The DNA ploidy and proliferative activity of transplantable melanoma cells in regard to their secretory function

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The study concerns DNA ploidy and proliferative activity of the cells of two hamster transplantable melanoma lines – differing in many biological features – in the light of possible changes in their secretory activity. DNA ploidy was determined from the index of propidium iodide (PI) stained DNA content (DI), while the proliferative activity was defined as the percentage of cells in S and G_2/M cell cycle phases. The secretory activity was described by determining total protein, interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), oncostatin M (OSM), interleukin 10 (IL-10) and nitric oxide (NO) content in the supernatants after 1, 6, 24 and 48 hours in cell culture.

Our results indicate that melanotic melanoma cells (Ma) have a near-tetraploid DNA content and about 18% of proliferating cells, while amelanotic melanoma cells (Ab) – have a near-triploid DNA content and almost twice as many proliferating cells. The Ab cells, in comparison with Ma cells, secreted *in vitro* less total protein and most of the cytokines examined except OSM, but a spontaneous alteration of transplantable melanoma was accompanied by an increase of the quantity and dynamics of NO secretion.

So, the cells of two melanoma lines have their own characteristic pattern of secretory function. But, the aneuploidy which accompanied the changes in cell differentiation of the studied melanoma lines, although seemed to reflect their changes in the proliferative activity, nevertheless did not reflect, in a direct way, differences in the secretion of the substances studied.

Key words: transplantable melanoma, ploidy DNA, proliferative and secretory activities

In spite of the numerous investigations of malignant melanoma, the biology of this tumor is not, as yet, fully understood, and its treatment is not fully efficacious [11, 12, 18].

Tumor cells (including melanoma), may be characterized by an incorrect, aneuploid DNA content, resulting from replication dissorders during the cell cycle [2, 9, 29, 36]. WASS et al [35] showed aneuploidy in 67% of studied human melanoma, but they did not find any differences in the occurrence of incorrect DNA content between primary and metastatic tumors, or between a melanotic and an amelanotic melanoma. At the same time, BÜCHNER et al [7] observed aneuploidy in 9% of naevus pigmentosus, in 54% of primary tumors and in 86% of metastatic tumors. Increased frequency of aneuploidy connected with tumor progression was described also by other authors who showed that it is associated with advanced stages of tumor development, thus with poorly differentiated tumor cells [2, 22]. SONDERGAARD et al [30] underlined that although an incorrect DNA content is not the absolute crtiterion of malignancy, its occurrence is most often accompanied by melanoma cell anaplasia. Simultaneously, it is indicated that an aneuploid DNA content in tumor cells is an unfavourable prognostic factor connected with an increased risk of tumor recurrence and shortened survival time of the patient [29].

Some authors point out that tumor development proceeds similarly, being independent of the type of aneuploidy [23] and that aneuploid melanoma cells, of primary and metastatic tumors alike, usually have a near-triploid DNA content [19, 31]. Other authors suggest, however, that the appearance of tetraploidy is connected with a better prognosis in tumor developments [33] while a triploid DNA content in melanoma cells indicates tumor progression, because these cells are a subpopulation which escapes from the genetic and metabolic control, tending towards of an unlimited growth [25].

An increased frequency of aneuploidy during tumor progression is most often accompanied by a higher proliferative activity [17, 24]. Many authors indicate that aneuploid tumors (including melanoma) have more cells in the S phase of

the cell cycle in comparison with diploid tumors, and they underline that an increased proliferative activity (expressed by a higher – 15% of cells in S phase) is related to an advanced stage of tumor development and this is directly proportionately correlated with a poor clinical outcome [2, 17]. Determination of the proliferative activity can be a significant prognostic procedure in the development of this tumor type [17].

It is also indicated that the structural and funcional cancer-specific phenotypes of tumor cells are the consequence of aneuploidy, which can be an image of their genetic instability [26, 28].

Besides, works of other authors show that tumor cells (including melanoma) can secrete *in vitro* and *in vivo* different substances, which modify the immunological response and enable the tumor to "escape" from the immunological control, thus permitting its further development [11, 18].

The secreted substances include many biologically active ones such as cytokines and nitric oxide [1, 21]whose local secretion increases during tumor progression [12, 18]. In relation to that it seems probable that changes of tumor cell DNA ploidy may have repercussions in the secretory activity of the cells.

Therefore in the present work we studied not only changes in DNA content in the cells of two transplantable melanoma lines differing phenotypically in many biological features, but we also estimated the secretion of total protein, cytokines (such as IL-6, OSM, TNF-α and IL-10) and NO by these cells. Across the pathways set by cytokines such as IL-6, OSM, TNF-α and IL-10 are initiated processes often completely opposite (such as proliferation or growth inhibition) [18, 21, 32]. IL-6 and OSM, according to the stage of melanoma progression, inhibit or stimulate melanoma cell proliferation [12, 20]. TNF-α and IL-10 action (connected with the influence on tumor growth, angiogenesis regulation, immunomodulation activity and on adhesion molecules), can also have different effects on tumor development [13, 21]. These cytokines can be synthesized by the tumor cells and released to the body fluids; among other this creates the possibility of their diagnostic use as markers serving for the recognition and monitoring of tumor development [10, 32].

It is also known that NO secretion by melanoma cells is a very important problem. This is indicated by reports concerning the ability of melanoma cells to secrete NO and its influence on tumor growth and the tumor-host interaction [1, 34].

Although, as it has been mentioned above, the various biological activity of IL-6, OSM, TNF-α, IL-10 and NO, is studied intensively, its importance for the biology of melanoma in connection with DNA ploidy and the proliferative activity of the cells of this tumor, is not yet fully understood.

Therefore, a comparative analysis of DNA ploidy and proliferative activity of the cells of two lines of transplantable melanoma with regard to their secretory activity was the subject of the present study.

Material and methods

Animals. 3–4 months old male Syrian (golden) hamsters *Mesocricetus auratus Waterhouse*, were purchased from the Central Animal Facilities of the Silesian Medical University, Katowice, Poland. The animals were then conventionally kept at the Department's animal facility and fed by standard diet and tap water *ad libitum*. The experiments' procedures were approved by the Animal Ethics Committee at Medical University of Gdansk and confirmed to the National Health and Medical Research Council's guide for the care and use of laboratory animals.

Transplantable melanomas. The tumors were transplantable melanotic and amelanotic melanomas. The melanotic melanoma line (Ma) derived from a spontaneous melanoma of the skin which had appeared spontaneously in a breed of golden hamsters in 1959 [3]. The amelanotic melanoma line (Ab) originated from the melanotic form by a spontaneous alteration differing in many biological properties [3, 4, 6, 14].

The hamsters were injected with a suspension of melanoma tissue obtained by mincing in a glass homogenizer. The tumor tissue was injected subcutaneously into the flank region in an amount of 200 mg of Ma per one hamster and 50 mg of Ab melanoma per one hamster. Hamsters with transplanted Ma melanoma were used for the experiments 21–24-days after the inoculation, and those with Ab melanoma 10–12 days after inoculation. Differences in the quantity of transplanted tumors and time of getting animals for experiments were adequate to the known rate of growth of these two melanoma lines.

Isolation of melanotic and amelanotic melanoma cells. Melanoma cells were isolated from solid tumors by a non-enzymatic method [5]. The suspension consisted 95–98% of viable cells (estimated by trypan blue test).

Ploidy and proliferative activity estimation. Ethanol-fixed $1x10^6$ melanoma cells were resuspended in 1 ml of staining solution (RNaseA 100 μg/ml and PI 40 μg/ml in PBS). Then cells were incubated for 30 min in 37 °C, in the dark and the fluorescence was analyzed on a FACSCalibour flow cytometer (Becton Dickonson, USA) [8]. Control diploid cells were cells isolated from hamster spleens. 10,000–20,000 events were stored from each stained sample and analyzed off-line using WinMDI2.6 software (obtained from J. Trotter, The Scripps Institute, La Jolla, CA, USA). Ploidy of melanoma cells was definited as index of DNA content DI = intensity of fluorescence for G_0/G_1 melanoma cells / intensity of fluorescence for G_0/G_1 control diploid cells. Proliferative activity was definited as percentage of cells in S and G_2/M phases of cell cycle.

Preparation of supernatants from melanoma cells. Melanoma cells at a concentration of $5x10^5$ /ml (for total proteins, IL-6, OSM, TNF-α and NO) and $3x10^6$/ml (for IL-10) were incubated in RPMI 1640 (culture medium, Biomed Lublin; with FCS – fetal calf serum, GIBCO) and antibiotics

(100 U/ml penicilin, 100 μg/ml streptomycin) for 1, 6, 24 and 48 h in 6-well plates (Sarstedt) in 5% CO_2 at 37 °C. After that time supernatants were harvested and stored at –70 °C until later use.

Total protein estimation. Total protein content in cultured melanoma cell supernatant was determined by the method described in detail in our earlier works [16, 39], using bovine albumin fraction V (Sigma) as standard.

IL-6, OSM, TNF-α and IL-10 determination by ELISA test. Level of cytokines in melanoma cells supernatants was determined by the Quantikine mouse cytokines immunoassays (Research and Diagnostic Systems, Mineapolis, MN, USA) which is a solid-phase ELISA. The assay was performed as described in the instructions. Absorbance at 450 nm was determined on a microplate reader (BioRad). Sensitivity limits of the ELISA for IL-6 was 3.1 pg/ml, for OSM 2.1 pg/ml, for TNF-α 5.1 pg/ml and for IL-10 4.0 pg/ml. Samples were assayed in triplicate.

Assay of NO concentration. Nitric oxide, quantified by the accumulation of nitrite (NO_2^-) (as a stable end product) in the melanoma cells supernatants, was measured by a microplate assay method described in detail in our earlier works [16, 39].

Statistical evaluation. Group data expressed as mean ±S.D. were statistically estimated by nonparametric Mann-Whitney's U test. The p value less than 0.05 was considered to represent a statistically significant difference.

Results

DNA ploidy and proliferative activity of transplantable melanoma cells. Cytometric assays of DNA content in transplantable melanoma cells indicated that Ma and Ab melanomas have cells with aneuploid DNA content (the DNA content differs from that in normal diploid cells). Simultaneously, we observed differences in nuclear DNA content between the cells of the two melanoma lines. The DNA index (DI) for the original melanoma cells is 1.9; this may indicate that these cells have a near-tetraploid DNA content.

The DI for Ab melanoma cells is 1.7, from which it can be assumed that these cells have a near-triploid DNA content (Fig. 1 and 2A, B).

The results of proliferative activity assays shown in Figures 1 and 2A, B indicate that about 18% of the Ma melanoma cells are in S and G_2/M phases of the cell cycle; for the Ab melanoma line – less differentiated, but growing faster – the number of cells in S and G_2/M phases is above twice as high (almost 40%).

The histograms present the percentage, different for each line, of cells with a lower DNA content (above 20% for Ma, about 9% for Ab), located below G_0/G_1 ($<G_0/G_1$) – it contains cells with a diploid DNA content as well as apoptotic cells (Fig. 1).

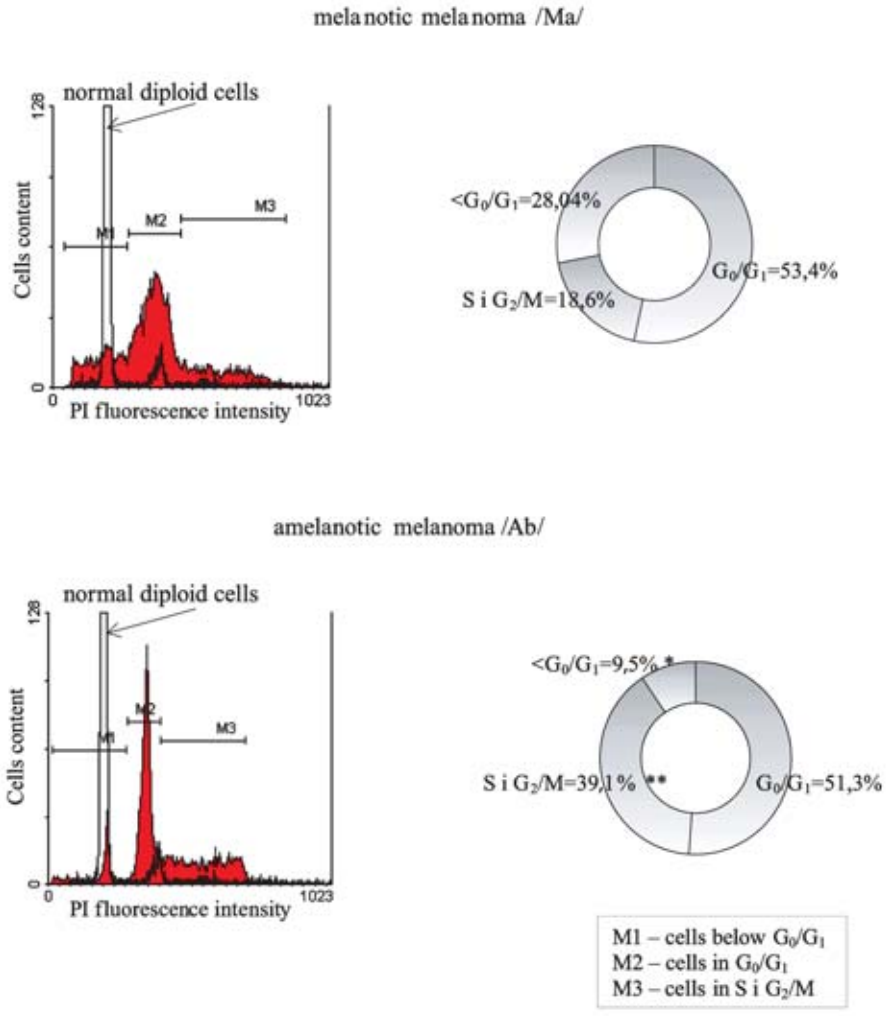

Figure 1. The DNA ploidy of cells of two transplantable melanoma lines with the content of these cell cycle phases (G_0/G_1, S and G_2/M) and below G_0/G_1($<G_0/G_1$), determined by the flow cytometric method. The values are the means ±SD of 7 experiments for Ma line and of 9 experiments for Ab line. P values were calculated using the Mann-Whitney U test; * statistically significant decrease of Ab melanoma cells content below G_0/G_1 in comparison with Ma melanoma cells, $p<0.001$; ** statistically significant increase of Ab melanoma cells content in S i G_2/M phases of cell cycle in comparison with Ma melanoma cells, $p<0.001$.

Content of total protein, IL-6, OSM, TNF-α, IL-10 and NO. The results, illustrated in Figures 2C and 2D, of protein content estimation in 1, 6, 24 and 48 hours transplantable melanoma cell culture supernatants, indicate that Ma and Ab melanoma cells secrete the protein *in vitro*. Ma melanoma cells secrete more protein than the cells of the less differentiated Ab line.

The results of the estimation of IL-6 content in the culture supernatants (Fig. 2E and 2F) indicate that independently of the culture time, Ma melanoma cells secrete a little more of this cytokine than the Ab line; after 1 hour culture this difference is statistically significant.

The results obtained show also that the Ma melanoma cells secrete during culture a little less OSM than the Ab line, but this decrease is not statistically significant (Fig. 2E, F).

Besides, in Figure 2E and 2F we can see that with the passing culture hours the Ab melanoma cells secrete definitely less TNF-α than the native Ma melanoma cells (the differences for 6–48 hours are statistically significant). Ma and Ab melanoma cells secrete also IL-10 to the culture supernatants, but only after a 48 hours culture (Fig. 2E, F). The secretion of this cytokine by Ab melanoma cells is 7 times lower than its secretion by the native line (the difference is statistically significant).

The results shown in Figures 2C and 2D indicate too that cells of the two transplantable melanoma lines spontaneously secrete NO already in a 1 hour culture. The observation of the dynamics of NO secretion by cells of the two transplantable melanoma lines indicates that after 1 and 6 hours culture Ma melanoma cells secrete similar amounts NO; after 24 hours its secretion level increases about 48.3% and after 48 hours – about almost 60% ($0.001<p<0.01$). However, for the Ab line, we observed an increase of NO secretion already in 6 hours culture, after 24 hours it rises dramatically up to 130% in comparison with its value after 6 hours culture ($0.0001<p<0.001$), and after 48 hours a further increase about 40% follows, in comparison with the 24 hours culture.

The results from the estimation of the content of the above-mentioned substances in the culture supernatants are presented in the Figure 2 (C–F) and have been described in detail in our earlier works [16, 39].

Discussion

The observed differences of DNA ploidy between cells of both melanoma lines during a spontaneous alteration of Ma line into Ab, which indicate changes in nuclear DNA content from near-tetraploid to near-triploid, agree with our previous cytogenetic analysis [38]. These results are in accordance with the suggestions of those authors, who indicate that melanoma cells have an aneuploid DNA content and the changes in their ploidy are connected with tumor progression, often accompanied by a decreased cell differentiation [2, 29]. Our present results are confirmed by statements according to which near-triploid cells originate from advanced tumor progression stages and are a subpopulation of melanoma cells which escape from genetic and metabolic control into unrestricted proliferation [25], as well as by statements of authors claiming that the appearance of tetraploidy is correlated with a better clinical prognosis [33].

The results related to the proliferative activity of the cells of both transplantable melanomas indicate that in the S and G_2/M phases of the cell cycle there are more than twice as many cells of the near-triploid Ab line – tumor characterized by a faster rate of growth and causing animal death within a shorter time – than in the original line; they also agree with the findings reported by other authors indicating that aneuploid tumors, have more proliferating cells than diploid tumors, and that a higher proliferative activity of melanoma cells is correlated with a more aggressive course of neoplastic disease [2, 17, 24].

Moreover, a higher proliferative activity of Ab melanoma cells permits the supposition that these cells are more sensitive than the cells of Ma line to genetic material damage, which according to some authors can be an indication of dissorders in cell differentiation [9].

This seems probable, especially in the light of the information that in the S phase of the cell cycle various DNA damages are very often found [9, 36]. DNA replication disorders during the S phase (which can result from the onset of the second replication round before the beginning of phase G_2) influence the formation of aneuploidy in tumor cells [36]. A higher proliferative activity of Ab melanoma cells – manifested by the percentage, higher than in the original Ma, of dividing cells (in S and G_2/M phases of cell cycle), is reflected by a more intensive rate of growth of Ab melanoma.

Our observations are indicating that along with a spontaneous alteration of transplantable melanoma, when the degree of cell differentiation decreases and there also occurs a decrease in their secretory activity and an increase in proliferative activity, corroborates the statements of other authors that the development of a neoplastic process is connected with the restriction of cell differentiation and all their functions in favour of the proliferation rate [6].

Besides, the observations of some authors showed that many of the changes related to the phenotype of tumor cells (such as the size and morphology of cells, level of substances produced, resistance to cytostatics, unrestricted ability to proliferate or loss of differentiation features) are a direct consequence of their aneuploidy [26, 28].

Our present results concerning cytokines secretion are not in agreement with the opinion of those authors who underline that with an advancing tumor progression the secretion of cytokines by melanoma cells increases [18]. It seems that transplantable melanoma progression is not determined by locally secreted cytokines, but the intensity of the growth of this tumor depends on other factors. Such a suggestion can be confirmed by our results on the decreased of TNF-α, IL-10 and IL-6 secretion and on the secretion of OSM which was similar in both melanoma cells – a cytokine previously de-

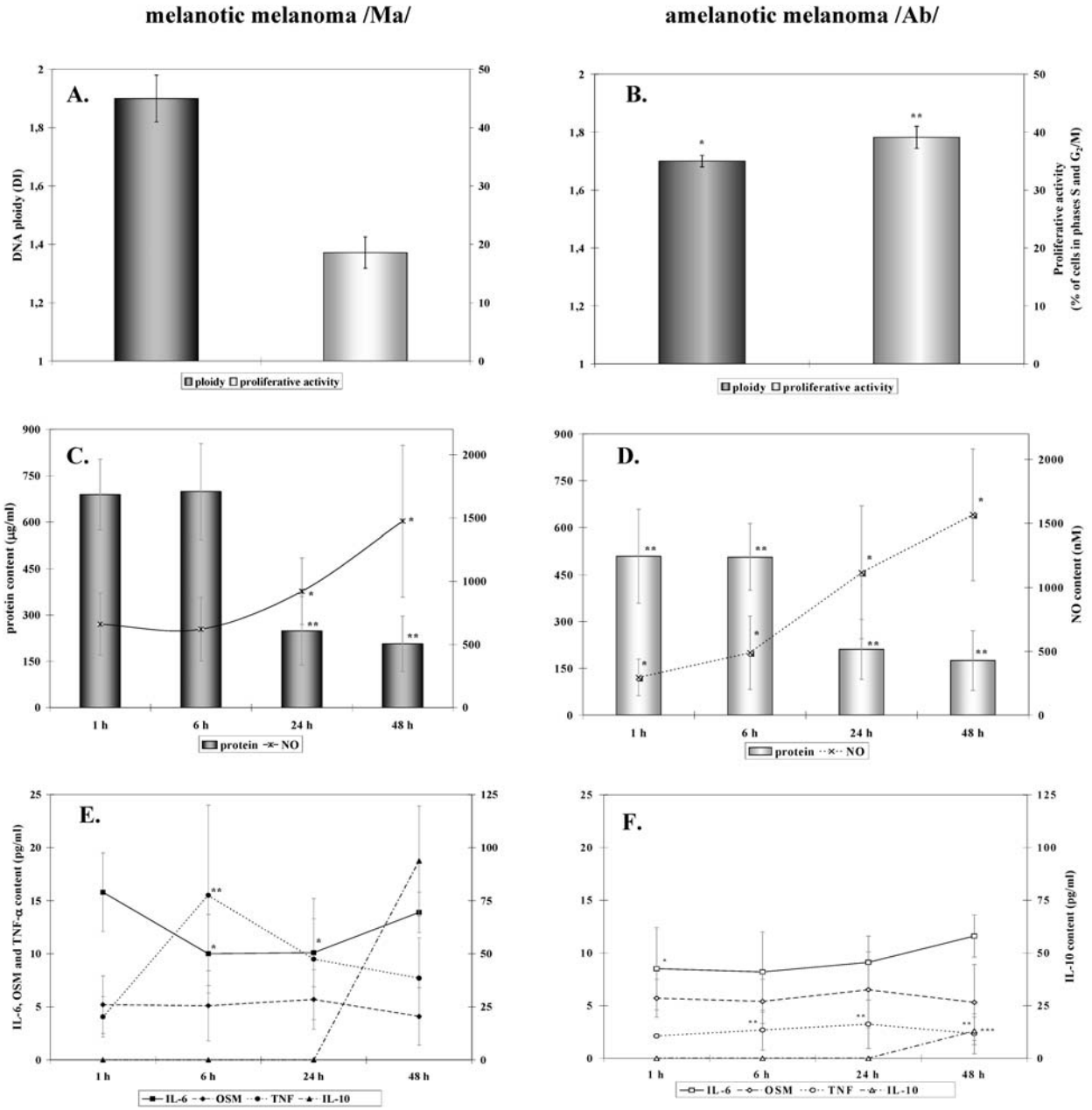

Figure 2. The DNA ploidy and proliferative activity of cells of two transplantable melanoma lines /A i B/ with the dynamics of total protein, nitric oxide (NO) /C i D/, interleukin 6 (IL-6), oncostatin M (OSM), TNFα and interleukin 10 (IL-10) /E i F/ secretion by these melanoma cells.
The values are the means ±SD of 7–9 experiments for DNA ploidy and proliferative activity, 6–12 for total protein, 9–16 for NO, 5–15 for IL-6, 4–12 for OSM, 4–7 for TNF-α, 5–6 for IL-10. Experiments done in triplicate. P values were calculated using Mann-Whitney U test.
B. *statistically significant decrease of DI for Ab cells in comparison with Ma cells, $p<0.001$; **statistically significant increase of proliferative activity for Ab cells in comparison with Ma cells, $p<0.001$.
C. *statistically significant increase of NO secretion by Ma cells after 24 and 48 hours culture in comparison with 1 and 6 hours, $0.001<p<0.01$; **statistically significant decrease of protein secretion by Ma cells after 24 and 48 hours culture in comparison with 1 and 6 hours, $p<0.01$.
D. *statistically significant decrease of NO secretion by Ab cells after 1 and 6 hours culture in comparison with Ma cells, $p<0.001$; **statistically significant decrease of protein secretion by Ab cells after 1 and 6 hours culture in comparison with Ma cells, $p<0.05$.
E. *statistically significant decrease of IL-6 secretion by Ma cells after 6 amd 24 hours culture in comparison with 1 hours, $p<0.01$; **statistically significant increase of TNF secretion by Ma cells after 6 hours culture in comparison with 1 hours, $p<0.05$.
F. *statistically significant decrease of IL-6 secretion by Ab cells after 1 hours in comparison with Ma cells, $p<0.01$; **statistically significant decrease of TNF-α secretion by Ab cells after 6, 24, and 48 hours in comparison with Ma cells, $p<0.01$; ***statistically significant decrease of IL-10 secretion by Ab cells after 48 hours in comparison with Ma cells, $p<0.01$.

scribed as a factor able to inhibit melanoma cell growth [37]. However the NO secretion is of some importance for the biology of this tumor, because it must be mentioned that with the prolongation of the time of Ab melanoma cell culture, the process of NO secretion becomes more dynamic than in the case of native line. The intensified dynamics of NO secretion by Ab line, less differentiated, but with a faster growth rate is probably connected with, as postulated by some authors, its antiapoptotic activity and its stimulatory influence on melanoma cell proliferation, which promote melanoma growth [27, 34]. Simultaneously, this would be supported by the results from our other work concering differences in the ability of the cells of two hamster transplantable melanoma lines to undergo apoptosis [15].

So, the cells of two melanoma lines have their own characteristic pattern of secretory function. Thus, we can suggest that the changes of the aneuploidy of melanoma cells did not reflect in direct way differences in their secretory activity, because the secretion varies depending on the substances examined. But it seems that the decrease of DNA content which accompanies a spontaneous melanoma alteration – from near-tetraploid to near-triploid – influenced the degree of biochemical, immunological and morphological differentiation of cells and also their proliferative activity. This is expressed by a more aggressive phenotype of Ab melanoma line.

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