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Melanization of Bomirski hamster amelanotic melanoma cells (Ab line) depends on the type of culture medium

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Abstract

Introduction. The effect of melanogenesis intensity on melanoma biology remains an open question, and the biological differences between melanotic and amelanotic melanoma cells have not yet been satisfactorily documented. As a result, the melanization of melanoma cells in *in vitro* cultures is not considered among experimental procedures. The aim of this study was to investigate the effect of the medium used to culture Bomirski amelanotic Ab melanoma cells on the melanogenesis process.

Material and methods. Amelanotic melanoma cells (Ab) were cultured in two media recommended for *in vitro* melanoma cell cultures, RPMI and DMEM. The melanization was evaluated by determining the melanin and tyrosinase presence in the cells using spectrophotometrical and western blot methods, respectively. Changes in Ab melanoma cells' ultrastructure were determined using electron microscopy (EM).

Results. The medium with higher level of tyrosine (DMEM) induced significant melanization of amelanotic melanoma cells (Ab) after only 24 h, while the RPMI medium, with a lower level of tyrosine, weakly affected melanin production. Melanization of Ab cells was paralleled by an increase in the amount of tyrosinase protein. Induced melanization was easily observed on EM-micrographs in the form of newly formed melanosomes containing melanin pigment. Melanosomes at stages from one (I) to four (IV) were observed.

Conclusions. Culture medium has an important effect on the *in vitro* biology of amelanotic melanoma cells, since it can affect the rate of cellular melanization. The appropriate medium should be carefully selected, taking into account the known biology of the melanoma cells being used. *(Folia Histochemica et Cytobiologica 2018, Vol. 56, No. 4, 207–214)*

Key words: Bomirski melanoma; amelanotic melanoma; melanogenesis; culture media; melanin; tyrosinase; western blotting; electron microscopy

Introduction

Melanoma cells are characterized by different amounts of melanin, and the color of a melanoma tumor can range from black through brown and blue, to white [1, 2]. Melanomas lacking or with very low levels of melanin are referred to as amelanotic, and can be difficult to identify [3, 4]. Amelanotic melanomas occur in 2–12% of all skin melanoma patients [2, 3, 5]. Additionally, it is estimated that from 14% to 60% of melanoma metastases are amelanotic, even when the primary melanoma is melanotic [6, 7], and the amelanotic form also dominates among melanomas in children [8]. Amelanotic melanoma cells are thus not so rare in general. The question of the effect of melanogenesis on the biology of melanoma cells remain controversial, mainly because there have been insufficient systemic studies of the comparative biology of melanotic and amelanotic melanoma cells. Melanotic and amelanotic melanoma forms thus receive the same treatment, despite suggestions that melanoma cells with melanin differ from those without melanogenesis [9–13]. In our laboratory, we use

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the Bomirski hamster melanoma model to compare the biology of melanotic and amelanotic melanomas; this consists of two basic lines, melanotic Ma and amelanotic Ab, which arose as a consequence of spontaneous alteration from the Ma form. The change of a melanotic into an amelanotic melanoma results in growth rate acceleration, a lack of melanosomes, ultrastructural changes in cells [14], and a low rate of spontaneous apoptosis, as well as high susceptibility to induced apoptosis [15–18]. The amelanization of melanoma cells is not well understood. It has been proposed that the lack of melanin in these cells is the result of disturbances in the biosynthesis of premelanosomes [14, 19]. The Ab line is a fast-growing, highly anaplastic, metastasizing malignant tumor. Słomiński *et al.* observed that Ab cells *in vitro* short cultures regained the ability to synthesize melanin [20, 21].

We have observed that the media recommended for *in vitro* cultivation of melanoma cells, RPMI-1640 and DMEM, induce the melanization of the amelanotic Ab line to different degrees. Tyrosinase (TYR) plays an essential role in the process of melanogenesis, carrying out tyrosine hydroxylation to L-3,4-dihydroxyphenylalanine (DOPA), which is rapidly oxidized to dopaquinone [22]. Brown–black eumelanin is produced by spontaneous cyclization of dopaquinone to dopachrome which, in the presence of the enzymes tyrosinase-related protein 1 (TRP1) and tyrosinase-related protein 2 (TRP2), can change into DHICA-melanin (DHI-2-carboxylic acid) or insoluble DHI-melanin (5,6-dihydroxyindole) [22]. Taking this into account, we wished to compare the rate of melanization of amelanotic Ab melanoma cells during culturing of up to 48 h in DMEM and RPMI media by determining tyrosinase protein and melanin levels with the cell ultrastructural changes.

Material and methods

Bomirski hamster melanoma model. The Bomirski hamster melanoma model has two basic melanoma lines: the melanotic Ma and the amelanotic Ab. The native melanotic Ma line was derived from a spontaneous melanoma of the skin (near the nose) in a male Syrian (golden) hamster in 1959; it has been maintained by serial transplantation through random-bred animals [12]. The amelanotic melanoma line (Ab) originated in 1963 from the Ma form by a spontaneous alteration [14]. The Ab amelanotic melanoma lost the ability to produce melanin as the result of disturbances in melanosome formation, but retains the tyrosinase activity at a much lower level than Ma line [14, 20]. The lack of melanin synthesis is accompanied by changes in many biological features, such as faster tumor growth rate, shorter animal survival, and changes in cells' ultrastructure [12].

The amelanotic melanoma was transplanted on 3–12 month-old male Syrian (golden) *Mesocricetus auratus* Waterhouse hamsters. The experimental procedures were approved by the Animal Ethics Committee at the Medical University of Gdansk (4/2011) and conducted in accordance with National Health and Medical Research Council's guide for the care and use of laboratory animals.

Isolation of melanoma cells. Both types of melanoma cells were isolated from the solid tumors by a nonenzymatic method reported previously [16]. The cell suspension contained 90–95% viable cells, as estimated by trypan blue dye exclusion assay. The primary cells were cultured at a density of 2.5×10^6 in 10 ml of basic medium (RPMI, Biomed-Lublin, Poland), supplemented with 10% FBS (fetal bovine serum, Sigma-Aldrich, St. Louis, MO, USA) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, Sigma-Aldrich) on Petri dishes. After isolation, the cells were precultured in the basic medium at 37°C, at 5% of CO_2 for 20 h before experiments.

Cell culture. There were two experimental groups of cell culture: the first was cultured in the basic RPMI-1640 (Roswell Park Memorial Institute) medium, and the second in DMEM (Dulbecco Modified Minimal Essential Medium), both supplemented with 10% FBS and antibiotics. DMEM has more (72 mg/l) L-tyrosine, the primary amino acid for the melanin synthesis, than RPMI (20 mg/l). The media differ also in their level of phenylalanine (66 mg/l in DMEM, 15 mg/l in RPMI), which is hydroxylated into L-tyrosine in the presence of L-phenylalanine hydroxylase. Both media are recommended for culturing melanoma cells *in vitro*. The melanization of Ab melanoma cells was determined by the cell ultrastructure, melanin content, and tyrosinase protein level after 24 and 48 h incubation in the media. Ma melanoma cells cultured for 24 h in RPMI were used as a positive control for the melanin content and tyrosinase protein level.

Ultrastructural changes. The collected cells were rinsed in PBS and the pellet immediately fixed in a solution of 2.5% glutaraldehyde (GA) in Na-cacodylate buffer at pH 7.4. Fixation was carried out at 4°C for 24 h, and the material was then rinsed three times in the same buffer. Postfixation was performed with 1% osmium tetroxide in an Na-cacodylate buffer for 2 h. Following fixation, the cells were dehydrated in a graded series of ethanol, immersed in propylene oxide, embedded in Epon812, and polymerized at 37°C. Sectioning was performed with a glass knife on an OmU2 ultramicrotome (Richert, Austria). Semithin sections (1.5 μm) were stained with toluidine blue and examined under a light microscope (Jenaval, Carl Zeiss Jena, Germany). Ultrathin sections were placed on formvar-covered copper grids and double stained with uranyl acetate and lead citrate. Observations were made using a 1200 EXII transmission electron microscope (Jeol, Tokyo, Japan) at an accelerating voltage of 80 kV.

Melanin content. The total amount of melanin in cells was estimated spectrophotometrically (Multiscan FC, Thermo Scientific). 2×10^6 cells were incubated with 1 N NaOH in 10% DMSO for 2 h at 60°C with shaking [23]. Samples were plated on 96-well plates and the absorbance was measured at 450 nm. Melanin content was determined using the standard curve of synthetic melanin (Sigma-Aldrich).

Immunoblotting of tyrosinase. Total cell lysates were obtained by incubating 2×10^6 cells in the lysis buffer RIPA (Sigma-Aldrich) for 1 h on ice, and then spinning for 15 min at 14,000 rpm. The supernatants were collected and stored at –70°C for further processing. The total protein was quantified using a Bradford assay (Bio-Rad, Hercules, CA, USA). 60 μg of lysate was subjected to electrophoresis in 10% SDS gel under reducing conditions and transferred to nitrocellulose membrane (Bio-Rad). After 1 h blocking in 5% nonfat milk, membranes were probed overnight with rabbit polyclonal antityrosinase antibody (1:5000, Abcam) at 4°C. After washing, horseradish peroxide (HRP)-conjugated anti-rabbit IgG was added for 1 h (1:10000, Sigma-Aldrich). The blots were reprobed with an antibody against β-actin (1:6000, Sigma-Aldrich) to confirm that there were equal amounts of protein in the lanes. A chemiluminescent signal was developed using the Super Signal West Pico system (Thermo Scientific, USA) and documented by a Chemi Doc XRS+System camera (BioRad). Band intensity was semiquantified using ImageLab 5.2.1 software (National Institutes of Health, USA) and was shown as a ratio to actin.

Statistical analysis. Statistical analysis was performed using the Statistica data analysis software system version 12 (Statsoft, Krakow, Poland). Data are displayed as arithmetical mean ± standard deviation (SD). The nonparametric Jonckheere and U Mann Whitney tests were used; differences with $p < 0.05$ were considered statistically significant.

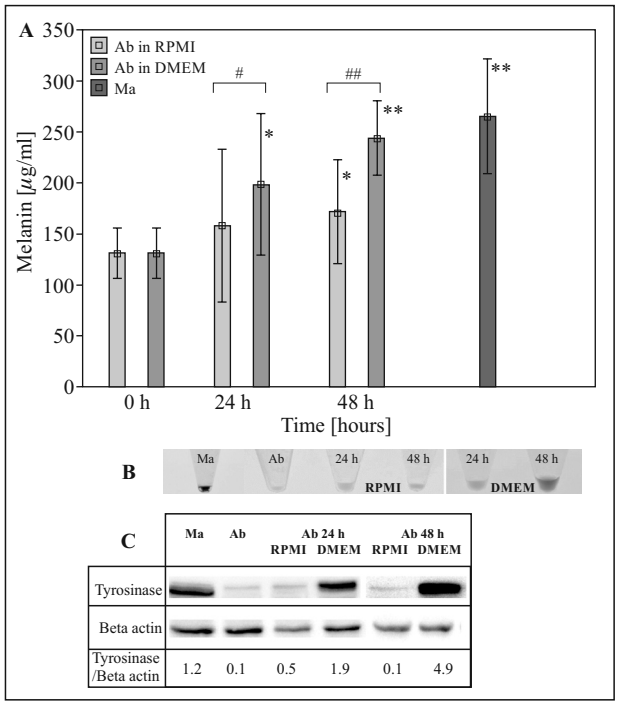

Figure 1. Parameters of Ab melanoma cell melanization during *in vitro* culturing for 24 h and 48 h in RPMI and DMEM. **A.** Comparison of melanin content of Ab amelanotic melanoma cells cultured in RPMI and DMEM media. Ma melanotic melanoma cells were used as a positive control for melanin production. Values are means ± SDs of 5 (0 h) and 7 (24 and 48 h) experiments. Statistical analysis was performed using the nonparametric Jonckheere and U Mann-Whitney tests. Statistically significant increases were observed in melanin levels in comparison to the 0 h time point (*) and between RPMI and DMEM (#); #, * $p < 0.05$; ##, ** $p < 0.01$. **B.** Macroscopic darkening of cell lysates from cultured cells. **C.** Tyrosinase protein levels in Ab melanoma cells after 24 h (Ab24h) and 48 h (Ab48h) of incubation in RPMI or DMEM, detected by western blotting. Ma melanoma cells were used as a positive control for tyrosinase presence. Beta actin was estimated as a protein loading control. The tyrosinase-to-beta-actin ratio reflects the relative changes of the tyrosinase content during culturing.

RESULTS

Melanin content

At the beginning of experiment (0 h, after overnight preincubation), the Ab melanoma cells had a low level of melanin (131 μg/ml) as the result of these cells' transfer from *in vivo* to *in vitro* growth conditions. The rate of melanization depends on the medium used, being much faster in DMEM than in RPMI. The Ab cells undergo very weak melanization in the RPMI medium; after 48 hours melanin levels increased by 32% ($p < 0.05$, Fig. 1A). Melanization in RPMI during the course of the whole incubation was not easily noticed macroscopically (Fig. 1B). As a result of melanization, amelanotic Ab cells in DMEM changed morphology, becoming rounded and detached. Pigmentation in DMEM was macroscopically observed after 24 h, when the melanin content increased by over 50%, reaching 198 μg/ml ($p < 0.05$). After 48 h melanin synthesis increased by an additional 20% ($p < 0.01$) and darkening of cells was observed macroscopically (Fig. 1A, B). After two days of incubation of Ab melanoma cells in DMEM, the melanin level (246 μg/ml) was similar to that in the melanotic Ma melanoma (265 μg/ml) (Fig. 1A).

Tyrosinase protein level determined by western blot

Tyrosinase protein from the Ma melanotic and Ab amelanotic melanoma cells forms bands at the same position (molecular weight 68 kDa). In the Ab amelanotic melanoma, tyrosinase was detected at a very

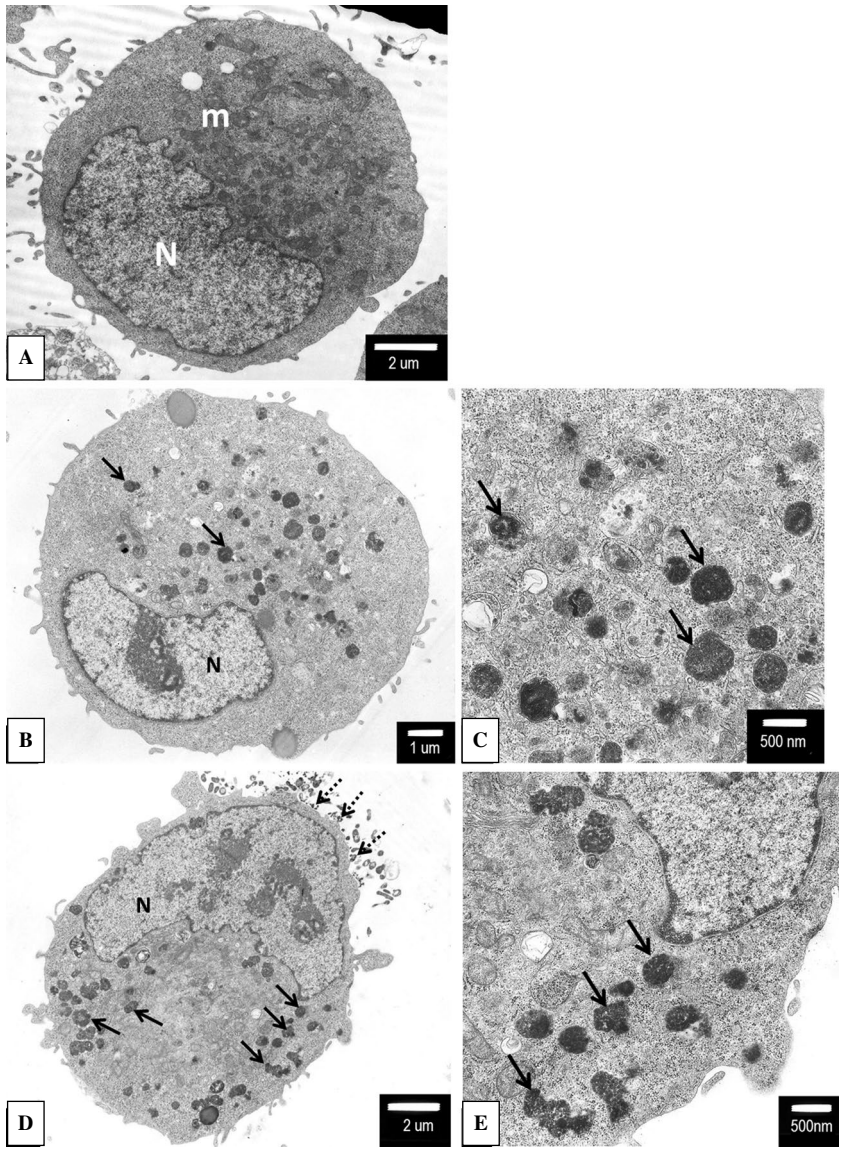

Figure 2. Ultrastructural changes in Ab amelanotic melanoma cells cultured in RPMI medium. At time 0, cells **(A)** showed no apparent melanosomes, had typical euchromatic nuclei (N) and cytoplasm occupied by numerous mitochondria (m); some microvilli were seen on the outline of the cell. Cells cultured for 24 h **(B, C)** and 48 h **(D, E)** showed very weak melanization. Granular melanosomes (arrows) were observed in the cytoplasm. After 48 h, melanin granules (dotted arrows) were seen outside the cell; these had plausibly been extruded from the cells (D).

low level — tenfold lower than in the Ma melanotic line. Cells cultured in RPMI showed an increase in tyrosinase level after 24 h, but then the protein level seemed to decrease (Fig. 1C).

The Ab amelanotic melanoma cells cultured in DMEM rapidly increased in tyrosinase content over cultivation time (Fig. 1C), and this was accompanied by the darkening of cell pellets (Fig. 1B). After the first day of culturing of Ab amelanotic melanoma cells their tyrosinase protein level reached a value similar to the melanotic Ma cells (Fig. 1C). After one additional day, the amount of enzyme more than doubled.

Ultrastructural changes in amelanotic melanoma cells determined by transmission electron microscopy

The ultrastructure of the Bomirski Ab amelanotic hamster melanoma cells was examined with particular reference to the melanization process. Cells cultured for 24 or 48 h in the medium with low tyrosine content (RPMI) (Fig. 2) demonstrated weak melanization as compared with cells incubated in DMEM medium containing higher level of tyrosine (Fig. 3). The latter showed more advanced melanization with higher content of melanosomes. Although different stages of granular melanosomes development were seen,

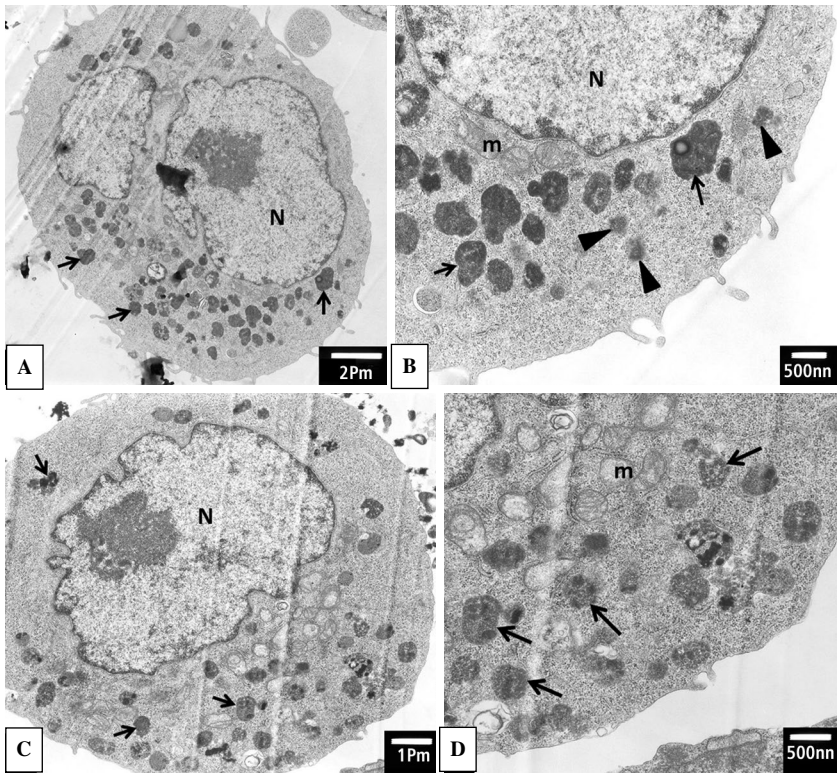

Figure 3. Induced melanization of Ab amelanotic melanoma cells incubated in DMEM for 24 h **(A, B)** and 48 h **(C, D)**. TEM images of cell cultures revealed an increase in the number of melanosomes with time. We observed melanosomes (arrows) as membrane-limited dense granules containing melanin, and occasionally some premelanosomes (arrowheads) with patterned matrices; N, nucleus.

the III and IV stages melanosomes dominated in cells cultured in DMEM for 48 h. These granular, rounded, 200–600 nm-wide melanosomes, with a diversity of architectures, demonstrated the deposition of electron-dense melanin granules (Fig. 3D). Melanotic Ma melanoma cells, which are native to the amelanotic Ab melanoma cells, cultured in the RPMI medium for 24 h, were used as a positive control for melanosome presence (Fig. 4). They showed the presence of typical four stages of melanosomes: stage I (early endosomes), stage II (premelanosomes with patterned lamellar matrices), stage III (partially melanized melanosomes with lamellar patterns), stage IV (melanosomes in the final stage of melanization, showing uniformly melanin-filled electron-dense granular cores). In the Ma cells granular melanosomes dominated whereas fibrillar melanosomes were rare (Fig. 4C).

Discussion

Amelanotic melanoma is one of the less known forms of melanoma, and is characterized by a lack of melanin, lower cell differentiation, higher malignancy, and worse prognosis than melanotic melanomas [2, 9–11]. Melanization of amelanotic melanoma cells *in vitro* is very important aspect, especially as the melanoma lines most easily grown *in vitro* are amelanotic forms, and are used as targets for testing new chemotherapeutics. The process of *in vitro* amelanotic melanoma melanization has been known for many years but is not fully understood [24–28]. However, the modulation of melanin levels in melanoma cells is perhaps a means by which melanoma cells become sensitized to therapy [29–32].

Two media are generally used in culturing amelanotic melanoma cells: RPMI and DMEM. The differences in their effects on melanization have been checked in human melanoma SKMEL188 cells [28] and mouse B16F10 cells [25]. In SKMEL188 cells, DMEM enhanced melanin production, while strongly stimulating the expression of tyrosinase and melanocortin 1 receptor (MC1R) genes. Additionally, DMEM was found to stimulate tyrosinase activity five times more strongly than L-tyrosine alone [28]. B16F10 cells cultured in DMEM showed tyrosinase activity, produced melanin, and underwent morphological changes (in the form of developing dendrites),

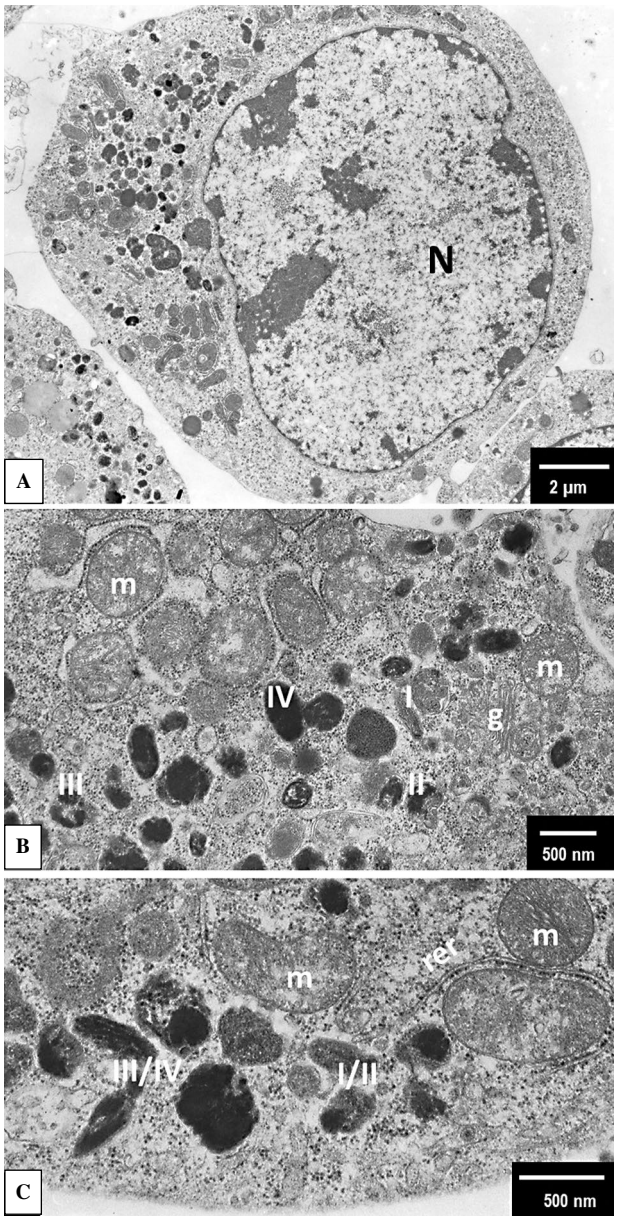

Figure 4. Ultrastructure of melanotic Ma melanoma cells cultured for 24 h in RPMI medium. **A.** General view of the Ma melanoma cell with melanosomes as electron-dense in a form of elongated to oval organelles. **B** and **C.** Melanosomes at four different stages of melanization. Stages: I. early endosomes; II. premelanosomes with patterned lamellar matrices; III. partially melanized melanosomes with lamellar patterns; IV. melanosomes in the final stage of melanization, showing uniformly melanin-filled electron-dense granular cores. Abbreviations: N — nucleus; m — mitochondria; g — Golgi apparatus; rer — rough endoplasmic reticulum.

but the elevated levels of tyrosine and phenylalanine in this medium were not the only factors responsible for phenotypic changes [25]. Additional factors that might enhance melanization include cAMP and αMSH [25]. Our results on the hamster amelanotic Ab line also confirm the observation that DMEM can induce melanogenesis in amelanotic melanoma cells. DMEM does not have the same effect on all human amelanotic melanoma lines — for example, it is the medium recommended by the American Type Culture Collection for the A375 melanoma line, which is widely used in many laboratories.

Over 30 years ago, hamster amelanotic Ab melanoma cells were cultured *in vitro* in Eagle's minimum essential medium, and from the third day begun melanin production, inhibiting proliferation [20, 21]. Our results indicate that the contemporary use of DMEM for melanoma cells *in vitro* cultures also induces melanization of the Ab melanoma line. This result confirms that amelanotic melanoma cells transferred to an *in vitro* environment could follow the melanization, leading to the appearance of melanosomes, which were not present in the Ab melanoma tissue *in vivo* [21].

Ab melanoma cells cultured in RPMI also underwent very weak melanization, which can be explained by the presence of small amounts of tyrosinase in the medium and fetal bovine serum (FBS) — an ingredient of both media which, according to Słomiński *et al.*, has a stimulatory effect on proliferation and melanization [33]. Apart from FBS, the L-tyrosine and L-DOPA (precursors of the melanogenic pathway) present in media could affect melanization [34].

In this experiment, we used two media that have significantly different levels of L-tyrosine: RPMI and DMEM. L-tyrosine stimulates melanosome synthesis and tyrosinase translocation to the melanosomes [35]. L-tyrosine hydroxylates into L-DOPA, which weakly stimulates the formation of premelanosomes [34, 36]. It is worth recalling that a lack of premelanosomes is regarded as the main reason for melanogenesis inhibition in the amelanotic Ab line. The effect of higher levels of L-tyrosine in the medium seems to be confirmed in our experiment: Ab cells cultured in DMEM undergo faster melanization because DMEM contains more L-tyrosine than RPMI-1640. Melanin was heavily produced in parallel with darkening of the cell pellets. The stimulatory effect of DMEM on melanosomes formation is also documented by the ultrastructural analysis, which showed that melanosomes were present in Ab cells after 24 hours of incubation, despite having been absent at time 0. During the progress of melanization, melanosomes were observed at different stages to contain melanin.

During culturing of the Ab melanoma cells in DMEM, the amount of tyrosinase protein increased, melanization did not always follow as a result of the increase in tyrosinase protein and tyrosinase activity [37]. According to Słomiński *et al.*, higher level of ty-

rosine, substrate for tyrosinase, protects the tyrosinase enzyme from degradation and enable it to accumulate in melanosomes [36].

The induction of melanization in amelanotic melanoma cells by a medium with higher level of L-tyrosine is an example of substrate influence on a metabolic pathway — in this case, melanogenesis — which can alter the biology of the target cells. L-tyrosine acts as a positive regulator of melanin synthesis, tyrosinase activity, and melanosome formation in a time-dependent manner. Thus, during the *in vitro* culturing of melanoma cells, it is very important to consider the content of tyrosine in the culture medium to avoid induction of melanogenesis. Melanogenesis is a functionally basic process for melanoma cells, and so any change may affect the biology of such tumor cells and their reaction to environmental factors, including drugs and radiotherapy.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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