Regulation of Melanin Synthesis of B16 Mouse Melanoma Cells by 1α,25-Dihydroxyvitamin D₃ and Retinoic Acid

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ABSTRACT

Melanin synthesis of B16 mouse melanoma cells was found to be stimulated dose and time dependently by 1α,25-dihydroxyvitamin D₃ [1α,25(OH)₂D₃], the hormonal form of vitamin D₃. The stimulation of melanogenesis resulted from an increase in the activity of tyrosinase, a key enzyme in melanin synthesis. The minimum dose required for this stimulation was as low as 0.05 ng/ml, or 0.12 nm, a physiological level of plasma 1α,25(OH)₂D₃. The stimulation by 1α,25(OH)₂D₃ was specific; other derivatives of vitamin D₃ caused no stimulation at a concentration of 500 ng/ml. When the cells were plated on agar plates, the proportion of dark or black colonies was not increased by the exposure to 1α,25(OH)₂D₃. Furthermore, this compound did not induce melanin synthesis of an amelanotic variant. Its stimulatory effect seemed to be due to stimulation of melanin synthesis of melanotic cells, rather than to conversion of amelanotic clones to melanotic ones. 1α,25(OH)₂D₃ did not induce intracellular cyclic adenosine 3':5'-monophosphate, while cholela toxin induced cyclic adenosine 3':5'-monophosphate and stimulated melanin synthesis and tyrosinase activity much more than did 1α,25(OH)₂D₃, suggesting that 1α,25(OH)₂D₃ stimulates melanin synthesis by a cyclic adenosine 3':5'-monophosphate-independent mechanism.

B16 melanoma cells contained specific receptors for 1α,25(OH)₂D₃. Scatchard plot analysis revealed two types of receptor: the high-affinity receptor had a Kᵦ of 18.3 pm and an Nₘₐₓ of 10.6 fmol/mg of protein. The specificity of receptor binding was demonstrated by studies showing that, for 50% displacement of 1α,25(OH)₂D₃ binding, other derivatives were required at 500 times higher concentrations or more.

In contrast to 1α,25(OH)₂D₃, retinoic acid inhibited melanin synthesis and tyrosinase activity of B16 melanoma cells dose and time dependently. On simultaneous treatment, 1α,25(OH)₂D₃ and retinoic acid caused mutual interference, and a balance between their respective stimulating and inhibitory effects was obtained at a molar ratio of 10:1; i.e., with 10 nm 1α,25(OH)₂D₃ and 1 nm retinoic acid.

INTRODUCTION

It is now well established that vitamin D₃ is metabolized first in the liver to 25(OH)D₃ and then in the kidney, mainly to either 24R,25(OH)₂D₃ or 1α,25(OH)₂D₃ (10). Of these metabolites, 1α,25(OH)₂D₃ is considered to be the hormonal form of the vitamin in regulating the blood Ca²⁺ level by enhancing intestinal calcium transport and bone mineral mobilization (10).

A receptor with high affinity for 1α,25(OH)₂D₃ has been detected in a wide variety of tissues and cells including cancer cells and cultured cell lines (Refs. 5, 7 to 9, 11 to 15, 19, 28, and 33 to 36; for a review, see Refs. 10 and 23). The existence of the receptor suggests that these tissues or cells may be targets for 1α,25(OH)₂D₃, although the physiological functions of this compound have been demonstrated only in certain tissues (10).

Recently, we have been studying the regulation of growth, differentiation, and tumor promotion by 1α,25(OH)₂D₃ (23). We found that 1α,25(OH)₂D₃ stimulates differentiation of the myeloid leukemic cells of humans (27, 35) and mice (1) and the epidermal keratinocytes of mice (14). This naturally occurring hormone was found to induce fusion of mouse alveolar macrophages by a direct mechanism and by an indirect mechanism mediated by spleen cells (2). We also found that this vitamin markedly enhances chemically induced transformation of BALB 3T3 cells (22) but that it paradoxically inhibits induction of ornithine decarboxylase caused by 12-O-tetradecanoylphorbol-13-acetate and teleocidin B (6). Its antitumor-promoting activity in skin carcinogenesis was reported by Wood et al. (39).

As for these collaborative studies, we report here that 1α,25(OH)₂D₃ stimulates melanin synthesis in B16 melanoma cells and that its effect is counteracted by another liposoluble vitamin, retinoic acid.

MATERIALS AND METHODS

Chemicals. Vitamin D₃ derivatives, i.e., 1α,25(OH)₂D₃, 24R,25(OH)₂D₃, 25(OH)D₃, and 1α(OH)D₃, were kindly donated by I. Matsunaga, Chugai Pharmaceutical Co. (Tokyo, Japan). They were dissolved in ethanol. All trans-retinoic acid was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in acetone. Cholela toxin was purchased from the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). [23,24,25]-H]-1α,25(OH)₂D₃ (specific activity, 85 Ci/mmol) and [3,5,6]-H]-tyrosine (specific activity, 54.6 Ci/mmol) were purchased from Amersham International, Ltd. (Amersham, Buckinghamshire, United Kingdom). All other compounds were of the purest grade available.

Cell Culture. A subline of B16 mouse melanoma cells, C2M (31), was provided by Dr. A. Okawa, Tohoku University. The cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO₂ in air. Colonies were formed on 0.5% agar plates (20) by plating 1000 cells and incubating them for 2 weeks. White and dark colonies were counted under a dissecting microscope. An amelanotic variant of B16 melanoma cells, H9 (16), was provided by Dr. K. Iwata, Central Research Laboratory, Japan Tobacco and Salt Public Corporation.

Measurement of Cytotoxic and Cytostatic Effects. Cytotoxic effects of 1α,25(OH)₂D₃ or retinoic acid were measured by plating 100 cells in a 60-mm Petri dish and adding the test compound 18 to 24 hr later (Day
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1. The cultures were fixed and stained with Giemsa on Day 7 for examination of plating efficiency. In agar plate culture, the cells were plated directly on an agar layer containing 1α,25(OH)2D3. For examination of the cytostatic effect, 5 x 10^6 cells were plated in a 35-mm Petri dish, and the test compound was added 18 to 24 hr later. Medium containing the compound was renewed on Day 3. The number of living cells was counted on Day 5 by dye exclusion test using 0.01% erythrocin B.

Measurement of Melanin Content. The melanin content was measured by a modification of the method of Oikawa and Nakayasu (29) and Meykens and Fuller (26). Cells grown in a 100-mm Petri dish were washed twice with phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 0.2 g KH2PO4, and 1.15 g Na2HPO4.2H2O per liter), scraped off with a rubber spatula, centrifuged at 300 x g for 10 min, and stock in a freezer until measurement. The cells were sonicated in 1 ml of water on ice, and the mixture was centrifuged at 1200 x g for 20 min. The supernatant was decanted, and acid-insoluble materials were recovered by 2 precipitations with 10% trichloroacetic acid and one washing with ethanol. The precipitate was dried and solubilized by treatment with 1 ml of 1 N NaOH and quickly with ice-cold phosphate-buffered saline, treated with 6% perchloric acid, and scraped off with a rubber spatula. The acid-soluble fraction was centrifuged, and the supernatant was treated with 50 mg of Norit SX3. Radioactivity of tritiated water derived from L-tyrosine was counted in a liquid scintillation counter.

Measurement of Intracellular cAMP. Intracellular cAMP was measured after succinylation of the cyclic nucleotides by radioimmunoassay with a kit from Yamasa Shoyu Co. (Chiba, Japan). The cells were washed quickly with ice-cold phosphate-buffered saline, treated with 6% perchloric acid, and scraped off with a rubber spatula. The acid-soluble fraction was sonicated and subjected to radioimmunoassay as described elsewhere (21).

Assay of 1α,25(OH)2D3 Receptor. The cytosol fraction of B16 melanoma cells was prepared and incubated with 0.015 to 1.0 nm [3H]-1α,25(OH)2D3 at 25°C for 60 min in buffer containing 0.3 μM KCl, 2 mM EDTA, 0.5 mM dithiothreitol, and 10 mM Tris-HCl (pH 7.4). Bound and free [3H]-1α,25(OH)2D3 were separated on hydroxyapatite (37). Details of the procedure were reported elsewhere (14).

RESULTS

Inhibition of Growth by 1α,25(OH)2D3. 1α,25(OH)2D3 slightly inhibited colony formation of B16 melanoma cells when added 18 to 24 hr after the cells were plated; at 5 ng/ml, it suppressed colony formation by 9%. Although 1α,25(OH)2D3 showed little cytotoxicity, it suppressed the growth dose dependently. It had a negligible cytostatic effect at 0.05 ng/ml but caused about 30% inhibition of the growth at 5 ng/ml on Day 5.

In the following experiments, 1α,25(OH)2D3 doses of 0.05 to 5 ng/ml were used because the physiological level in plasma is about 0.05 ng/ml (4) and because at these concentrations 1α,25(OH)2D3 was only moderately toxic, and we had found previously that it induced differentiation of mouse epidermal keratinocytes and of mouse and human myeloid leukemia cells (1, 14, 27, 35).

Stimulation of Melanin Synthesis by 1α,25(OH)2D3. Chart 1A shows time-dependent stimulation of melanin synthesis by 1α,25(OH)2D3. To avoid possible effects of the length of culture period on melanin synthesis, B16 melanoma cells were cultured for the same period, i.e., 6 days in all experimental groups, and 1α,25(OH)2D3 (4 ng/ml) was added for the indicated periods during the last part of cultivation. Melanin synthesis was stimulated time dependently, reaching a maximum of about 3-fold the control level on treatment for 4 days. Chart 1B shows the dose-dependent stimulation of melanin synthesis on exposure for 4 days to 1α,25(OH)2D3 at 0.05 to 5 ng/ml. Even 0.05 ng/ml caused a significant increase. Some variation in the extent of the stimulation was observed among experiments. The stimulation was 2.1-fold on the average (SD, 0.5; range, 1.4 to 3.2) in 9 independent experiments in which 4 or 5 ng of 1α,25(OH)2D3 per ml were added for 4 days.

Melanin synthesis is catalyzed by tyrosinase which oxidizes tyrosine to dopaquinone and converts 5,6-dihydroxyindole to melanochrome (18). Therefore, tyrosinase is considered as a key enzyme in melanin synthesis. As shown in Chart 2, the tyrosinase activity of B16 melanoma cells was stimulated by 1α,25(OH)2D3 dose dependently at 0.05 to 5.0 ng/ml. This result suggests that...
1α,25(OH)2D3 stimulates melanin synthesis through activation of tyrosinase.

For assessment of the specificity of the effect of 1α,25(OH)2D3, B16 melanoma cells were cultured in the presence of other derivatives of vitamin D3, i.e., 25(OH)D3, a precursor of 1α,25(OH)2D3 formed in the liver; 24R,25(OH)2D3, a metabolite formed in the kidney in parallel with 1α,25(OH)2D3; and 1α(OH)D3, a synthetic derivative of vitamin D3. No increase in tyrosinase activity was observed with these derivatives at concentrations of 500 ng/ml (Chart 2).

The population of B16 melanoma cells is heterogeneous with respect to melanin synthesis; when plated on the surface of agar plates, white colonies and colonies showing various degrees of melanin synthesis were observed under a dissecting microscope. Stimulation of melanin synthesis by 1α,25(OH)2D3 might therefore be due to stimulation of melanin synthesis of melanotic clones and/or population change by conversion of amelanotic clones to melanotic ones. For examination of these possibilities, B16 melanoma cells were plated on agar plates containing 1α,25(OH)2D3 at concentrations of 0.05 to 5 ng/ml, and dark and white colonies were counted. As seen in Table 1, colony formation decreased dose dependently to a greater degree than in liquid medium as described above. This toxicity might have been due to plating of the cells directly on plates containing 1α,25(OH)2D3. It was found that 1α,25(OH)2D3 did not increase the proportion of melanin-synthesizing clones. The majority (54 to 69%) of colonies were dark in both the absence and presence of 1α,25(OH)2D3. Consistent with this observation, we found that exposure of cells of an amelanotic variant (H9) of B16 melanoma cells to 1α,25(OH)2D3 (5 ng/ml) for 4 days did not induce melanin synthesis. These results provide evidence for the first possibility that 1α,25(OH)2D3 stimulates melanin synthesis of melanotic cells rather than of amelanotic cells.

Intracellular cAMP Level after Treatment with 1α,25(OH)2D3. The amount of intracellular cAMP was measured by radioimmunoaassay after exposure of the cells to 1α,25(OH)2D3 (5 ng/ml). During incubation for 6 h, there was no increase in the intracellular cAMP level, which remained at 0.8 pmol/mg protein. In contrast, addition of cholera toxin (1 ng/ml) markedly increased the amount of cAMP, and a level of 35 pmol per mg protein, 44-fold the control value, was observed after 3 h incubation. Cholera toxin also stimulated melanin synthesis to a greater extent than did 1α,25(OH)2D3. The stimulation was dose dependent at concentrations above 0.01 ng/ml, and about a 6-fold increase was obtained at 1 ng/ml. These results indicate that there are 2 mechanisms of stimulation of melanin synthesis, cAMP-dependent and -independent mechanisms, and that 1α,25(OH)2D3 affects the latter.

### Table 1

<table>
<thead>
<tr>
<th>1α,25(OH)2D3 (ng/ml)</th>
<th>Plating efficiency</th>
<th>No. of colonies&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Proportion of black/dark colonies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>26.2 ± 1.7</td>
<td>91.0</td>
<td>79.2</td>
</tr>
<tr>
<td>0.05</td>
<td>25.7 ± 1.7</td>
<td>97.0</td>
<td>72.0</td>
</tr>
<tr>
<td>0.5</td>
<td>21.1 ± 3.1</td>
<td>134.8</td>
<td>67.2</td>
</tr>
<tr>
<td>5.0</td>
<td>10.1 ± 3.1</td>
<td>46.8</td>
<td>53.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average for 6 dishes.

<sup>b</sup> Mean ± SD.

Presence of Specific Receptors for 1α,25(OH)2D3. The specific receptor for 1α,25(OH)2D3 was assayed by incubating cortisol with [3H]-1α,25(OH)2D3 and then separating the bound and free vitamin. Binding was corrected for nonspecific binding, which was determined by parallel incubation with a large excess of cold 1α,25(OH)2D3. This assay was repeated 3 times, and a typical result is shown in Chart 3. Specific binding increased with increase in the dose of radioactive vitamin but did not reach a plateau at a concentration of 1 nM (Chart 3A). This specific binding was examined by Scatchard analysis. Two straight lines were obtained (Chart 3B). There are 2 types of receptor in B16 melanoma cells: a high-affinity receptor with a $K_d$ of 18.3 pm and $N_{max}$ of 10.6 fmol/mg protein; and a low-affinity receptor with a $K_d$ of 287 pm and $N_{max}$ of 19.7 fmol/mg protein. The $N_{max}$ of the high-affinity receptor is of the same order as but smaller than those obtained for epidermal keratinocytes (14), BALB 3T3 cells (22), and myeloid leukemia cells (35).

The specificity of the binding to the high-affinity receptor was assessed by testing the competitive binding of other vitamin D3 derivatives; i.e., 25(OH)D3, 1α(OH)D3, and 24R,25(OH)2D3. As seen in Chart 4, these derivatives bound to the receptor but had much lower affinities than did 1α,25(OH)2D3. Concentrations of 500-fold or more being required for 50% displacement.

![Chart 3](image-url)

**Chart 3.** Analysis of binding of 1α,25(OH)2D3 to cytosol receptor of B16 melanoma cells. A, dose-dependent binding of [3H]-1α,25(OH)2D3. Specific binding (D) was defined as the difference between the 2 values: B, Scatchard analysis of the specific binding data from A.

![Chart 4](image-url)

**Chart 4.** Competition of vitamin D3 derivatives with [3H]-1α,25(OH)2D3 for binding. Binding of [3H]-1α,25(OH)2D3 was assayed in the presence of 1α,25(OH)2D3 (6), 25(OH)D3 (2), 1α(OH)D3 (1), or 24R,25(OH)2D3 (2). The cytosol fraction of B16 mouse melanoma cells was incubated with 0.063 nM [3H]-1α,25(OH)2D3 in the presence of various concentrations of unlabeled 1α,25(OH)2D3, 25(OH)D3, 24R,25(OH)2D3, or 1α(OH)D3.
Inhibition of Melanin Synthesis by Retinoic Acid. A number of reports had indicated the involvement of vitamin A and its analogues in the regulation of cellular differentiation. We therefore investigated its effects, alone and in combination with 1α,25(OH)2D3, on melanin synthesis under the same conditions. As shown in Chart 5, retinoic acid inhibited melanin synthesis both dose and time dependently. The inhibition was maximum on treatment with retinoic acid (3 ng/ml) for 3 days or more. Retinoic acid at concentrations of 0.03 to 3 ng/ml was not toxic to the cells, although it slightly inhibited growth dose dependently (data not shown).

Retinoic acid also inhibited melanin synthesis induced by 1α,25(OH)2D3. For the results shown in Chart 6, B16 melanoma cells were exposed to 1α,25(OH)2D3 (10 nm or 4 ng/ml) in the presence and absence of retinoic acid (0.1 to 1000 nm or 0.03 to 300 ng/ml) for 4 days, and the amounts of melanin synthesized were measured. Retinoic acid inhibited melanin synthesis induced by 10 nm 1α,25(OH)2D3 dose dependently. A balance of stimulation and inhibition by the 2 vitamins, i.e., A and D3, was obtained at a molar ratio of 1:10. Tyrosinase activity induced by 1α,25(OH)2D3 and cholera toxin was also inhibited by retinoic acid (data not shown).

DISCUSSION

In the present study, we demonstrate that 1α,25(OH)2D3 stimulates melanin synthesis of B16 mouse melanoma cells as a result of increasing the activity of tyrosinase, a key enzyme in melanin synthesis. The presence of a specific receptor, together with specific stimulation by 1α,25(OH)2D3, suggests a receptor-mediated mechanism of stimulation.

There are 2 previous reports suggesting the possible regulation of melanin synthesis by vitamin D3. Oikawa and Nakayasu (30) reported that tyrosinase activity and melanin synthesis of B16 melanoma cells were stimulated by vitamin D3 (ergocalciferol) and vitamin D2 (cholecalciferol), but only at a much higher concentration of 1α,25(OH)2D3, i.e., 10 μg/ml. Colston et al. (7) demonstrated that 1α,25(OH)2D3 inhibited the growth and DNA synthesis of human melanoma cells (Hs 69ST) which contained a specific receptor for 1α,25(OH)2D3 with a Kd of 180 pm and an Nmax of 176 fmol/mg protein. These 2 reports, although on different melanoma lines, suggest regulation of melanin synthesis by 1α,25(OH)2D3, and this possibility was supported by the present results.

It is known that MSH, a pituitary hormone, stimulates melanin synthesis of melanocytes by causing activation of tyrosinase. Wong and Pawelek (38) reported that MSH also stimulates melanin synthesis of Cloudman S91 melanoma cells. MSH binds to a membrane receptor of melanocytes or melanoma cells and activates adenylyl cyclase, resulting in elevation of the intracellular level of cAMP. Körner and Pawelek (17) found that the tyrosinase activity of melanoma cells is activated by cAMP-dependent protein kinase, possibly due to inactivation of an inhibitor of tyrosinase. Stimulation of melanin synthesis by cholera toxin may be governed by a similar cAMP-mediated mechanism. In contrast, 1α,25(OH)2D3 did not increase the intracellular cAMP level of B16 melanoma cells, suggesting that unlike MSH and cholera toxin it stimulates tyrosinase activity by a mechanism not mediated by cAMP.

Vitamin A and its derivatives are known to act as regulators of differentiation in various cell types, including melanoma cells. Lotan and Lotan (24, 25) reported that retinoic acid induces melanogenesis in S91 mouse melanoma cells and Hs931 human melanoma cells. Inconsistent with this observation, we found that melanin synthesis and tyrosinase activity of B16 mouse melanoma cells were suppressed by retinoic acid. This apparent discrepancy suggests that the regulatory mechanism of melanin synthesis differs among different melanoma cell lines. Of particular interest is the finding that retinoic acid counteracted the stimulatory effect of 1α,25(OH)2D3 and that the balance of these 2 lipophilic vitamins, A and D3, was attained at a molar ratio of 1:10.

It is well known that vitamin D3 is formed from provitamin D3 in the skin, principally in the malpighian stratum, on exposure to sunlight. Pigmentation of the skin prevents penetration of sunlight into the epidermis, thus resulting in reduced synthesis of vitamin D3. Stimulation of melanin synthesis by 1α,25(OH)2D3, if it occurs in epidermal melanocytes in vivo, may act as a mechanism of negative feedback of the metabolism of vitamin D3 by preventing conversion of provitamin D3 to vitamin D3. Colston et al. (7) also proposed the same hypothesis from the receptor study of human melanoma cells.

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