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Quantitative Cytochemical Analysis by Microdensitometry of Spontaneous or α-MSH-stimulated Melanogenesis in B16 Melanoma Cells Cultivated *in Vitro**

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Abstract—*A spontaneous cyclic phenomenon characterized by successive waves of either high proliferative rate or intense melanogenesis is described in non-confluent B16 melanoma cells subcultivated during* 2 months *(or more). Dopa-oxidase activity is quantified in individual cells after* L-*dopa reaction, by an original method of visible light absorption cytophotometry. A* 24-*hr treatment with α-MSH increases dopa-oxidase activity. This increase is also noted during the following* 14 hr, *in a fresh medium devoid of α-MSH, in which cell proliferation resumes after* 24 hr. *Phenylthiourea, cycloheximide or actinomycin D inhibit dopa-oxidase activity, but also cell proliferation in α-MSH pre-stimulated cells. The effects of the two latter agents suggest that* de novo *synthesis of the enzyme takes place following α-MSH treatment.*

INTRODUCTION

CELLS from B16 melanoma of the mouse can be cultivated *in vitro* while keeping their ability to produce melanin [1]. Melanogenesis is controlled by dopa-oxidase, which converts L-tyrosine into L-dopa and then into dopaquinone during the first steps of melanogenesis. Inhibitors of dopa-oxidase activity have been used by different authors [1-4]; they act at various levels: transcriptional (actinomycin D), translational (cycloheximide) or enzymatic (phenylthiourea). The stimulating activity of α-MSH on melanogenesis has been related either to activation of pre-existing dopa-oxidase [5] or to its *de novo* synthesis [6]. Cyclic AMP seems to act as a chemical mediator (second messenger) because its concentration rapidly increases in cells treated in culture by α-MSH; 6-9 hr after this increase dopa-oxidase activity is intensified, and 16 hr later melanin granule formation is observed [6].

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In fact, it is not known that MSH acts only to increase dopa-oxidase synthesis. We are interested in the study of stimulating or inhibiting factors of dopa-oxidase activity and of their action on proliferation and differentiation in B16 melanoma cells cultivated *in vitro*. We report here results concerning the spontaneous behaviour of these cells in culture (pigmentation, proliferation) and their reactions to α-MSH treatment. Dopa-oxidoreductase (dopa-oxidase) activity is estimated with an original cytophotometric method (visible light absorption after L-dopa reaction) applied to individual cells. To date, such a method has been used only for dopa-oxidase activity measurements on whole small ascidian embryos [7]. Our results suggest that α-MSH induces *de novo* synthesis of dopa-oxidase, which is maintained for some time even in a medium devoid of α-MSH. The latter can also inhibit cell proliferation and increase cell necrosis. Probably, the toxicity of melanin precursors produced during melanogenesis is responsible for cell death and for the cyclic spontaneous behaviour of B16 melanoma cells *in vitro*, characterized by successive waves of active proliferation and weak pigmentation, and vice versa.

MATERIALS AND METHODS

Culture conditions

Small explants from primitive pigmented tumors implanted in C57BL mice were cultivated in T 75 Falcon dishes. Culture medium: 80% Eagle's minimal medium, 20% foetal calf serum and penicillin 100 U/ml. Culture dishes are incubated at 37°C in a mixture of 5% CO_2-air. Three times a week, fresh medium was given to washed cells. When cells became confluent they were trypsinized (trypsin solution at 0.05% during 10 min at 37°C), and then subcultivated either in T 75 Falcon dishes or in small Petri dishes.

Treatments

Cells cultivated in Petri dishes were maintained for 14 or 24 hr in nutritional medium containing a potential inhibitor (phenylthiourea 0.1 or 1 mM, cycloheximide 0.1 or 1 μg/ml, or actinomycin D 1 or 10 μg/ml) or a stimulating agent of melanogenesis (α-MSH 0.2 μg/ml).

Cytological and cytochemical methods

Fixed (ethanol-acetone 1:1) cells were stained by Feulgen reaction (hydrolysis for 20 min in HCl 3.5 N at 37°C) for DNA measurements by visible light absorption cytophotometry (545 nm; Vickers M 86 microdensitometer); 50 individual nuclei were measured in each case. Mitotic activity was expressed as mitotic index (‰); a minimum of 3000 cells were observed in each case. Melanin production was estimated by cell visual observation and expressed in pigmented cells ‰; a minimum of 1000 cells was used in each case. The L-dopa reaction [8] was performed on cells fixed with formol-calcium 10% and L-dopa-oxidoreductase activity was shown in individual cells by absorption cytophotometry (Vickers M 86 microdensitometer; 100 cells in each case). The wavelength used was 475 nm (absorbance maximum of the dopachrome produced by the enzymatic reaction [9]).

Statistical analysis

Student's *t* test was used for comparing means (mitotic index, and light absorption values in arbitrary units, after L-dopa reaction). The χ^2 test was used for comparing DNA values (percentages of cells in different classes).

RESULTS

Proliferative activity of B16 cells is clearly decreased when intense pigment synthesis is noted. Such a behaviour was also described in other types of pigmented cells [10–12]. However, original observations were performed by analysing proliferation and pigment production for long periods of cultivation (2 months; Fig. 1). Clearly,

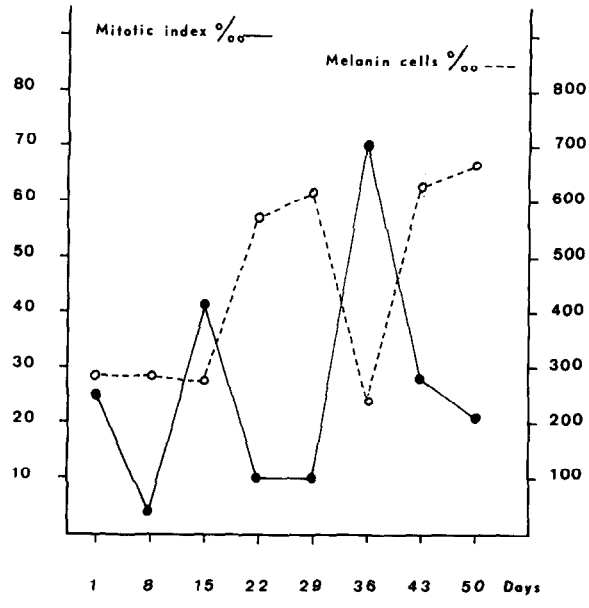

Fig. 1. Evolution of mitotic activity and frequency of pigmented cells as a function of duration of cultivation (minimum Eagle's medium supplemented with foetal calf serum).

peaks of melanin accumulation in the cells coincide with low proliferation rates and vice versa. Such a cyclic behaviour spontaneously takes place in the usual nutrient medium and in non-confluent cell populations.

B16 cell populations can be characterized by light absorption measurements in individual cells (475 nm after L-dopa reaction). Histograms of this kind are shown in Fig. 2(A and B) (control cells).

Effect of α-MSH treatment during 14 *or* 24 hr

α-MSH (0.2 μg/ml) induces significant mitotic inhibition (mitotic index: 19.5 ‰ after 24 hr of treatment against 30 ‰ in control cells; significant differences: $P < 0.001$). DNA measurements in α-MSH-treated cells indicate a significant increase in percentage of cells with intermediate values between 2 DNA and 4 DNA (18 vs 7% in controls) but a decrease of 8 DNA cells (24 hr of treatment: 16 vs 28% in controls; $P < 0.05$).

If we compare such α-MSH-treated cells to control cells (Fig. 2) for absorbance after the L-dopa reaction, we note an increase in the percentage of cells with relatively high absorption values. Mean values (arbitrary units) calculated from histograms are: 8.5 (2A); 10.6 (2B); 15.1 (2C); and 15.2 (2D); significant differences between treated and control cells, $P < 0.01$.

Effect of a treatment with α-MSH followed by cultivation in fresh medium devoid of α-MSH

B16 cells are stimulated for melanogenesis with α-MSH (0.2 μg/ml for 24 hr) and then cultivated in a fresh medium devoid of α-MSH for a further

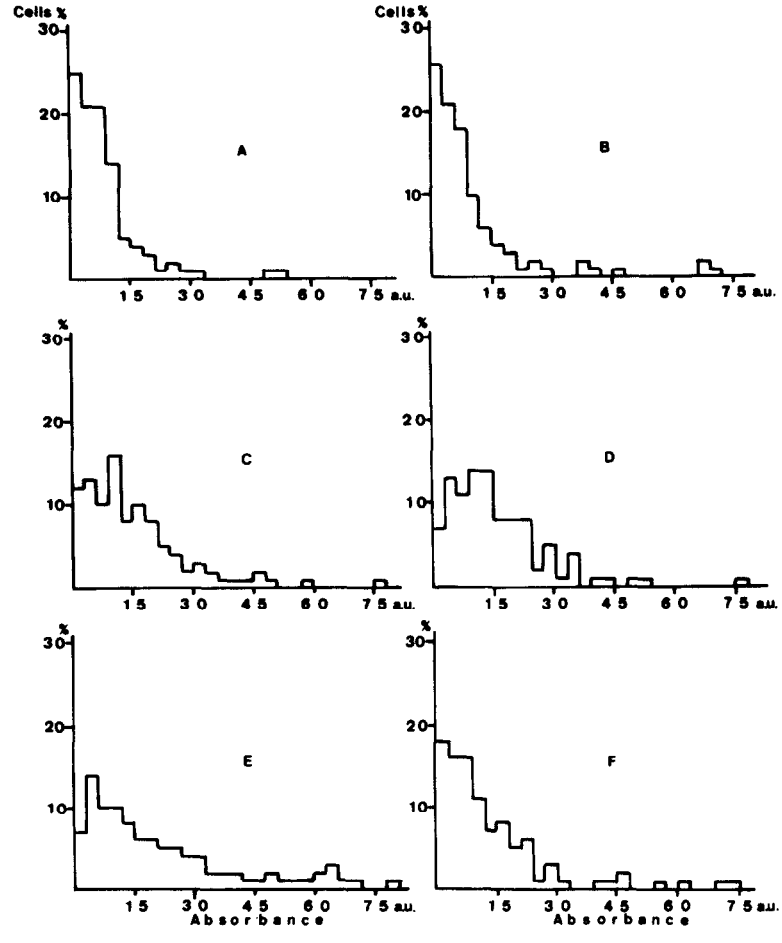

Fig. 2. Histograms obtained from absorption measurements (at 475 nm) after the L-dopa reaction in 100 individual B16 cells in each case. a.u.: arbitrary units. Controls at 0 hr (A) or 24 hr later (B). α-MSH treatment (0.2 μg/ml) during 14 (C) or 24 hr (D). α-MSH treatment (0.2 μg/ml) during 24 hr, followed by cultivation in a fresh medium without α-MSH during 14 (E) or 24 hr (F).

24 hr. During the latter, the mitotic index increases from 19.5 to 33 ‰ ($P < 0.001$) and DNA measurements show no differences between treated and control cells. Thus, the effect of α-MSH on cell proliferation is temporary and reversible.

Histograms given in Fig. 2 (E and F) represent light absorption values measured after the L-dopa reaction; the increase in percentage of cells with high absorption values (arbitrary units) is still more evident than in 2D after 14 hr of cultivation in fresh medium without α-MSH (2E). Twenty-four hours later (2F) the percentages of these highly pigmented cells fall, probably because of their detachment from the substrate. Mean absorption values calculated from histograms are 21.7 (2E; $P < 0.001$) and 13.7 (2F; not significantly different from 15.2).

Effects of phenylthiourea, cycloheximide or actinomycin D added to the culture medium after a 24-hr treatment with α-MSH

For each agent two concentrations and two times of action (14 or 24 hr) were tested. They are added to fresh culture medium devoid of α-MSH after a 24-hr stimulation for melanogenesis with α-MSH (0.2 μg/ml).

Mitotic inhibition is significant ($P < 0.01$) after 24 hr of treatment except for phenylthiourea at a low concentration (mitotic index: 33 ‰ for controls; 28 and 10 ‰ for phenylthiourea 0.1 and 1 mM; 10 and 8 ‰ for cycloheximide 0.1 and 1 μg/ml; 12 and 1.5 ‰ for actinomycin D 1 and 10 μg/ml). DNA measurements show increased percentages of cells with 2 or 4 DNA, but lowered percentages of 8 DNA cells ($P < 0.05$; phenylthiourea or cycloheximide at a high concentration, actinomycin D at a low concentration); these results suggest that the progression in the cell cycle is slowed down. At high concentrations the latter agent induces some block in G_2.

Histograms obtained for absorption values measured after the L-dopa reaction following a 14-hr treatment with one of these inhibitors indicate no increase in the percentage of cells with high absorption values. These histograms remain analogous to those obtained after a 24-hr

stimulation with α-MSH (0.2 μg/ml; Fig. 2D). Mean absorption values have been calculated and are given in Table 1. For each treatment significant differences are observed ($P<0.05$) after 14 hr (non-treated: 21.7 a.u.; Fig. 2E). After the 24-hr treatment with one of the agents only two values (cycloheximide 1 μg/ml; actinomycin D 10 μg/ml) are significantly different from non-treated controls (13.7, $P<0.05$; Fig. 2F).

DISCUSSION

Estimation of dopa-oxidase activity

Generally, dopa-oxidase activity in pigmented cells is measured by analysis of the release of ^3H_2O in culture medium after incorporation of radioactive L-tyrosine (Pomerantz method; [13]). L-dopa is also a substrate for dopa-oxidase [14] and is more rapidly oxidized by this than by tyrosine. Histochemical tests are classically based upon formation of melanin in the presence of L-dopa when dopa-oxidase is active in cells. Microdensitometry has not yet been used for quantifying this histochemical reaction for dopa-oxidase in individual cells. Recently, however, such an application was described in whole small ascidian embryos [7]. Under our experimental conditions, absorption measurements performed at 475 nm are related not only to dopa-oxidase activity, but also to melanin granules present in various quantities in the individual cells. Some measurements have been performed with a u.v.-visible spectrophotometer on fixed B16 cells in suspension. They indicate that the mean initial natural absorption before the L-dopa reaction is of the order of 10% of the final absorbance values measured after L-dopa reaction in controls or α-MSH-stimulated cell populations. Results obtained with our method based upon microdensitometry are in good correlation with frequencies of pigmented cells as calculated from visual microscopical observations in control or treated cells. More numerous classes of more or less pigmented cells are, however, detected with microdensitometry.

Table 1. Mean values (arbitrary units) of absorption measured after the L-dopa reaction in 100 individual B16 cells in each case

	Mean absorption values (arbitrary units) ± confidence interval
Controls	
0 hr	8.5 ± 1.7
24 hr	10.6 ± 2.7
α-MSH 24 hr	15.2 ± 2.4
Fresh medium without α-MSH	
14 hr	21.7 ± 3.8
24 hr	13.7 ± 2.9
Phenylthiourea 0.1 mM	
14 hr	14.6 ± 2.4
24 hr	12.1 ± 2
Phenylthiourea 1 mM	
14 hr	14.1 ± 2.6
24 hr	10.4 ± 1.8
Cycloheximide 0.1 μg/ml	
14 hr	13.6 ± 2.3
24 hr	12.2 ± 2.3
Cycloheximide 1 μg/ml	
14 hr	12.9 ± 2.6
24 hr	10.3 ± 1.8
Actinomycin D 1 μg/ml	
14 hr	13.8 ± 2.1
24 hr	10.9 ± 1.9
Actinomycin D 10 μg/ml	
14 hr	13.7 ± 2.1
24 hr	9.7 ± 1.5

The cells are treated during 24 hr with α-MSH (0.2 μg/ml), then incubated in a fresh culture medium without α-MSH but containing or not phenylthiourea, cycloheximide or actinomycin D at two concentrations for 14 or 24 hr.

Effects of α-MSH on cell proliferation

In our pigmented B16 cell cultures, mitotic inhibition is observed when α-MSH is maintained for 24 hr in the medium; our DNA measurements suggest that some inhibition of DNA synthesis is induced. Other authors have shown that α-MSH can provoke (in Cloudman S 91) arrest of the cell cycle [15], sometimes in G1 phase [1]. We show that these effects are, however, reversible 24 hr later if the cells are incubated in a fresh medium devoid of α-MSH. This reactivation of proliferation is accompanied by the disappearance of highly pigmented cells, but is preceded by increased dopa-oxidase activity (after 14 hr in the fresh medium). These variations seem somewhat analogous to the spontaneous cyclic process of proliferation-melanogenesis we have observed but which occurs over longer periods of time and without any α-MSH stimulation. We suggest linking these cyclic activities to the presence in the cells of high concentrations of toxic chemical precursors of melanin, particularly 5,6-dihydroxyindole, L-dopa and dopachrome [16–18]. Some of these molecules, accumulating in highly pigmented cells and in the culture medium, most probably provoke some mitotic inhibition and some degree of cell death. The renewal of nutritional medium, by eliminating these cytotoxic molecules, enables mitotic activity to recover [19]. It must be noted that in our material mitotic activity again reaches normal values after

removal of α-MSH from the medium, although dopa-oxidase activity is increased (and thus probably toxic precursors also). In α-MSH-treated cells high or low levels of toxic precursors, but also probably high or low levels of intracellular cyclic AMP, are responsible for mitotic inhibition or stimulation [11, 16, 20–22].

Effects of α-MSH on dopa-oxidase activity and melanin synthesis

Our original results based on microdensitometry in individual cells after the L-dopa reaction show the well-known α-MSH stimulating effect on melanogenesis in B16 melanoma cells cultivated *in vitro*. We also show that this stimulation is maintained 14 hr later if cells prestimulated by α-MSH are cultivated in a medium devoid of this hormone. Treatment with phenylthiourea prevents such a prolonged stimulation; it is a chelating agent of copper which is necessary for dopa-oxidase activity.

Our results obtained with cycloheximide or actinomycin D suggest that transcriptional and translational processes are necessary for continued α-MSH stimulation of melanogenesis as observed in B16 cells incubated in fresh medium containing no α-MSH. Requirements of this kind have been demonstrated in the presence of α-MSH in mouse skin melanocytes [23] and in non-synchronized Cloudman S 91 cells [2, 6]. Thus similar requirements for melanogenesis are observed in the presence of α-MSH as well as after its removal from the culture medium.

Highly pigmented cells tend to disappear following treatment with phenylthiourea, cycloheximide or actinomycin D, but only when the cells are pre-treated with α-MSH. Mitotic inhibition is also stronger than that observed after similar treatment but in the absence of α-MSH pre-treatment, as shown by other personal experiments. This increased sensitivity could be tentatively explained by α-MSH inactivating a blocking factor considered to be able to protect non-stimulated cells, as postulated by other authors in Cloudman S 91 cells [18].

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