Stimulation of Tyrosinase Activity and Melanin Formation of Cultured Melanoma Cells by Serum Deprivation Alone or in Combination with Dibutyryl Cyclic AMP and Theophylline

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(Z. Naturforsch. **32 c**, 567–571 [1977]; received February 24, 1977)

Melanoma, Cell Culture, Serum Removal, Tyrosinase, Melanin

Serum removal from the media of serial monolayer cultures of the Harding-Passey melanoma during an incubation period of 3 days resulted in an exponentially declining DNA synthesis rate (measured by the incorporation of [^{14}C]thymidine) and in an inhibition of cell proliferation. Protein synthesis, as measured by the incorporation of radioactive leucine, was less affected than DNA synthesis. Incubation in serum-free culture medium resulted in significant rises of tyrosinase activity and cellular melanin content. Addition of dibutyryl adenosine 3′:5′ monophosphate (Bu_2cAMP, 5×10^{-4} M) and theophylline (5×10^{-4} M) to serum-free cultures caused a further striking increase of tyrosinase activity and melanin formation, while treatment of serum containing cultures with Bu_2cAMP and theophylline showed only a slight rise in melanogenesis. It is suggested that these stimulatory effects are mediated by an increased intracellular cAMP level, since a correlation between the degree of melanogenesis and cellular cAMP content was indicated. Treatment of serum-free or serum-containing cultures with the phosphodiesterase inhibitor theophylline (5×10^{-4}–1×10^{-3} M) alone revealed only a slight enhancement (about 20%) of melanogenesis. Because augmentation of melanogenesis by serum-free medium alone or together with Bu_2cAMP and theophylline was prevented by cycloheximide (or actinomycin D), de novo protein synthesis seems to be required for these stimulatory effects.

Introduction

The nature of the biological effects of serum or serum factors on growth and expression of differentiated functions of mammalian cells in culture is not well understood. A primary role of serum is to provide hormones and growth factors, and serum deprivation from culture medium reduces DNA synthesis and cell growth (for review see ref. 1).

An inverse relationship between cell growth and melanin synthesis has been observed to occur in melanin producing mammalian cells (for review see ref. 2) and previous studies have shown a preferential melanogenesis of cultured melanoma cells during non-proliferating growth conditions [3, 4]. Recent evidence indicates that enhanced melanogenesis and decreased cell proliferation of melanoma cells in culture occur as a result of increased levels of cAMP [5–7], for review see ref. 8).

Serum deprivation has been reported to result in growth inhibition and increased cAMP concentrations in cultured non-melanogenic mammalian cells [9–11]. So far, no studies have been undertaken on the effect of serum respectively serum omission in culture media on growth, melanogenesis and cAMP level of melanin producing cells. Such investigations might provide insight in the role of serum (respectively serum factors) for the regulation of melanin synthesis. We now report on the effect of serum deprivation (and for the purpose of comparison also on the effect of exogenous Bu_2cAMP and the phosphodiesterase inhibitor theophylline) on growth, the activity of tyrosinase and on melanin formation of serially cultured monolayer cells of the Harding-Passey mouse melanoma.

Abbreviations: cAMP, adenosine 3′:5′ monophosphate; Bu_2cAMP, N^6,$O^{2'}$ dibutyryl adenosine 3′:5′ monophosphate; tyrosinase, *o*-diphenol: O_2-oxidoreductase (EC 1.10.3.1); TCA, trichloric acetic acid; L-DOPA, 3,4-dihydroxy-L-phenylalanine.

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Material and Methods

Cells (designated HPM-73) were isolated in culture in 1973 from a Harding-Passey melanoma bearing NMRI-mouse (the melanoma bearing mice were kindly provided by Dr. Gericke, Hoechst AG, Frankfurt) according to methods previously described [12]. These monolayer-cells were kept in permanent culture since that time and passaged (about 1 : 4 splits) every 6–8 weeks. Subcultures were prepared by seeding 5 ml volumes (approx. 200 μg cell protein/culture) of a pooled cell suspension (derived by dislodging cells from stock cultures by

means of a "rubber policeman") in 2-ounce pharmacy bottles (Brockway Glass Comp., Brockway, Pa.). Culture medium was Eagle's Basal Medium (BME) (Gibco, Grand Ilsand, N. Y.), containing 100 IE/ml penicillin-G-sodium (Gruenenthal, Stolberg) and 135 μg/ml streptomycin sulfate (Hoechst AG, Frankfurt). L-tyrosine (Merck AG, Darmstadt) was added to a final concentration of 4×10^{-4} M. Serum containing medium was supplemented with 10% fetal calf serum (Gibco, Grand Island, N. Y.). Incubation was performed in a gas phase of 7% CO_2, 20% O_2 and 73% N_2. The first medium change with regular serum containing medium was made after 1 day. Three days after subcultivation the medium of all cultures (average cellular protein content per culture about 350 μg) was replaced-following 2 washes with 5 ml serum-free medium-with 10 ml serum-free or 10 ml 10% serum containing medium. Treated cultures obtained medium with 5×10^{-4} M Bu_2cAMP (Boehringer, Mannheim) and 5×10^{-4} M theophylline (Sigma, St. Louis, Mo.) prepared by distribution of 5×10^{-3} M stock solutions (in serum-free medium). Cycloheximide (Sigma, St. Louis, Mo.) or actinomycin D (Boehringer, Mannheim) were added to cultures from fresh stock solutions (100 μg/ml).

After incubation periods of 1, 2 or 3 days the culture medium was decanted and the cell monolayer treated with consecutive washes (10 ml each) with Earle's salt solution (3 times, 1 min, 0 °C), with 5% TCA (2 times, 5 min, 0 °C) and with 96% ethanol (3 times, 1 min, 0 °C). The airdried monolayer was dissolved in 1 N NaOH. An aliquot of the solution was used for determination of cell protein [13], using bovine serum albumin (Sigma, St. Louis, Mo.) as reference. Another aliquot was dissolved in Bray's scintillation liquid [14] and used for determination of incorporated radioactivity (Beckman Scintillation Counter Model LS 100). Melanin content of the solubilized cell residue was measured by determination of the extinction at 400 nm (E_{400}) [3]. An average E_{400}/mg cell protein/ml for cell cultures containing 300 – 500 μg protein was 0.12.

Tyrosinase activity was measured according to the method of Oikawa *et al.* [15]. Following 3 washes with Earle's salt solution (10 ml each) cells were frozen at – 20 °C. For determination of enzyme activity the thawed cells were scraped off from the glass surface in aqua bidest. and sonicated at 0 °C for 1 minute (position 5, Branson Sonifier B 12). An aliquot of the cell homogenate (30 – 50 μg protein) was incubated with 250 nmol (2 μCi) of L-[3,5-^{3}H]tyrosine, 25 nmol L-DOPA (Merck AG, Darmstadt), 25 IE penicillin-G-sodium and 35 μg streptomycin sulfate in 250 μl of 0.1 mM sodium phosphate buffer (pH 6.8) for 5 h at 37 °C. The reaction was stopped by freezing at – 20 °C. Charcoal treatment and counting of the released tritiated water was done as described by Oikawa *et al.* [15]. Under the conditions used, tritium release in the presence of cell homogenate was linear over a period of about 24 h and there was a direct relationship between protein content and tritium release. Tritium release of 10% serum containing control cultures was about 7×10^5 cpm/mg cell protein. Tyrosinase activity was also determined according to the method of Chen and Chavin [16]. In this case an aliquot of non-frozen cells suspended in Earle's salt solution (about 50 μg protein) was incubated with 400 nmol (1 μCi) of L-[U-^{14}C]tyrosine and 40 nmol L-DOPA in 1 ml 0.1 mM sodium phosphate buffer (pH 6.8) containing 100 IE penicillin-G-sodium and 135 μg streptomycin sulfate for 2 hours at 37 °C. Incubation was stopped by addition of ice cold TCA (final concentration 5%). Following centrifugation and 4 washes with 5% TCA the pellet was dissolved in 1 N NaOH and radioactivity of incorporated tyrosine determined (under these conditions tyrosine was nearly exclusively incorporated into melanin). A typical incorporation result of control cultures was 7×10^3 cpm/mg cell protein. If one calculates the results (cpm/mg protein) of the ^{3}H-release method [15] and the [^{14}C]tyrosine incorporation method [16] for the same experimental conditions, there is about a 10 fold higher release of ^{3}H than incorporation of [^{14}C]tyrosine into melanin. This difference is probably due partly to differences in the rate of tyrosine hydroxylation and incorporation into the acid insoluble melanin and partly to experimental influences, *e. g.*, freezing of cells, differential effects of the DOPA concentration in both tests [17]. Though we did not find a stoechiometric relationship between both methods the relative results obtained with both methods were in good agreement.

Radioactive studies: L-[U-^{14}C]tyrosine (522 mCi/mmol), L-[3,5-^{3}H]tyrosine (42 Ci/mmol), L-[1-^{14}C]leucine (59 mCi/mmol) and [methyl-^{14}C]thymidine (59 mCi/mmol) were obtained from Amersham-Buchler, Braunschweig. For radioactive studies 0.2 μCi [^{14}C]thymidine (plus 48 μg unlabelled thymidine), 0.2 μCi [^{14}C]leucine or 0.5 μCi [^{3}H]tyrosine were added per 10 ml medium. For determination of incorporated radioactivity, see above.

Results

Cell growth

The average population doubling time of the cells grown in the presence of 10% serum was about

4 days. Incubation of serum containing cultures in the presence of Bu_2cAMP (5×10^{-4} M) and theophylline (5×10^{-4} M) for 3 days resulted in a slight inhibition of cell proliferation (20 – 30%), as determined by cell counting and protein content of treated and control cultures.

Following omission of serum there was still a slight increase of cell number but without any significant increase of protein content per culture during an incubation period of 3 days. As judged from metabolic activity (glucose utilization and lactate production) cells stayed viable under serum-free conditions for at least 3 days. The DNA synthesis rate, as measured by incorporation of [^{14}C] thymidine into DNA, declined exponentially under serum-free conditions and reached about 15% of the controls after an incubation-time of 15 hours (Fig. 1). Protein synthesis as measured by L-[^{14}C] leucine incorporation was less affected by the same treatment than DNA synthesis (Table I). Incorporation of tyrosine which can be utilized for protein and melanin synthesis of the cultures was inhibited to a lesser degree than leucine incorporation, especially under serum-free conditions (Table I). The ratio of incorporated tyrosine: leucine increased from 1.0 (serum-containing state) to 1.5 (serum-free state) to 3.3 (serum-free state + Bu_2cAMP + theophylline) indicating that relatively more tyrosine might have been utilized for melanin formation in serum-free medium (without or with addition of Bu_2cAMP + theophylline) [Table I].

Melanin production and tyrosinase activity

Measurements of the melanin content of cultures incubated in media with different serum concentrations revealed an increasing cellular melanin accumulation in the presence of low or no serum content (Fig. 2). This increase of the melanin content started after about 1 day in low serum or serum-free medium (Table II, Fig. 3). Therefore, the question arose wether this increased melanin content was related to an increased activity of tyro-

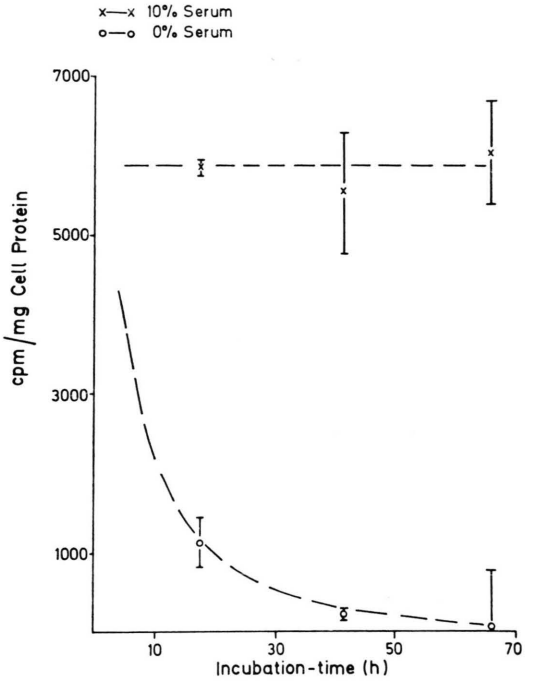

Fig. 1. Influence of serum deprivation on incorporation rate of [^{14}C]thymidine into the DNA of Harding-Passey melanoma cells in culture. Medium renewal (with or without addition of serum) was performed at 0-time. [^{14}C]thymidine pulse during the last 12 h before termination of the cultures. Results: mean (± SD) of 4 cultures for each point.

Table I. Influence of serum-free medium with or without addition of theophylline and dibutyryl cAMP on incorporation of thymidine, leucine and tyrosine into the TCA-insoluble fraction of Harding-Passey melanoma cells in culture.

Medium	Relative incorporation * of			
	[^{14}C]Thymidine	[^{14}C]Leucine	[^{3}H]Tyrosine	Tyrosine / Leucine
10% Serum ("control")	100 ± 10	100 ± 19	100 ± 2	1.0
0% Serum	5 ± 2	36 ± 7	53 ± 10	1.5
0% Serum + Bu_2cAMP (5×10^{-4} M) + Theophylline (5×10^{-4} M)	3 ± 1	27 ± 5	89 ± 7	3.3

* Expressed in % of control cultures (=100%). N of each point: 4 – 8 cultures; average ± SD. Incorporation into controls (cpm/mg cell protein): [^{14}C]thymidine: 19360; [^{14}C]leucine: 27060; [^{3}H]tyrosine: 6070. Incubation-time: 3 days. Labelling period during the last 24 h. For details of the measurements see under Material and Methods.

Table II. Influence of serum-free medium with or without addition of dibutyryl cAMP and theophylline on tyrosinase activity and melanin content of Harding-Passey melanoma cells in monolayer culture (incubation-time: 1 or 3 days).

Medium	Tyrosinase-activity *		Melanin-content *	
	1 day	3 days	1 day	3 days
10% Serum ("control")	100± 4	100± 2	100± 4	100± 6
10% Serum + Bu_2cAMP (5×10^{-4} M) + Theophylline (5×10^{-4} M)	126± 9	136±26	107±18	146±13
0% Serum	102±20	175±33	105±22	185±34
0% Serum + Bu_2cAMP (5×10^{-4} M) + Theophylline (5×10^{-4} M)	147±10	305±59	112±19	326±65

* Tyrosinase activity (cpm/mg cell protein: measured both by [^{3}H]release from [^{3}H]tyrosine or by incorporation of [^{14}C] tyrosine into melanin) and melanin content (E_{400}/mg cell protein) expressed in % of control (=100%). For details of the measurements see under Material and Methods.

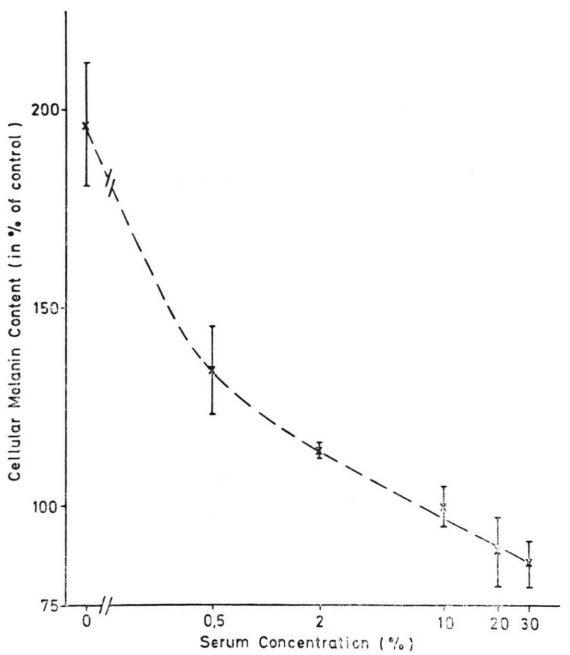

Fig. 2. Influence of serum concentration in media of Harding-Passey melanoma cell cultures on cellular melanin formation. Melanin content (E_{400}/mg cell protein) of control cultures (10% serum) =100%. Incubation time: 3 days, number of cultures analyzed for each point: 4–8; results: mean ± SD.

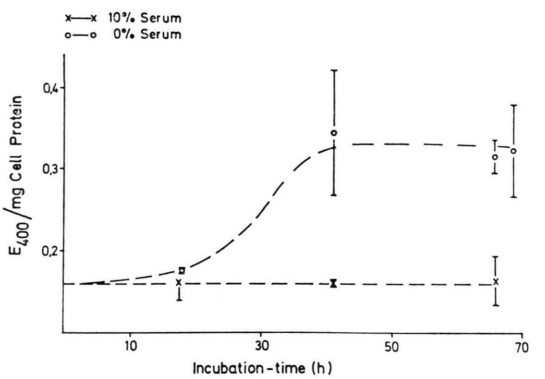

Fig. 3. Influence of media with 10% or 0% serum concentration on melanin formation of Harding-Passey melanoma cells in monolayer culture as a function of incubation-time. Results: mean (± SD) of 4 cultures for each point.

sinase which is regarded as the rate-limiting eznyme in melanin synthesis. Determination of tyrosinase activity of the cells by two different methods revealed that serum omission resulted in an enhanced activity after incubation times of more than 1 day. Presence of Bu_2cAMP and theophylline in serum-free media led to a further striking increase of the enzyme level (Table II).

Contrary to the striking effect of serum removal alone (and its significant enhancement by exogenous cyclic AMP and theophylline) on tyrosinase activity and melanin formation we found a lower effect (20 – 50%) on these two parameters, if the cultures were treated with the combination of Bu_2cAMP and theophylline (each $5-10^{-4}$ M) in the presence of 10% fetal calf serum (Table II). Also theophylline addition alone to serum-free or serum-containing cultures resulted in only a slight (20%) stimulatory effect on melanogenesis.

Effects of cycloheximide

In order to ascertain whether new protein synthesis is required for the increase of melanogenesis exerted by serum-deficient medium, serum-free cultures were incubated in the presence of cycloheximide (0.2 μg/ml) for two days. Under these conditions protein synthesis measured by [^{14}C]leucine incorporation was inhibited by more than 90%. No increase of melanin formation was found after 2 days. Also, stimulation of [^{3}H]tyrosine incorporation into melanin in the presence of serum-free medium (with and without addition of Bu_2cAMP and theophylline) [Table I] was prevented by cycloheximide. Actinomycin D ($0.05 - 0.5$ μg/ml) addition to serum-free cultures for 1 and 2 days resulted in rather severe cytotoxic effects (approx. 80% cell loss). Therefore, these experiments must be interpreted with some caution. At any case, the remaining cells (approx. 20%) did not show an increase of melanin content following serum removal.

Discussion

In accordance with the findings of other authors in non-melangogenic cells [1, 9, 11, 18, 19] it was shown in the present study that serum omission resulted in a cessation of cell proliferation of melanin producing melanoma cells in monolayer culture. The DNA synthesis rate, as measured by incorporation of [^{14}C]thymidine into DNA, declined exponentially under serum-free conditions and reached about 15% of the control cells after an incubation time of 18 hours (Fig. 1). Also, cellular protein synthesis was inhibited, but to a lesser degree than DNA synthesis (Table I).

In previous studies it was shown that melanization occurs primarily under conditions of nearly stationary growth in crowded monolayer cultures [3] (for review see ref. 2). Therefore, the question arose whether the proliferation restricting state of serum omission has an influence on melanin formation. Indeed, an effect of the serum concentration on melanin formation was found (Fig. 2). A striking rise of the celluar melanin content occured after $1 - 2$ days in serum-free medium (Fig. 3). This increase could either be due to an enhanced rate of synthesis or to a decreased breakdown. If an increase of the melanin synthesis rate would have happened, one could expect a rise of the activity of cellular tyrosinase, which is regarded as the rate limiting enzyme in melanin formation. Actually, there was a significant enhancement of cellular tyrosinase activity under serum-free conditions (Table II). This increase started about 1 day following serum omission and it appeared to precede melanin accumulation. The latter was strikingly higher than in control cells after about 2 days (Fig. 3). This observation of an increased tyrosinase activity and enhanced melanin formation in serum-free medium is of particular interest.

A possible mediator of the effect of serum restriction on melanogenesis could be cAMP. Thus, serum removal or addition of Bu_2cAMP and theophylline to serum-free or serum containing cultures resulted in increased intracellular cAMP levels, whereby the strongest effect was observed in the case of serum omission together with treatment of Bu_2cAMP (5×10^{-4} M) and theophylline (5×10^{-4} M). Thus, short-term treatment with serum-free medium alone or together with Bu_2cAMP and theophylline resulted in about a 2-fold respectively 4- to 12-fold increase of the intracellular cAMP content (unpublished results). Other investigators have previously shown a stimulating effect on melanization of serum containing mammalian pigment cell cultures by addition of exogenous cAMP (respectively its dibutyryl derivative) or of agents which elevate the intracellular cAMP level by activating the synthesis or inhibiting the breakdown of cAMP [5–8, 20–26]. Since serum removal combined with addition of exogenous Bu_2cAMP and theophylline resulted not only in the strongest effect on intracellular cAMP level but also in a greater stimulation of tyrosinase activity and melanin formation than each treatment alone (Table II), these findings support the concept of a direct relationship between cAMP and melanogenesis.

We cannot confirm the recent observation [7] that addition of theophylline alone to melanoma cultures produced a greater stimulation of melanogenic activity than the simultaneous treatment with Bu_2cAMP and theophylline. We only found a slight stimulatory effect (approx. 20%) by theophylline ($5 \times 10^{-4} - 1 \times 10^{-3}$ M) alone added up to three days to serum-free or serum containing cultures. Assuming that this stimulatory effect results from the inhibition of cAMP breakdown, then the enhanced effects of serum-free treatment with or without addition of theophylline and Bu_2cAMP suggest that not only the degradation but also the synthesis or the storage

of cAMP are affected in serum-free cultures. However, the possibility of an altered responsiveness of the melanin synthesising machinery towards cAMP under serum-free conditions cannot be ruled out, at present.

In regard to influences of cAMP on the enzymic expression of mammalian cells, the following observations are noteworthy. Thus, treatment of cultured cells with cAMP or the dibutyryl derivative was found to increase the activities of phosphoenol pyruvate carboxykinase [27], tyrosine aminotransferase [27–31], tyrosine hydroxylase [32], serine dehydratase [33], alkaline phosphatase [34], and acetylcholinesterase [35]. Somewhat reminiscent of the stimulatory effect of serum-free medium on melanogenesis are findings on an increase in the proportion of morphologically differentiated neuroblastoma cells [36], in the rate of acetylcholinesterase synthesis in cultured neuroblastoma cells [36, 37] and about a severalfold rise of the glia-specific acidic protein S-100 in a glia cell line [38], when the culture medium lacks serum.

Studies are in progress to elucidate whether the increase of tyrosinase activity induced by serum removal (with or without addition of Bu_2cAMP and theophylline) is due to an activation of a preexisting enzyme or to a stimulation of de novo synthesis. Preliminary experiments showing cycloheximide (or actinomycin D) to prevent the stimulation of melanin formation under the influence of serum-free medium (with and without Bu_2cAMP and theophylline) favor the interpretation that de novo protein synthesis is required for this stimulatory effect, in spite of the fact that overall protein synthesis is reduced (Table I) under the influence of serum removal.

This study was supported in part by a research grant from Stiftung Volkswagenwerk. We wish to thank Mrs. H. Bonner for her skillful technical assistance.

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