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Effects of all-*trans* retinoic acid on melanogenesis in pigmented skin equivalents and monolayer culture of melanocytes

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Abstract

The effects of all-*trans* retinoic acid (RA) on melanogenesis and the mechanism of its action in topical treatment have not been elucidated. The purpose of this study was to determine the effects of RA on melanogenesis in the pigmented skin equivalent as well as in monolayer culture of melanocytes, and to determine whether RA, hydroquinone (HQ), and hydrocortisone (HC) show synergistic depigmenting effects in combined treatments of each other. The suppressing effect of RA on melanogenesis was not observed in pigmented skin equivalents and monolayer culture of murine and human melanocytes, although HQ showed strong inhibition of melanogenesis. The synergistic effects between RA, HQ, and HC were not particularly seen. The results suggested that RA neither has direct inhibitory effects on melanogenesis of melanocytes, nor influences the cell–cell interactions between melanocytes, keratinocytes and fibroblasts, such as paracrine actions with regard to melanin production. The role of RA in bleaching treatments appears to be in other specific actions, such as promotion of keratinocytes proliferation and acceleration of epidermal turnover.

Keywords: All-*trans* retinoic acid; Melanogenesis; Pigmented skin equivalents; Melanocytes

1. Introduction

All-*trans* retinoic acid (tretinoin; RA) has been clinically used for acne and photoaged skin for more than two decades. Topical application of RA is also known to be effective for melasma and some other skin hyperpigmentation in a single [1–4] or combined use [5–8] with some other

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reagents such as hydroquinone (HQ) and corticosteroids. Since Kligman et al. [5] proposed a combined use of RA, HQ and dexamethasone as a topical depigmenting formula, a number of products based on the formula have become commercially available and widely used for bleaching of hyperpigmented skin lesions.

Hydroquinone is the most widely used depigmenting agent at present and has been shown to inhibit tyrosinase-mediated conversion of tyrosine to dopa [9] and dopa to dopaquinone [10]. Corticosteroids are also known to have a depigmenting effect in single use [11], and this is one of the reasons why Kligman et al. [5] employed corticosteroid in his formula.

Although it is known that topical RA improves a variety of skin hyperpigmentation, the mechanism underlying these depigmenting effects of RA has not been elucidated. Furthermore, although a number of studies have been performed on effects of RA on pigment cells, the experimental results remain contradictory [12]. Some studies with mouse melanoma cell lines and human melanocytes indicated a pigmentation-promoting effect of RA [13–16]. On the other hand, there was no melanogenic effect on K-1735P ultraviolet-irradiated, transformed murine melanoma cells, which remained amelanotic following RA treatment [17]. Under conditions which supported proliferation, RA at concentrations of 0.25–0.1 μg/ml inhibited growth of human melanocytes, while there was no significant change in the amount of melanin per cell or in tyrosinase activity [18]. It is known that tyrosinase gene expression and activity, if already stimulated by MSH, is inhibited by RA in murine and hamster melanoma cells [19], whereas RA treatment caused a marked increase in MSH binding capacity for both cell surface and internal MSH binding sites [20]. Another report indicated that RA significantly decreased the UVB-stimulated melanogenesis through suppression of tyrosinase and TRP-1 synthesis at the post-transcriptional level in mouse melanoma cells and human melanocytes [21]. Thus, it is difficult to establish an unequivocal effect of RA on melanogenesis in pigment cells.

Several growth factors have been identified as the paracrine factors from keratinocytes and fibroblasts, showing effects on melanocyte proliferation and functions [22–25]. Three-dimensional pigmented skin equivalent is a useful strategy to investigate the cell–cell interactions in the regulation of in vivo melanogenesis, and the effects of RA has never been examined with the pigmented skin equivalent. The purpose of this study is to determine the effects of RA on melanocytes in monolayer culture and also in pigmented skin equivalents consisting of melanocytes and keratinocytes grown on a dermal equivalent, and to determine whether RA and/or HC show synergistic depigmenting effects in combined use with HQ.

2. Materials and method

2.1. Melanocyte culture

The murine melanocyte cell lines, melan-a, and conditions for its culture have been described previously [26]. Melan-a was cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 5% fetal calf serum (FCS), 100 μM β-mercaptoethanol, 2 mM L-gultamine, and 200 nM TPA (tumor promoter 12-*O*-tetradecanoylphorbol acetate). Normal human melanocytes obtained from Asian foreskin and serum-free growth medium were purchased from Morinaga Institute of Biological Science (Yokohama, Japan). Human melanocytes were cultured in MM-4 medium [27] supplemented with 10 μg/l phorbol-12-myristate 13-acetate, 10 μg/l cholera toxin, and 150 mg/l bovine pituitary extract.

RA was obtained from Sigma (St. Louis, MO), dissolved at 10^{-3} M in ethanol, and kept in foil-wrapped containers protected from light at 4 °C. HQ and hydrocortisone (HC) were obtained from Wako (Osaka, Japan).

Murine melanocytes (melan-a) or human melanocytes were seeded in 3.5 cm dishes (1.5×10^5 cells/dish) for melanin and protein assay. Human melanocytes were cultured in MM-4 with 0.5% serum for 24 h, after which the medium was exchanged for MM-4 without serum. Melan-a was

cultured in DMEM with TPA for 2 days, after which the medium was exchanged for DMEM without TPA. After murine or human melanocytes had been cultured for 4 days, every assay was performed. RA (10^{-6} M), HQ (10^{-5} M), and HC (10^{-4} M) were individually or in combination of each other added to each medium for 4 days, and seven kinds of culture groups were prepared as follows; none (control), RA alone (RA), HQ alone (HQ), HC alone (HC), RA + HQ, RA + HC, HQ + HC, and RA + HQ + HC. At least four data were collected for each group.

2.2. Pigmented skin equivalents

Human keratinocytes and fibroblasts were isolated from skin sections obtained from skin surgery for young patients. Human keratinocytes were grown in a modified serum-free KGM (Kyokuto Seiyaku, Tokyo), which consists of MCDB153 with high concentrations of amino acids, transferrin (final concentration, 10 μg/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), phosphorylethanolamine (14.1 μg/ml) and bovine pituitary extract (40 μg/ml). Human fibroblasts were grown in DMEM supplemented with 10% FCS. Keratinocytes and fibroblasts at population doubling levels of 2–4 and 10–15, respectively, were used for experiments.

Human keratinocytes and murine melanocytes were cultured in a three-dimensional fashion at the air–liquid interface on top of a dermal equivalent consisting of cultured fibroblasts (10^6/gel) and type I collagen. The dermal equivalents (contracted collagen gels) were prepared according to the method described by Tsunenaga et al. [28]. Murine melanocytes were plated at $2 \times 10^5/cm^2$ inside a glass ring (12 mm diameter) on the surface of the dermal equivalent, which was then placed on a stainless steel mesh. Melanocytes were grown in DMEM plus 10% FCS on day 1. On day 2, keratinocytes were additionally plated at $8 \times 10^5/cm^2$ inside the glass ring on the dermal equivalent, and the medium was changed to 1:1 mixture of KGM and DMEM plus 10% FCS, in which the Ca^{2+} concentration was adjusted to 0.18 mM. After day 4, the medium in the glass ring was removed and the surface of the dermal equivalent on which keratinocytes and melanocytes were cultured were allowed to face the air. The discharged medium in the ring was aspired twice a day after day 4. The medium in the dish was changed every other day. The medium including the designated compounds was used from day 4. On day 9, skin equivalents were fixed with 4% paraformaldehyde in PBS.

2.3. Measurement of pigment on the skin equivalents

The melanin pigments of each sample were photographed and each digital image was analyzed with Image-Pro Plus (version 3.0, Media Cybernetics, Silver Spring, MA). Difference between an original image and its extracted background was estimated as the melanin pigments, and area and intensity of each pigment were measured. Total sum of melanin pigments data of each sample was referred to as 'total melanin' of individual samples in this paper.

2.4. Melanin and protein assay

Melanin content was determined according to the method described by Oikawa and Nakayasu [29], which we modified. Briefly, melan-a or human melanocytes were cultured at 37 °C for 4 days with the various types of medium. Cell pellets were lysed in 1.0 ml extraction buffer (50 mM Tris buffer; pH 7.5, 2 mM EDTA, 150 mM NaCl, and 1% Triton-X). Supernatants were used for protein assay, which was performed with a BCA protein assay kit (Pierce, Rockford, IL). After resuspending melanin pellets in 1.0 ml extraction buffer and centrifuging, the melanin pellets were incubated with 0.5 ml 2 N NaOH containing melanin 20% DMSO for 30 min. The optical absorbance of each sample was measured at 470 nm with an ELISA plate reader (model 550 microplate reader, Bio-Rad Laboratories, CA).

2.5. Tyrosinase assay

Compounds were tested for direct effects on tyrosinase activity using a modified radiometric tyrosinase assay as previously described [30].

Briefly, the melanogenic tyrosinase assay was performed in quadruplicate in 96-well microtiter plates by adding 10 μm compound and 20 μl purified murine or human tyrosinase, in that order. After 30 min preincubation at 23 °C, 10 μl L-[^{14}C]tyrosine was added along with 10 μl 0.25 mM L-dopa cofactor in 1 M sodium phosphate buffer, pH 7.2, containing 0.01% albumin. Reactions were incubated for 1 h at 37 °C, after which 100 μl 0.1 M HCl with excess unlabelled L-tyrosine was added to each well. The contents of each well were removed with a multichannel pipettor to a dot-blot apparatus (Bio-Rad, Hercules, CA) and acid-insoluble radioactive melanin and melanin precursors were bound to ZetaProbe blotting membranes (Pharmacia, Piscataway, NJ) for 15 min at 23 °C. The membranes were then dried under vacuum and washed three times with 250 μl 0.1 M HCl with excess unlabelled tyrosine; they were then removed from the apparatus and washed three more times for 20 min each with 100 ml 0.1 M HCl. Membranes were then air-dried and exposed to a Storm phosphor screen; quantitation of radioactive melanin production on those blots was performed using a Strom 860 PhosphorImager and ImageQuant software (Molecular Dynamics).

2.6. Statistics

Significant differences were sought using two way analysis of variance (ANOVA). Post hoc comparison of individual group was performed only if the F ratio for the overall ANOVA was significant; appropriate Bonferonni corrections were applied to all post hoc comparisons. Differences were considered significant when P-values were < 0.05.

3. Results

3.1. Melanin pigments on pigmented skin equivalents

The representative histology of the control sample was shown in Fig. 1, and representative samples of each group were demonstrated in Fig. 2. Total melanin was significantly reduced in HQ-, RA + HQ-, HQ + HC-, and RA + HQ + HC-treated groups (Fig. 3). Total melanin of RA-treated group was elevated by 10% in average, but the difference was not statistically significant. Total melanin was significantly less in HQ-, HC-, RA + HQ-, RA + HC-, HQ + HC-, and RA + HQ + HC-treated groups than in RA-treated group.

3.2. Melanin content and total protein in murine melanocytes

Total protein in any group except for the RA + HQ + HC-treated group was not significantly different from that in the control (Fig. 4). Total melanin (absolute value) did not show a large change after RA-, HC-, or RA + HC-treatment. The relative melanin in RA-, HC-, and RA + HC-treated groups was elevated by 10–35% in average value, but the difference was not statistically significant. The absolute and relative values of melanin were significantly reduced in HQ-, RA + HQ-, and RA + HQ + HC-treated groups.

3.3. Melanin content and total protein in human melanocytes

Total protein in any group was not significantly different from that in the control (Fig. 5). In the RA-treated group, the absolute and relative melanin was elevated by 20–30% in average

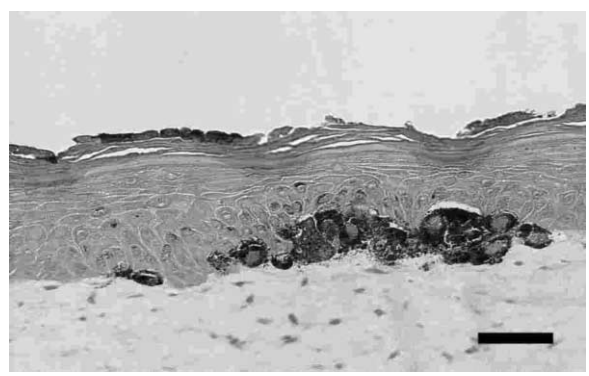

Fig. 1. Histological view of pigmented skin equivalents (one of the control samples) at the site of intense pigmentation. Bar = 50 μm.

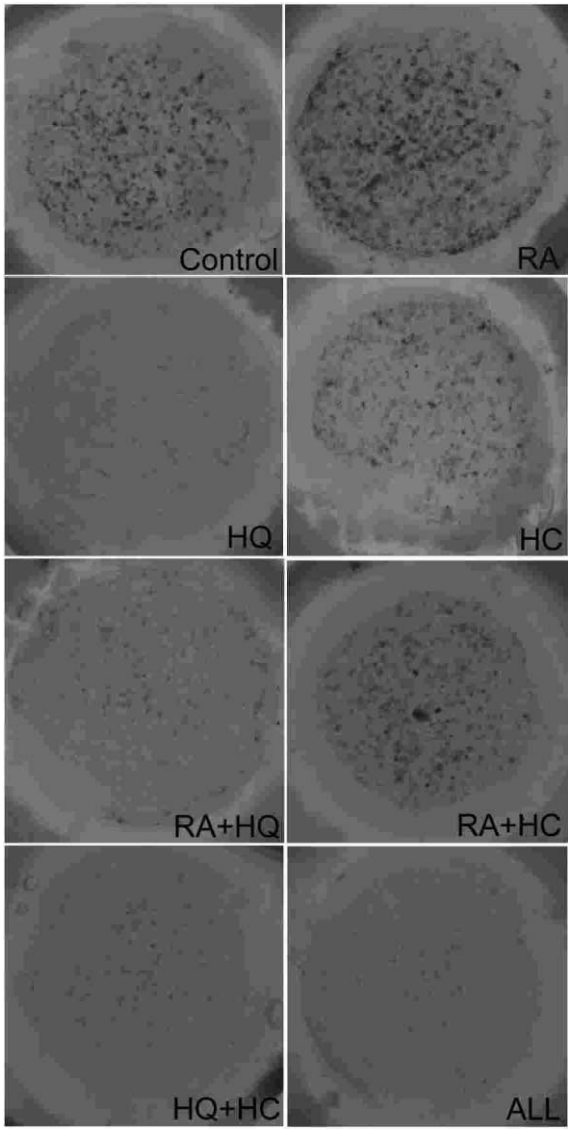

Fig. 2. Representative samples of pigmented skin equivalents. Pigmented spots were produced on the dermal equivalents inside glass rings (12 mm in diameter).

value, nevertheless without statistical significance. The absolute and relative values of melanin were significantly reduced in HQ- and RA + HQ-treated groups.

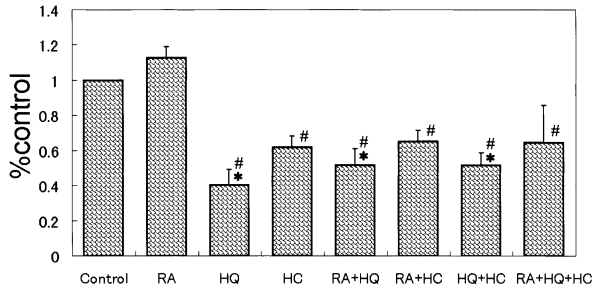

Fig. 3. Melanin production on pigmented skin equivalents measured with a computed image analyser. Values were shown as mean + S.E. $*P < 0.05$ to control, $\# P < 0.05$ to RA.

3.4. Tyrosinase activity in murine melanocytes

HQ had an obvious inhibition on tyrosinase activity up to 70% (Fig. 6). RA and HC also had also slight inhibition on tyrosinase activity. Synergistic effects of three compounds were not seen in this study.

3.5. Tyrosinase activity in human melanocytes

RA appeared to show no suppressing effects (Fig. 7). HQ showed a 60% decrease in melanogenic activity. HC also showed a decrease in melanogenic activity, but no synergistic effects in the three compounds.

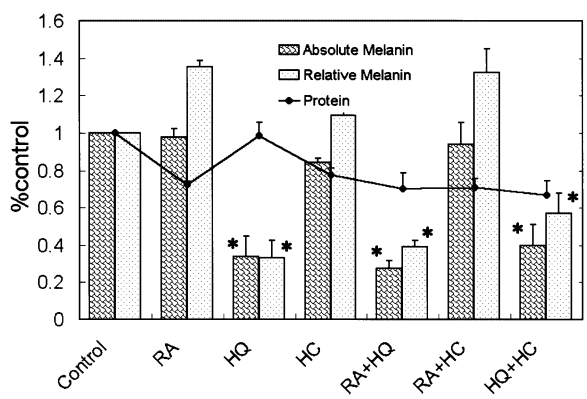

Fig. 4. Melanin and protein assay in monolayer culture of murine melanocytes (melan-a). The relative values to control were shown as mean + S.E. Relative melanin was calculated as (absolute melanin)/(protein). $*P < 0.05$ to control.

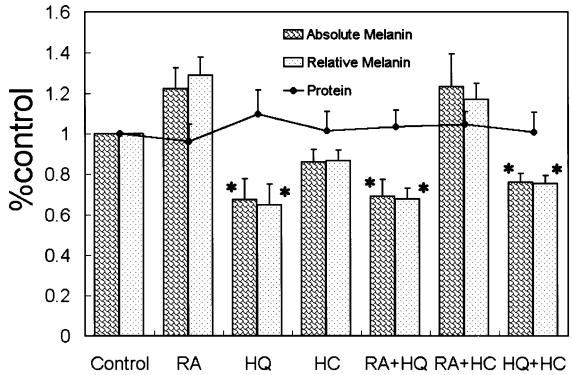

Fig. 5. Melanin and protein assay in monolayer culture of human melanocytes. The relative values to control were shown as mean + S.E. Relative melanin was calculated as (absolute melanin)/(protein). $*P < 0.05$ to control.

4. Discussion

The mechanisms by which RA and HQ act in the combined bleaching protocols such as in Kligman's regimen and others are still to be elucidated. Although a number of studies have been performed concerning the effects of RA on skin in vivo or in vitro, there are some contradictory results the reasons for which remain unknown [31–33]. Although it is reported that even the topical application of RA alone has a clinically depigmenting effect [1–3], the suppressive effects of RA on melanocyte growth and melanogenesis have not been established in vitro [12]. Welsh et al. [34] reported that the topical application of RA to mouse skin increases the number of activated epidermal melanocytes and makes melanocytes more sensitive to activation by ultraviolet B radiation.

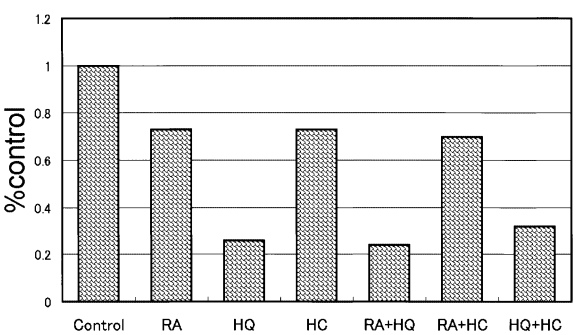

Fig. 6. Effects on tyrosinase activity in monolayer culture of murine melanocytes (melan-a).

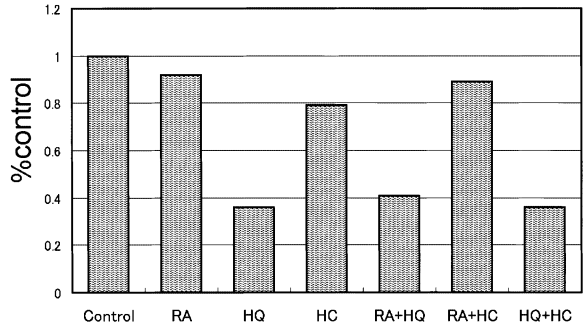

Fig. 7. Effects on tyrosinase activity in monolayer culture of human melanocytes.

In the present study, suppressing effects of RA on melanogenesis of melanocytes were not observed both in monolayer culture and organotypic culture. Melanin production per cell was not significantly changed in the monolayer culture of the murine melanocyte cell line and human melanocytes, although proliferation of melanocytes and tyrosinase activity were somewhat suppressed by RA treatment in the monolayer culture of murine melanocytes. In pigmented organotypic culture consisting of murine melanocytes and human keratinocytes grown on a dermal equivalent, melanogenesis was even promoted, although the difference in total melanin production between the control and RA-treated groups was not statistically significant. The results suggested that RA neither has direct inhibitory effects on melanogenesis of melanocytes, nor influences the cell–cell direct and indirect interactions between melanocytes, keratinocytes and fibroblasts in a paracrine manner with regard to melanin production.

In the monolayer culture of murine and human melanocytes, all experimental groups with treatment of HQ suppressed melanin production and tyrosinase activity. HC did not show apparent suppressing effects on melanogenesis and tyrosinase activity in the monolayer culture. Neither RA nor HC show synergistic suppressing effects of HQ on melanogenesis and tyrosinase activity.

The results of the present study suggested that RA does not have suppressing effects on melanogenesis of melanocytes in spite of its clinical whitening efficacy on some pigmented skin lesions, nor synergistic suppressive effects with HQ. Therefore, it is suggested that the depigmenting effects in vivo of RA in a single or combined use with HQ and/or HC are derived from other mechanisms than direct effects on melanocytes. A recent report suggested a role for cellular retinoic acid binding protein (CRABP)-I in mediating RA effects on melanogenesis and involvement of keratinocytic and dermal influences on CRABP activity in melanocytes [35]. However, in our pigmented skin equivalents, in which interaction between keratinocytes, melanocytes and fibroblasts can be examined, inhibitory effects of RA on melanogenesis were not observed.

RA promotes not only proliferation but also turnover of keratinocytes in vivo, and compaction of the horny layer and hyperplasia of the epidermis are characteristic changes after the topical application of RA [31]. It is also known that RA can promote collagenogenesis in dermis and wound healing [36,37]. On the other hand, skin becomes atrophic after the application of corticosteroid [38,39] and corticosteroid suppresses collagenogenesis and wound healing [40]. Thus, corticosteroid appears to be antagonistic in skin to retinoids in some aspects [36,37,39,41]. In our clinical experiences, depigmenting effects of a combined treatment of RA and HQ were suppressed by corticosteroid [7,8], although corticosteroids are known to have a depigmenting effect with single use [11]. Taken together, it is speculated that the promotion of keratinocyte proliferation and acceleration of keratinocyte differentiation are most likely to be the roles of RA in the depigmenting treatment. Melanin granules may be washed out of the epidermis by the fast and strong stream of keratinocytes in the epidermis induced by the mechanism above. These effects could not be reproduced in our pigmented skin equivalents, which could be cultured for only 1 week after they were exposed to air.

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