Tyrosinase activity in primary cell culture of amelanotic melanoma cells

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After transfer of the Ab amelanotic melanoma cells from in vivo to in vitro growth conditions tyrosinase activity in their soluble fraction rapidly increased. This increase lasted to the middle of the logarithmic phase of growth and was followed by a decrease of tyrosinase activity, which was accompanied by accumulation of melanin in the cells. Calf serum stimulated simultaneously tyrosinase activity, melanin synthesis, and proliferation of the melanoma cells. Acrylamide-gel electrophoresis patterns of soluble tyrosinase from the Ab melanoma cells cultured in vitro consisted of two bands, similarly as soluble tyrosinase from the Ma melanotic melanoma cells freshly isolated from solid tumors.

Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) plays an essential role in the process of melanogenesis. This enzyme catalyzes the following reactions: i) hydroxylation of tyrosinase to dihydroxyphenylalanine (dopa); ii) oxidation of dopa to dopaquinone; iii) conversion of 5,6-dihydroxyindole to melanochrome (9).

The Ab amelanotic melanoma originated as a result of a spontaneous alteration of melanotic melanoma. The alteration involved an increase in the growth rate and the loss of the ability for melanosome and melanin synthesis (1,2,3). In spite of the lack of melanin, a low tyrosinase activity has been detected in Ab amelanotic melanoma tumors maintained by serial passages in Syrian hamsters (6). Tyrosinase in the Ab melanoma differs from that of the Ma melanotic melanoma in the gel-electrophoresis pattern of the soluble cell fraction, which consists of one band, while that of the melanotic melanoma consists of two bands (16). After transfer of the Ab amelanotic melanoma cells from in vivo to in vitro growth conditions a rapid melanin synthesis occurred in their cytoplasm (15). In this paper we describe changes of the activity and the electrophoretic pattern of soluble tyrosinase in Ab cells cultured in vitro.
Materials and Methods

The Ab amelanotic melanoma (passages 412-416) and the Ma melanotic melanoma (passages 171-173) were used for experiments. The melanomas were transplanted in random-bred, conventional, male Syrian hamsters by the suspension method (1).

For starting primary cell cultures the cells were isolated from solid tumors of the Ab amelanotic melanoma by means of the non-enzymatic method described earlier (15).

The cultures were kept in an incubator (Assab, Sweden) under the following conditions: 37°C, 5% CO₂ in air, 80% humidity. The cell cultures were started with 10⁶ cells, suspended in 10 ml of culture medium consisting of Eagle's minimal essential medium (MEM) supplemented with 10% calf serum (Biomed, Poland), 100 µg/ml streptomycin, and 100 units/ml penicillin and were placed in Petri dishes of 80 mm diameter. The medium was changed after 1 and 3 days of culture. In type-I culture the changed medium was the same as at the start, while in type-II culture the changed medium consisted of 100% MEM supplemented with antibiotics as described above, but devoid of calf serum. Six cultures of each type were made. To determine growth kinetics the cells were counted daily.

The relative melanin content in melanoma cells was determined by the electron-spin-resonance (e.s.r.) method (11). The samples of 4 x 10⁶ cells were examined using a Varian E-4 spectrometer, equipped with 100 kHz magnetic field modulation and working in X band, as described previously (15). The relative degree of melanization of the cultured cells was monitored by recording the amplitude of the e.s.r. signals with a microwave power of 10 mW and modulation amplitude of 8 G.

The soluble fraction of the melanoma cells for tyrosinase assays or for gel electrophoresis was obtained by a method described earlier (16).

Tyrosinase (dopa oxidase and tyrosinase hydroxylase) activity in the soluble fraction was determined colorimetrically at 475 nm in a Specord UV VIS spectrophotometer (Carl Zeiss Jena, GDR), using as substrates 1 µmol of L-dopa or 1 µmol of L-tyrosine + 0.05 µmol of L-dopa (BDH Chemicals Ltd., England) in 0.1 M phosphate buffer, pH 6.8, in a total vol. of 1 ml in a cuvette with a 1-cm light path (16). Tyrosinase activity was expressed as µmol of dopachrome produced by 1 mg of protein in 1 or 2 h. Protein concentration was determined by the method of Spector (17).

Acrylamide-gel electrophoresis was carried out according to the method described by Scisłowski et al. (18). Gels were stained for tyrosinase activity with 1 mM L-dopa or 1 mM L-tyrosine + 0.05 mM L-dopa in 0.1 M phosphate buffer, pH 6.8. The stained gels were traced at 475 nm using the densitometer (Carl Zeiss Jena, GDR).

Results

Growth kinetics of the Ab melanoma cells in cell culture are presented in Fig. 1. The growth rate of the cells cultured in the medium with serum was higher than that of the cells cultured in the serum-free medium. In type-II cultures, the cells started to detach
gradually from the glass on day 2, after the medium containing serum had been replaced with the serum-free medium. In type-I cultures, this process started on day 3, and it was accompanied by an accumulation of melanin in the cells.

The e.s.r. spectra of the Ab cells are presented in Fig. 2. In the Ab melanoma cells isolated directly from solid tumors and derived from cell cultures on days 1 and 2, melanin was not detectable by the e.s.r. method. A melanin e.s.r. signal in the cells appeared on day 3, and later its amplitude increased, reaching a maximum on days 5 and 6. The amplitudes of the e.s.r. signals, reflecting differences in the degree of melanization of the melanoma cells, were greater in the cells detached from the glass than in those attached to it, and they were greater in the cells from type-I cultures than in the cells from type-II cultures.

Gel-electrophoresis patterns of the soluble fraction of melanoma cells stained for dopa oxidase and tyrosine hydroxylase activity are presented as densitometric traces in Fig. 3. In both the soluble fraction of Ab cells cultured in vitro and the Ma melanoma cells freshly isolated from solid tumors, stained for dopa oxidase and tyrosine hydroxylase activity, two bands with the same locations were present.

The levels of dopa oxidase and tyrosine hydroxylase activity in the soluble fraction of Ab cells are presented in Table 1. Both these activities increased in the early phase of growth and reached their maximum at day 3 of culture. On this day the soluble dopa oxidase and tyrosine hydroxylase activities were significantly higher in the cells from a monolayer of type-I culture than in the cells from type-II culture. As soon as the cytoplasm of the cells filled with melanin, both these activities decreased.
Fig. 2. E.s.r. spectra of Ab melanoma cells.
1, Cells isolated from solid tumor;
2, cells growing upon glass at day 2 of type-II culture;
3, cells growing upon glass at day 2 of type-I culture;
4, cells growing upon glass at day 3 of type-II culture;
5, cells growing upon glass at day 3 of type-I culture;
6, cells detached from the glass at day 3 of type-II culture;
7, cells detached from the glass at day 3 of type-I culture;
8, cells detached from the glass at day 5 of type-II culture;
9, cells growing upon glass at day 5 of type-I culture;
10, cells detached from the glass at day 5 of type-I culture.

Discussion
In different melanotic melanomas there usually occur two isozymic forms of soluble tyrosinase (4,8,19). Gel-electrophoresis patterns of the soluble fraction of melanoma cells isolated freshly from solid tumors and stained for tyrosinase activity consisted of two bands in the case of the Ma melanotic melanoma, but of only one band in the case of the Ab amelanotic melanoma (16). The soluble fraction of the Ab cells cultured in vitro gave two electrophoretic bands of
Fig. 3. Densitometric traces of the gel-electrophoresis patterns of the soluble fraction of cultured Ab melanoma cells and Ma melanotic melanoma cells isolated from solid tumor, both stained for tyrosinase activity.

1, Ab cells growing upon glass at day 2 of culture + 1 mM L-dopa;
2, Ab cells detached from the glass at day 3 of culture + 1 mM L-dopa;
3, Ab cells growing upon glass at day 4 of culture + 1 mM L-dopa;
4, Ma cells + 1 mM L-dopa;
5, Ab cells growing upon glass at day 2 of culture + 1 mM L-tyrosine + 0.05 mM L-dopa;
6, Ab cells detached from the glass at day 3 of culture + 1 mM L-tyrosine + 0.05 mM L-dopa;
7, Ab cells growing upon glass at day 4 of culture + 1 mM L-tyrosine + 0.05 mM L-dopa.

Tyrosinase activity located in the same position as the two bands of the soluble fraction of the Ma melanoma. The identical gel-electrophoresis patterns of the soluble fractions of the Ab cells cultured in vitro and the Ma cells freshly isolated from solid tumors, both stained for tyrosinase activity, suggest that soluble tyrosinase in these types of cells does not differ qualitatively. The appearance of the second band of soluble tyrosinase after transfer of Ab cells from in vivo to in vitro growth conditions might be caused by a chemical modification of a part of soluble tyrosinase molecules or by an increase in the activity of this enzyme in cell culture.

During prolonged transplantation of Ab amelanotic melanoma in Syrian hamsters tyrosinase activity gradually diminished and the growth rate increased (1). At present in the soluble fraction of the cells freshly isolated from this tumor, dopa oxidase activity is 0.21 μmol of
Table 1. Tyrosinase activity in the soluble fraction of Ab melanoma cells cultured in vitro

The values are means of determinations from two cultures, each performed in duplicate. The differences between separated assays were below 10%. g, cells growing upon glass; d, cells detached from the glass.

<table>
<thead>
<tr>
<th>Type</th>
<th>Day</th>
<th>Dopa oxidase activity (μmol x mg⁻¹ x h⁻¹)</th>
<th>Tyrosinase activity of tyrosine hydroxylase activity (μmol x mg⁻¹ x h⁻¹)</th>
<th>Tyrosine hydroxylase activity (μmol x mg⁻¹ x 2h⁻¹)</th>
</tr>
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<tr>
<td></td>
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<td>g</td>
<td>d</td>
<td>g</td>
</tr>
<tr>
<td>I</td>
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<td>0</td>
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<td></td>
<td>2</td>
<td>3.11</td>
<td>-</td>
<td>0.21</td>
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<tr>
<td></td>
<td>3</td>
<td>18.71</td>
<td>6.72</td>
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<td></td>
<td>4</td>
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<td>0.56</td>
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<tr>
<td>II</td>
<td>3</td>
<td>6.41</td>
<td>5.29</td>
<td>0.71</td>
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Dopachrome x mg⁻¹ of protein x h⁻¹, but tyrosine hydroxylase activity is detectable only after 2 h of measurement and it amounts to 0.011 μmol of dopachrome x mg⁻¹ of protein x 2h⁻¹ (16). In the soluble fraction of the Ma melanotic melanoma, which is related to the Ab melanoma by a common origin, dopa oxidase and tyrosine hydroxylase activities amount to 1.5 and 0.19 μmol of dopachrome x mg⁻¹ of protein x h⁻¹, respectively (16). During the first day of type-I culture, tyrosine hydroxylase and dopa oxidase activities increased 14- and 64-fold, respectively, in comparison with the solid Ab melanoma tumor. Afterwards tyrosinase activity increased still more, reaching its maximum in the middle of the logarithmic phase of growth, i.e. at day 3 of culture. On this day tyrosine hydroxylase and dopa oxidase activities were 300 and 900 times higher, respectively, than in the solid Ab tumor and 10 and 12 times higher than in the solid Ma tumor. These results indicate that in the Ab melanoma, which has been amelanotic for 20 years of in vivo maintenance, a biochemical potential for melanogenesis has persisted, being even higher than that of the melanotic melanoma in vivo. The increase in tyrosinase activity during the early phase of growth up to the middle of the logarithmic phase of growth corresponds to the results published by some authors for melanotic melanomas (5,12). Melanin content, tyrosinase activity, and rate of cell proliferation were higher in the cells cultured in the medium with serum than in those cultured in the serum-free medium. A similar influence of serum on melanogenesis and proliferation was observed in some clones of melanotic melanomas (7,10). A simultaneous stimulation of cell proliferation and tyrosinase activity by calf serum and an increase in activity of this enzyme during the exponential phase of growth suggest that the increase in tyrosinase activity might be a result of a general stimulation of anabolic processes connected with proliferation of Ab cells in vitro.

In the primary Ab cell culture, tyrosinase activity decreased after reaching its maximum. This decrease was accompanied by accumulation of melanin in the cells. This effect may be caused by blocking
of tyrosinase by products of melanogenesis and/or by this enzyme's own action (15,16).

Melanin, melanosomes, and tyrosinase activity are markers of differentiation of normal and malignant melanocytes. After transfer of the Ab cells from in vivo to in vitro conditions tyrosinase activity rapidly increased at day 1 of culture, but melanin was detected by histologic, histochemical, and e.s.r. methods at day 3 (15). In mammalian melanocytes, the process of melanin synthesis occurs in melanosomes. The time when the processes of melanosome synthesis and melanization start in cultured Ab melanoma cells remains to be elucidated by ultrastructural studies.

It is not astonishing that an increase of tyrosinase activity precedes melanin synthesis in amelanotic melanoma cells after their transfer to in vitro growth conditions, but it is striking that the increase in tyrosinase activity is so great. The above results indicate that a change in the extracellular environment may rapidly affect the biochemical apparatuses of differentiated function of malignant melanocytes.

References


