STIMULATION OF MELANOGENESIS IN CULTURED MELANOMA CELLS BY CALCIFEROLS

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Received 11 February 1974

1. Introduction

UV-irradiation of skin has two well-known results, skin darkening and conversion of 7-dehydrocholesterol to cholecalciferol [1]. Although the latter reaction is a simple photochemical reaction demonstrable in a quartz tube [2], the molecular mechanism of stimulation of melanin synthesis by UV-irradiation, resulting in skin darkening, has not been elucidated.

The establishment of cell culture system of melanotic melanoma [3] made it possible to examine this mechanism in a simplified model system. Results described here show that 1) cholecalciferol and ergocalciferol stimulate melanogenesis, 2) this stimulation results from an elevated level of tyrosinase activity of cells, and 3) the melanogenesis is stimulated specifically by calciferols but not by their precursors before photoconversion.

2. Materials and methods

2.1. *Chemicals*

Cholecalciferol, 7-dehydrocholesterol, cholesterol, ergocalciferol and ergosterol were purchased from Sigma Chemical Co., St. Louis, USA and their purities were checked by thin layer chromatography [4]. Stock solutions of these chemicals were prepared immediately before each experiment by dissolving the compounds in ethanol (20 mg/ml) and were stored under N_2 in a freezer.

Eagle's MEM was purchased from Chiba Serum Institute, Chiba, Japan, calf serum from the Medical Biological Laboratory, Ltd., Nagoya, Japan, and 'Soluene 100', a 0.5 N solution of dimethyl *n*-undecyl dodecyl ammonium hydroxide in toluene, from Packard Instrument Co., Downers Grove, USA. L- [3,5-^{3}H]Tyrosine (47 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, England. All other chemicals used were of reagent grade.

2.2. *Cell cultures*

The melanotic mouse melanoma cell line, C_2M, used in the present experiments is a subline of B 16-XI [3]. Eagle's MEM supplemented with 10% calf serum and 50 μg ml of kanamycin was used throughout. Cells were maintained as described previously [5].

Experimental cultures were made by seeding cells into Leighton tubes (flat area, 5 cm^2) with exceptions of experiments 2 and 3 in table 1, where large modified T-flasks were used, and when the cultures started exponential growth, usually after culture for one day, the culture medium was changed to medium containing calciferol or one of the related chemicals cited above. These media were prepared immediately before use by adding stock solutions of the respective chemicals to the standard medium. As control culture medium, the same amount of ethanol was added to the standard medium. Culture media were changed every 24 hr during experiments.

2.3. *Assay methods*

Tyrosinase activity of living cells in culture (TyC) was assayed as described previously [5,6]. This method determines tritiated water released into culture medium from L- [3,5-^{3}H]tyrosine (2 μCi and 190 nmoles per

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Table 1
Effects of cholecalciferol and ergocalciferol on melanin synthesis

Expt.	Addition	Melanin/culture* (A_{400} · ml)		Increase in melanin (A_{400} · ml)	Stimulation by calciferol
		0-time	Day 4		
1	–	0.016	0.062	0.046	
	CC		0.096	0.080	1.7
2	–	0.072	0.172	0.100	
	CC		0.225	0.153	1.5
3	–	0.108	0.525	0.417	
	CC		1.12	1.01	2.4
	EC		0.706	0.598	1.4
			Day 9		
4	–	0.013	0.137	0.124	
	EC		0.233	0.220	1.8

* Figures are mean of values in 2 or 3 cultures.

Cells were seeded in standard medium in culture vessels at an inoculum size of about 0.2×10^5 cells/cm^2. After one day (0-time) incubation was continued with and without calciferols (10 μg/ml) for 4 or 9 days. Washed cells were precipitated and solubilized in 'Soluene 100' [7], and their absorbance at 400 nm was measured. CC, cholecalciferol; EC, ergocalciferol.

ml of medium) and gives the amount of tyrosine hydroxylated by living cells in culture per unit time without interferring cell culture conditions. Melanin was assayed spectrophotometrically at 400 nm after solubilizing cells with 'Soluene 100' for 2 hr at 37°C [7]. This method gives a solution which shows an absorbance stable for several hours and follows Beer's law up to an absorbance of 1.6 for crude and protein-free melanins. Cell numbers in cultures were counted with a hemocytometer after treating the cells with trypsin. Protein was determined by the method of Lowry et al. [8].

3. Results

C_2M cells were cultured in the presence of cholecalciferol or ergocalciferol for 4 days. The cells from cultures containing calciferol were darker than those from control cultures, and their higher melanin content was demonstrated spectrophotometrically (table 1).

This higher melanin content of cells cultured with calciferols was due to high tyrosinase activity (TyC) in these cells (fig. 1A). Namely, TyC in control cultures was very low at the high population density as expected from the type of melanogenesis of this cell line [3], while TyC in cultures grown with calciferol was still high at the same high population density. Due to this difference in TyC, cells in cultures containing calciferol accumulated about twice as much melanin as those in control cultures by the end of cultivation (expt. 4 in table 1).

Six experiments like that shown in fig. 1, using ergocalciferol or cholecalciferol, gave similar results with deviations ranging from 3 to 10 times more TyC in cultures with these compounds than in control cultures in the confluent cultures, and from 0 to 40% stimulation of the maximum TyC. The maximum TyC in cultures with these compounds was usually on the day after that when the TyC of control cultures reached a maximum.

The optimal concentration of ergocalciferol for this stimulation was around 10 μg/ml (fig. 2), and at this concentration ergocalciferol reduced the growth rate only slightly if at all (fig. 1B). At a concentration of 25 μg/ml the stimulation of TyC was delayed and not clear (fig. 2), and the doubling

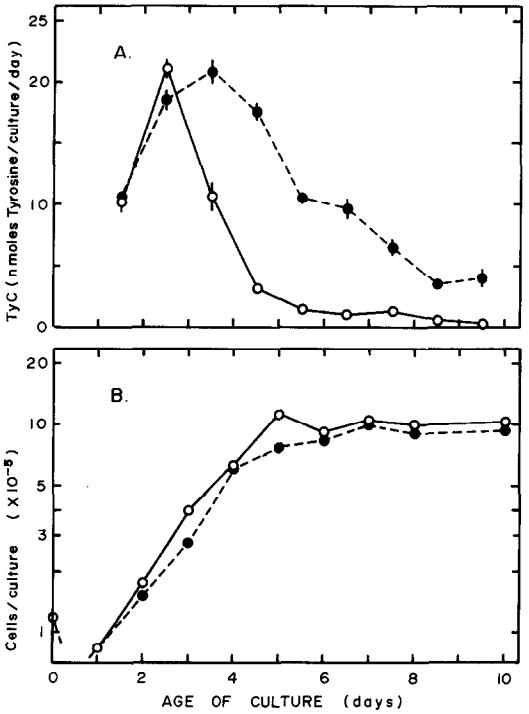

Fig. 1. Effect of ergocalciferol on tyrosinase activity of cells in culture (TyC). 1.2×10^5 cells were seeded into volumes of 1 ml of standard medium in Leighton tubes. After incubation for one day the medium in half the cultures was changed to medium containing L-[3,5-^{3}H]tyrosine (2 μCi/ml) and ergocalciferol (10 μg/ml), and in the other half to medium supplemented only with tritiated tyrosine. These media were changed every 24 hr. Four cultures in each group were used for assay of TyC (A) and others for determination of the cell proliferation (B). Points and bars in A represent means and standard deviations of values in 4 cultures. Points in B are the mean of two determinations. (o–o–o) Control cultures; (•–•–•) cultures with ergocalciferol.

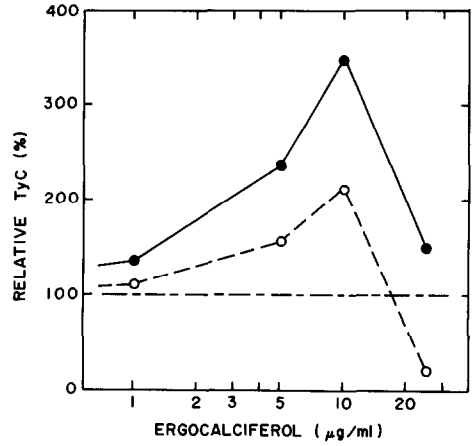

Fig. 2. Effect of ergocalciferol concentration on TyC. The concentrations of ergocalciferol indicated on the abscissa were added to cultures one day after the start of culture. TyC's (nmoles tyrosine/mg protein/day) on day 4 (the fourth 24 hr period of cultivation, (o–o–o) and day 5 (•–•–•) were assayed and the activities relative to those of the respective control cultures are plotted as percentages.

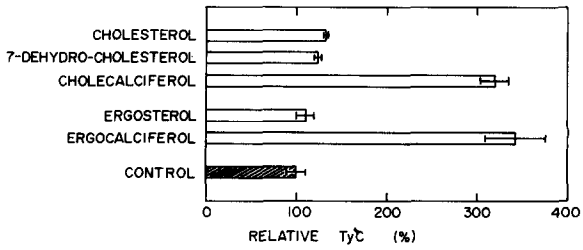

Fig. 3. Effect of calciferols and related compounds on TyC. After culture of cells for 2 days 10 μg/ml of calciferols or related compounds were added, and culture was continued for 3 days more with two changes of the media. On day 5, TyC was assayed and expressed as in fig. 2. Columns show means of values in 3 cultures and bars show standard deviations.

time of culture was approximately twice that in control culture.

7-Dehydrocholesterol and ergosterol, which are precursors of cholecalciferol and ergocalciferol, respectively, before photoconversion, had little effect on TyC (fig. 3), as well as on growth rate. These results make it unlikely that the stimulated melanogenesis resulted from the non-specific reduction of growth rate caused by calciferol, and suggest a specific relationship between calciferol and melanogenesis.

4. Discussion

The present results on malignant melanocytes suggest that skin darkening caused by UV-irradiation is due to calciferol-mediated elevation of tyrosinase activity in melanocytes. In the cultured cell system used here the effective concentration of calciferol for this stimulation was 10 μg/ml (fig. 2). After

exposure to sunlight, the local concentration of cholecalciferol in the basal cell layer of the skin, where melanocytes are located, may be similar to that in the culture medium for the following reasons: (a) According to Reinertson and Wheatley [9] human epidermis contains 400–500 μg of 7-dehydrocholesterol per gram of dry tissue, and (b) epidermal Malpighian layer, immediately above the basal cell layer, has the highest 7-dehydrocholesterol content in the epidermis. (c) Photoconversion of 7-dehydrocholesterol to cholecalciferol seems to be efficient [2,9,10], and this is supported indirectly by the recent observation of 50% elevation of the 25-hydroxycholecalciferol level in the blood after exposure to sunlight [11]. (d) Irradiation products could be retained for a while at a high local concentration due to lack of capillaries in the epidermis. (e) In the present study the mean concentration of calciferol in culture media during the 24 hr incubation period was probably considerably lower than the initial concentration because in a preliminary test no calciferol could be detected after incubation of medium containing calciferol for 24 hr at 37° C. Thus, under the suitable environmental conditions the cells in skin may have the necessary calciferol concentration for this mechanism.

The specificity of calciferols for stimulation of tyrosinase activity (fig. 3) also suggests a physiological role of cholecalciferol in melanogenesis. The cellular component(s), which interacts with calciferol, is under investigation.

Acknowledgement

This work was supported in part by research grants Nos. 801006 and 801032 from the Ministry of Education, Japan.

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