Stimulation of Human Melanocytes by Vitamin D_3 Possibly Mediates Skin Pigmentation After Sun Exposure

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We found an increased amount of immunoreactive tyrosinase in human melanocytes after 6-d culturing with vitamin D_3 (cholecalciferol). Most of these melanocytes became more dendritic and swollen in a fashion similar to that noted in the skin after ultraviolet irradiation. However, 7-dehydrocholesterol (pro-vitamin D_3) or 1α,25-dihydroxy-vitamin D_3 (activated vitamin D_3) were found to have little effect on the same system. Because vitamin D_3 is known to be photochemically converted from pro-vitamin D_3 in the skin by ultraviolet irradiation, the mechanism of human skin pigmentation after ultraviolet irradiation, thus far unknown, may be at least partly explained by this stimulating effect of vitamin D_3 on melanocytes. *J Invest Dermatol 90:882–884, 1988*

The basic mechanism underlying human skin darkening induced by ultraviolet (UV) irradiation has not yet been elucidated. Oikawa and Nakayasu noted that ergocalciferol and cholecalciferol (vitamin D_3) increased the activity of tyrosinase, a melanin-forming enzyme, in the cultured cells of B-16 mouse melanoma [1]. Because it is well known that these calciferols can be converted photochemically from ergosterol and 7-dehydrocholesterol (pro-vitamin D_3) by ultraviolet B (UVB) irradiation [2,3], they speculated that cholecalciferol might play a physiological role in photoinduced melanogenesis in the human skin [1]. However, it has been impossible to obtain direct evidence of the effect of vitamin D_3 on human melanocytes, because of the lack of a pure culture system of melanocytes isolated from normal human skin. Recently, using the roof of a suction blister raised on human skin, we have developed a simple and reliable technique to culture normal melanocytes without contamination of other cells [4]. By using this melanocyte culture method as well as a monoclonal antibody against tyrosinase [5], we have examined whether or not vitamin D_3 stimulates human melanocytes.

MATERIALS AND METHODS

Materials A 20 W FL 20 SE lamp obtained from Matsushita Electric Co. (Osaka, Japan) was used as a source of UVB; its irradiance at 305 nm was 1.0 mJ/cm^2/sec at a distance of 10 cm from the lamp. PMA, bovine serum albumin (BSA), 7-dehydrocholesterol, and cholecalciferol were obtained from Sigma Chemical Co. (St. Louis, MO), and 1α,25-dihydroxy-vitamin D_3 (1α,25-$(OH)_2$-vitamin D_3) was generously donated by Dr. M. Fukushima (Chugai Pharmaceutical Co., Tokyo, Japan).

Melanocyte Culture Four oriental male volunteers provided melanocytes for each of the four different series of experiments mentioned below, respectively. The procedures used to isolate human melanocytes from the roof of suction blisters raised on the skin of the volunteers was as described previously [4]. Melanocytes were cultured in Eagle's MEM (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 10 ng/ml phorbol 12-myristate 13-acetate (PMA) for the first 6 d, and then the medium was changed to a PMA-free medium. For the evaluation of the effects of vitamin D_3 or its various derivatives, these test agents were added to the culture medium 3 d after culturing in the latter medium, and the culture was continued for 6 d.

Immunofluorescence Staining The cultured melanocytes were stained immunohistologically according to a previously described method [5] using monoclonal antibody against tyrosinase [6] and fluorescein isothiocyanate (FITC)-conjugated second antibody against the monoclonal antibody (TAGO Inc., CA). The intensity of fluorescence of FITC in each melanocyte was measure at 520 nm by an Olympus multimicrospectrophotometer (model MMSP).

Morphometry Stained melanocytes were photographed microscopically with a Vanox-T (Olympus, Tokyo, Japan). From the enlarged photographs, the shapes of all the melanocytes were transferred to a sheet of tracing paper, and the cell perimeter, area, and number of dendrites of each melanocyte were determined with an image scanner (NEC, PC-IN 502) connected to a microcomputer (NEC, PC-9801VM) employing appropriate software as described elsewhere [7]. The perimeter of a melanocyte was measured as a substitute for the sum of the length of all dendrites, although the perimeter of the cell reflects not only the length of each dendrite but also the cell size.

RESULTS

Effect of UVB on Human Melanocytes The amount of immunoreactive tyrosinase as well as the area of the cell, i.e., cell size, was increased in cultured melanocytes that were isolated from the skin after UVB irradiation administered as described in the footnote of Table I, although the number of dendrites and the perimeter of the cell decreased more than those of the control, as shown in Exp. 1 of

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Abbreviations:
1α,25-$(OH)_2$-vitamin D_3: 1α,25-dihydroxy-vitamin D_3
BSA: bovine serum albumin
FBS: fetal bovine serum
FITC: fluorescein isothiocyanate
PMA: phorbol 12-myristates 13-acetate
UV: ultraviolet

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Table I. The effects of various vitamin D derivatives and UVB irradiation on cultured normal human melanocytes.

Treatment	Number of Cells Examined	Immunoreactive Tyrosinase (Relative Fluorescent Intensity at 520 nm) Mean	S.D.	Perimeter (μm) Mean	S.D.	Area (μm^2) Mean	S.D.	Number of Dendrites Mean	S.D.
Exp. 1									
none	50	25.9	7.5	238.6	126.4	564.1	222.8	3.5	1.5
UVB irradiation in vivo	50	52.6	19.5	182.8	105.4	722.3	307.3	2.5	1.2
Exp. 2									
none	50	25.7	12.4	219.0	70.1	784.2	262.6	2.7	0.9
UVB irradiation in vitro	50	36.9	13.5	206.9	75.0	896.4	342.6	2.3	0.8
Exp. 3									
none	100	28.6	6.8	191.2	59.3	835.5	235.0	2.1	0.2
1.3 μM 7-dehydrocholesterol	100	27.2	6.4	194.2	75.7	720.7	252.0	2.2	0.5
1.3 μM cholecalciferol	100	39.9[a]	11.8	241.9[a]	90.7	1043.7[a]	409.9	2.7[a]	0.9
10 nM 1α, 25-$(OH)_2$vitamin D_3	100	27.1	7.3	194.0	63.3	865.5	266.9	2.2	0.4
Exp. 4									
none	100	18.4	3.4	169.4	51.1	628.2	262.3	2.2	0.4
1.3 μM 7-dehydrocholesterol	100	20.7	2.7	211.3	46.9	738.7	206.2	2.3	0.7
1.3 μM cholecalciferol	100	26.6[b]	3.6	287.7[a]	113.5	1113.3[a]	426.3	3.5[a]	1.7
10 nM 1α, 25-$(OH)_2$vitamin D_3	100	17.8	2.3	188.7	60.2	616.7	249.3	2.2	0.5
1.3 μM ergocalciferol	100	19.2	2.9	194.3	49.8	742.3	266.3	2.4	0.7

Exp. 1: The flexor surface of the left forearm of an oriental volunteer was irradiated by a FL 20 SE lamp for 4.5 min (about 260 mJ/cm² at 305 nm) on days 1 and 3. On day 4, melanocytes were obtained both from the irradiated skin and the nonirradiated skin. Two days after the start of the culture with 10 ng/ml PMA, these melanocytes were fixed for immunofluorescence staining. Exp. 2: Melanocytes were cultured with PMA for 6 d just after isolation, and thereafter cultured without PMA for 3 d until the start of the following treatment, during which they were cultured in PMA-free medium. One group of these melanocytes was irradiated by a FL 20 SE lamp both 3 and 6 d before the fixation; the total amount of irradiated UVB at 305 nm was 30 mJ/cm². Exp. 3: Melanocytes were prepared in the same way as those in Exp. 2. They were cultured for 6 d in MEM with 10% FBS without PMA in the presence of various vitamin D_3 derivatives. Exp. 4: Experimental conditions were the same as those in Exp. 3 except for replacement of 10% FBS by 0.5% BSA during a 6-d culturing of melanocytes with various vitamin D derivatives. For each of the 4 experiments melanocytes were obtained from 4 different oriental males, respectively (i.e., one volunteer provided melanocytes for one experiment). After the fixation, the melanocytes were stained immunohistochemically with anti-tyrosinase monoclonal antibody and FITC-conjugated second antibody.

[a] cholecalciferol vs none, $p < 0.001$ by Welch-t-test.

[b] cholecalciferol vs none, $p < 0.001$ by Student-t-test.

Table I. When cultured melanocytes were irradiated by UVB in vitro twice, 3 and 6 d after the start of culture without PMA (Exp. 2), an increase in the amount of immunoreactive tyrosinase associated with morphologic changes similar to the result in Exp. 1 was observed 3 d after the second irradiation.

Effect of Various Vitamin D Derivatives on Human Melanocytes To confirm whether the UVB effect on melanogenesis is mediated by vitamin D_3 as suggested by Oikawa & Nakayasu [1], we next examined the effects of various vitamin D derivatives on cultured melanocytes. The amount of immunoreactive tyrosinase increased only in those melanocytes cultured with 1.3 μM of cholecalciferol (Exp. 3). The average perimeter and area of the melanocytes showed an increase of 25%, and half of the melanocytes exhibited an increase in dendrites, as shown in Table I and Fig 1. In contrast, 1.3 μM of 7-dehydrocholesterol and 1 or 10 nM of 1α,25-$(OH)_2$-vitamin D_3 did not have any effect on either the fluorescence intensity or the cell morphology (Table I and Fig 1). At a concentration of 50 nM or higher, 1α,25-$(OH)_2$-vitamin D_3 was found to be rather toxic with regards to the melanocytes (data not shown).

Melanocytes used in Exp. 3 were cultured in the presence of 10% FBS. Thus, to exclude the possible effect of some unknown factor(s) contained in the FBS, Exp. 4 was carried out by using MEM with an addition of 0.5% BSA in place of 10% FBS. The reason for selecting 0.5% BSA is that albumin is thought to be a carrier protein for various types of vitamin D_3 and that the concentration of 0.5% BSA is equivalent to the albumin fraction contained in the 10% FBS. The results obtained in Exp. 4 were almost the same as those of Exp. 3, although the stimulatory effect by cholecalciferol was rather enhanced by the absence of 10% FBS. Figure 1 shows a histogram of the number of dendrites of the melanocytes cultured with and without cholecalciferol in Exps. 3 and 4. Conversion of fusiform melanocytes, which were counted as having two dendrites, into multi-dendritic melanocytes was clearly demonstrated to occur after treatment with cholecalciferol (Fig 2).

DISCUSSION

It has been found in vivo that melanocytes in the epidermis become swollen with elongated dendrites after UV irradiation of the skin and that their tyrosinase activity is increased with a resultant deposition of the enzyme product, melanin, in the epidermis several days after irradiation [8-11]. We found that vitamin D_3 induced features similar to those noted in such UV irradiated skin, i.e., it increased

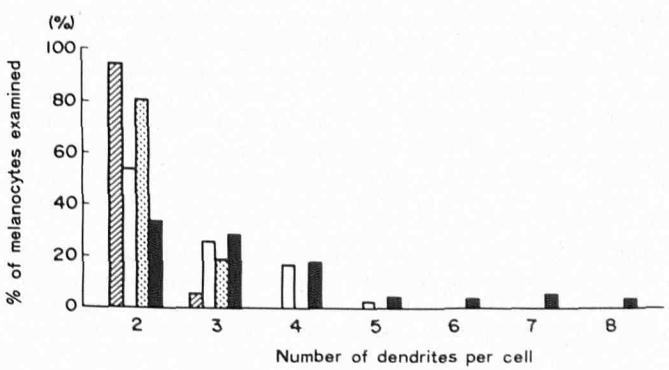

Figure 1. Number of dendrites in melanocytes cultured with or without cholecalciferol. The number of dendrites in cholecalciferol-treated or control melanocytes counted in Exp. 3 and 4 of Table I were histogrammed. ▨, cultured with 10% FBS; □, cultured with 10% FBS and 1.3 μM cholecalciferol; ▩, cultured with 0.5% BSA; ■, cultured with 0.5% BSA and 1.3 μM cholecalciferol.

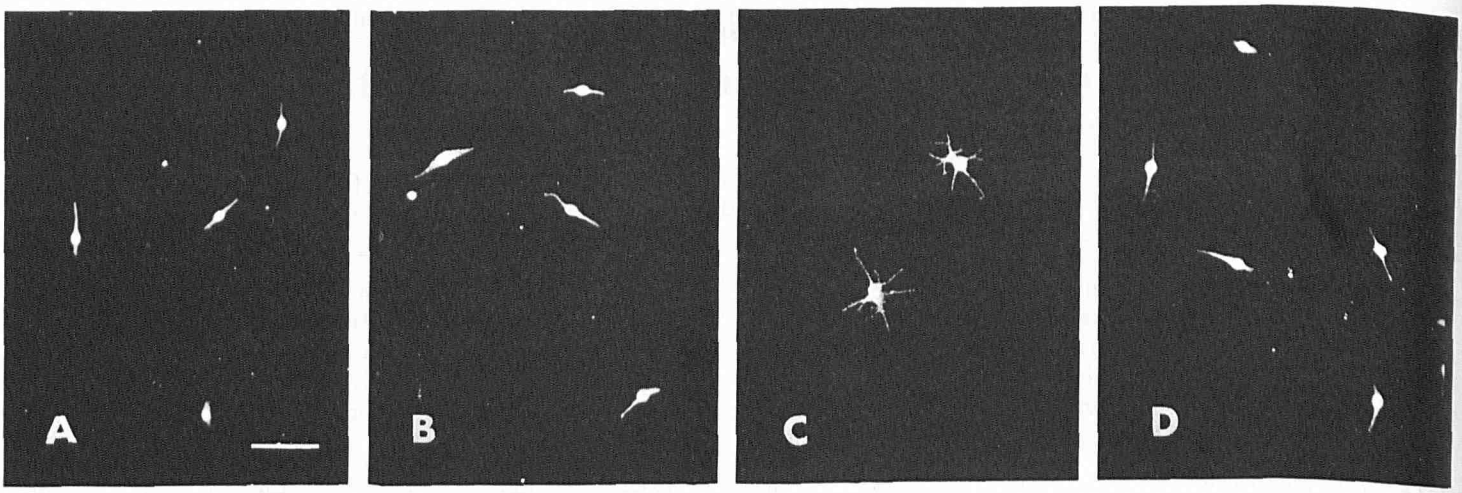

Figure 2. Human melanocytes cultured with various vitamin D_3 derivatives. The melanocytes cultured with none *(A)*, with 7-dehydrocholesterol *(B)*, with cholecalciferol *(C)*, or with 1α,25-$(OH)_2$-vitamin D_3 (D) in Exp. 4 of Table I were photographed. Magnification is the same in *A–D*. *Bar* = 0.1 mm.

cell size, number of dendrites and amount of immunoreactive tyrosinase.

Photochemically-induced cholecalciferol (vitamin D_3) is enzymatically converted to activated vitamin D_3 such as 1α,25-$(OH)_2$-vitamin D_3 in the liver and kidney to regulate the concentration of calcium in the body fluid. Recently Hosoi et al showed the 1α,25-$(OH)_2$-vitamin D_3 increased tyrosinase activity in B-16 mouse melanoma cells [13]. Also, Oikawa and Nakayasu observed a twofold increase in tyrosinase activity in cultured cells of B-16 mouse melanoma both with cholecalciferol and ergocalciferol, vitamin D_2. In contrast to these previous reports, we found that neither 1α,25-$(OH)_2$-vitamin D_3 nor ergocalciferol induced any significant effect in cultured normal human melanocytes. Such discrepancy in obtained results is thought to reflect the difference in the source of melanocytes; that is, between our normal human melanocytes and their mouse melanoma cells.

The high concentration of cholecalciferol required to affect human melanocytes as well as mouse B-16 melanoma cells in vitro [1] suggests that such concentrations capable of stimulating melanocytes are attained only in the skin where vitamin D_3 is produced photochemically by UVB irradiation. It is, therefore, reasonable to presume that sharply confined pigmentation occurs in the skin only where the dosage of irradiation by sunlight is sufficient to produce such a high concentration of vitamin D_3.

We found the UVB irradiation alone could increase the amount of tyrosinase in cultured melanocytes without any supplementation of the medium with cholecalciferol. Thus, it appears that the 7-dehydrocholesterol that remained in the melanocytes was converted into cholecalciferol by UVB, which in turn, stimulated the activity of the melanocytes. But unlike those cultured with cholecalciferol, most melanocytes irradiated with UVB, either in vivo (Exp. 1) or in vitro (Exp. 2), became only swollen and exhibited a somewhat decreased number and length of dendrites. Hence, at present we cannot exclude the possibility that other mechanisms not mediated by vitamin D_3 are involved in melanogenesis resulting from UVB irradiation.

The results of the present study provide direct evidence for the first time that cholecalciferol, vitamin D_3, has a stimulatory effect on melanocytes with regard to changes taking place in photoinduced skin pigmentation; namely, the de novo synthesis of tyrosinase, the increase in cell size, and the elongation of cell dendrites which facilitates the transfer of melanin granules to the surrounding keratinocytes in the skin.

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