Yoshida H. Murine cutaneous mastocytosis and epidermal melanocytosis induced by keratinocyte expression of transgenic stem cell factor. J Exp Med 1998;187:1565–1573.

[26] Kunisada T, Yoshida H, Yamazaki H. Transgene expression of steel factor in the basal layer of epidermis promotes survival, proliferation, differentiation and migration of melanocyte precursors. Development 1998;125:2915–2923.

[27] Grichnik JM, Burch JA, Burchette J, Shea CR. The SCF/KIT pathway plays a. critical role in the control of normal human melanocyte homeostasis. J Invest Dermatol 1998;111: 233–238.

[28] Chakraborty AK, Funasaka Y, Pawelek JM, Nagahama M. Enhanced expression of melanocortin-1 receptor (MC1R) in normal human keratinocytes during differentiation: Evidence for increased expression of POMC peptides near suprabasal layer of epidermis. J Invest Dermatol 1999;112:853–860.

[29] Hirobe T. Basic fibroblast growth factor stimulates the sustained proliferation of mouse epidermal melanoblasts in a serum-free medium in the presence of dibutyryl cyclic AMP and keratinocytes. Development 1992;114:435–445.

[30] Hirobe T. Melanocyte stimulating hormone induces the differentiation of mouse epidermal melanocytes in serum-free culture. J Cell Physiol 1992;152:337–345.

[31] Hirobe T. Keratinocytes are involved in regulating the developmental changes in the proliferative activity of mouse epidermal melanoblasts in serum-free culture. Devel Biol 1994;161:59–69.

[32] Hirobe T. Endothelins are involved in regulating the proliferation and differentiation of mouse epidermal melanocytes in serum-free primary culture. J Invest Dermatol 2001;6:25–31.

[33] Bertolotto C, Abbe P, Hemesath TJ, Bille K, Fisher DE, Ortonne JP, Ballotti R. Microphthalmia gene product as a signal transducer in cAMP-induced differentiation of melanocytes. J Cell Biol 1998;142:827–835.

[34] Haddad MM, Xu W, Schwahn DJ. Activation of a cAMP pathway and induction of melanogenesis correlate with association of p16 (INK4) and p27 (KIP1) to CDKs, loss of E2F-binding activity, and premature senescence of human melanocytes. Exp Cell Res 1999;253:561–572.

[35] Goding CR. Mitf from neural crest to melanoma: Signal transduction and transcription in the melanocyte lineage. Genes Dev 2000;14:1712–1728.