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Characterization of NO and Cytokine Release by Transplantable Melanoma Cell Lines in Relationship to Their Differentiation

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Abstract. Changes in the secretory activity of two transplantable melanoma lines (differing in many biological features) as regards nitric oxide (NO) release and interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and oncostatin M (OSM) secretion were the subject of the present study. The results obtained show that a spontaneous alteration of the hamster native melanoma line into an amelanotic one is accompanied by a global change of the secretory activity, but there was not a distinct correlation between the endogenous cytokine secretion and NO secretion by cells of both melanoma lines.

Key words: NO; interleukin 6; tumor necrosis factor α; oncostatin M; transplantable melanomas.

Introduction

The ability of melanoma cells to secrete biologically active substances such as nitric oxide (NO)[11] and cytokines has been described since the late 80's. These substances modifying, many of the biological functions of melanoma, influence the tumor-host' interaction[14, 16, 26–30].

We still do not know the exact mechanism of this phenomena and to what extent the secretory activity of tumor cells as regards cytokine and NO secretion depends on the biological features of the tumor, such as the degree of cell differentiation and the rate of growth[9, 22, 23].

The biological role of many cytokines endogenously secreted by melanoma cells and their participation in the autocrine regulation of NO synthesis has not been explained either, although there are reports indicating an influence of exogenous cytokines on *in vitro* NO synthesis[1, 8, 11, 15]. Having at our disposal two transplantable melanoma lines of common origin which spontaneously changed their levels of differentiation, growth rates and immunogenicity[5, 6, 18], we attempted to find out to what extent a spontaneous alteration of the hamster native melanoma line into an amelanotic one, less differentiated but with a higher growth rate, is related to changes in the secretion of NO and such cytokines as interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and oncostatin M (OSM). Also, we decided to establish if the NO secretion by cells of both melanoma lines is correlated to any degree with the their cytokine secretion.

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Materials and Methods

Animals. 3-4 month-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 reared at the Department's animal facility and fed a standard diet and tap water *ad libitum*. The experimental procedures were approved by the Animal Ethics Committee of the Medical University of Gdańsk and conformed to the National Health and Medical Research Council's guide lines for the care and use of laboratory animals.

Transplantable melanomas. The tumors were transplantable melanotic and amelanotic melanomas.

The **melanotic melanoma line** was derived from a melanoma of the skin which had appeared spontaneously in a breed of golden hamsters in 1959 and was described by BOMIRSKI in 1977 as Ma line[5, 6].

The **amelanotic melanoma line** originated from the melanotic form by a spontaneous alteration described in 1977 as Ab line[5]. These melanoma lines differ in many biological features[6, 18].

Isolation of melanotic and amelanotic melanoma cells. Melanoma cells were isolated from solid tumors by a non-enzymatic method, described previously, to obtain single-cell suspensions[7]. The suspension consisted of 95–98% viable cells (estimated by testing with trypan blue).

Preparation of supernatants of melanoma cell culture. Isolated melanoma cells at a concentration 5×10^5/ml were incubated in RPMI 1640 (culture medium, Biomed, Lublin) with 10% fetal calf serum (FCS, Gibco) and antibiotics (100 U/ml penicilin, 100 μg/ml streptomycin) for 1, 6, 24, 48 h in 6-well plates (Corning) in 5% CO_2 at 37°C. After that, supernatants were harvested and stored at –70°C until later use.

Protein estimation. Total protein content in 1-, 6-, 24- or 48-hour cultured melanoma cell supernatants was determined by the method of LOWRY et al.[21], using bovine albumin fraction V (Sigma) as standard. 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 1-, 6-, 24- and 48-hour melanoma cell supernatants, was measured by a microplate assay method according to DING et al.[10]

NO concentration was calculated from a sodium nitrite ($NaNO_2$, Sigma) standard curve. In all experiments, the nitrite content in wells containing medium without melanoma cells was also measured and substracted from the experimental values. Data are expressed as nM nitrite/0.5×10^6 cells.

The specific inhibitor of NO synthase N^G-methyl-L--arginine (LNMMA, Sigma) at 0.5 mM/ml concentration was used for the inhibition of NO production.

Cytokine determination by ELISA test. The levels of IL-6, TNF-α and OSM in melanoma cell supernatants were determined by the Quantikine mouse IL-6, TNF-α and OSM immunoassays (Research and Diagnostic Systems, Minneapolis, MN, USA) which is a solid-phase ELISA. The assay was performed as described in the instructions. The concentrations of the cytokines in the supernatants were determined by comparing the optical density of the samples to the standard curve (2–1500 pg/ml). Sensitivity limits of the ELISA for IL-6, TNF-α and OSM were 3.1, 5.1 and 2.1 pg/ml respectively. Samples were assayed in triplicate.

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

Results

The results we obtained made it possible to determine that the cells of both the transplantable melanomas spontaneously secreted NO (from 300 to 1500 nM) already after 1 h of incubation (Fig. 1).

Both lines also produced more NO over a prolonged incubation. It should be noted that after 1 h the amelanotic melanoma cells secreted about 53% and after 6 h 21.3% less NO than the native line, this differences being statistically significant.

But after a longer up to 24 and 48 h incubation, these cells secreted 21.2 and 6.3%, more NO, respectively. This difference in the amount of secreted NO by cells of both melanomas was most evident after 1 h incubation ($p<0.001$).

On the basis of the dynamics of NO secretion by melanotic melanoma cells it can be stated that NO secretion was about the same, after 1 h and 6 h but it increased by about 48.3% after 24 h and by almost 60% after 48 h ($0.001<p<0.01$). However, the amelanotic melanoma cells, less differentiated morphologically but with a higher growth rate, showed an increase of NO secretion already after 6 h. After 24 h this increase was dramatically up to 130% of the value recorded after 6 h ($0.001<p<0.01$), and after 48 h it was about 40% greater than the value for 24 h.

Our results on the inhibtion of NO secretion by N^G--methyl-L-arginine showed that the inhibition effect was observed in both melanoma cells, being a little

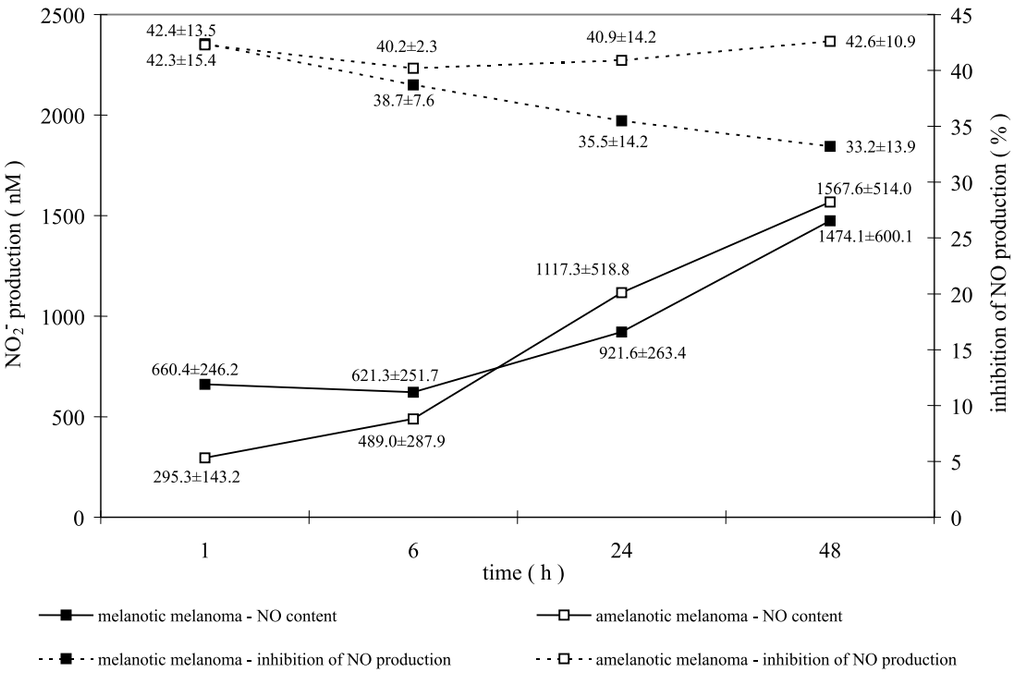

Fig. 1. Dynamics of NO secretion by transplantable melanoma cells cultured with and without N^G-methyl-L-arginine

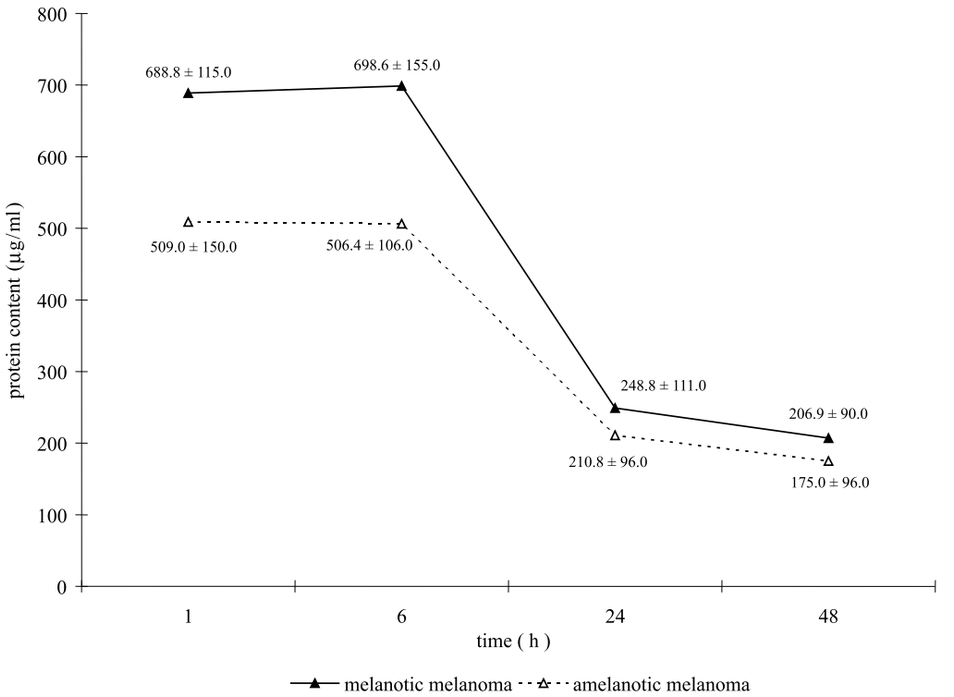

Fig. 2. Dynamics of protein secretion by transplantable melanoma cells

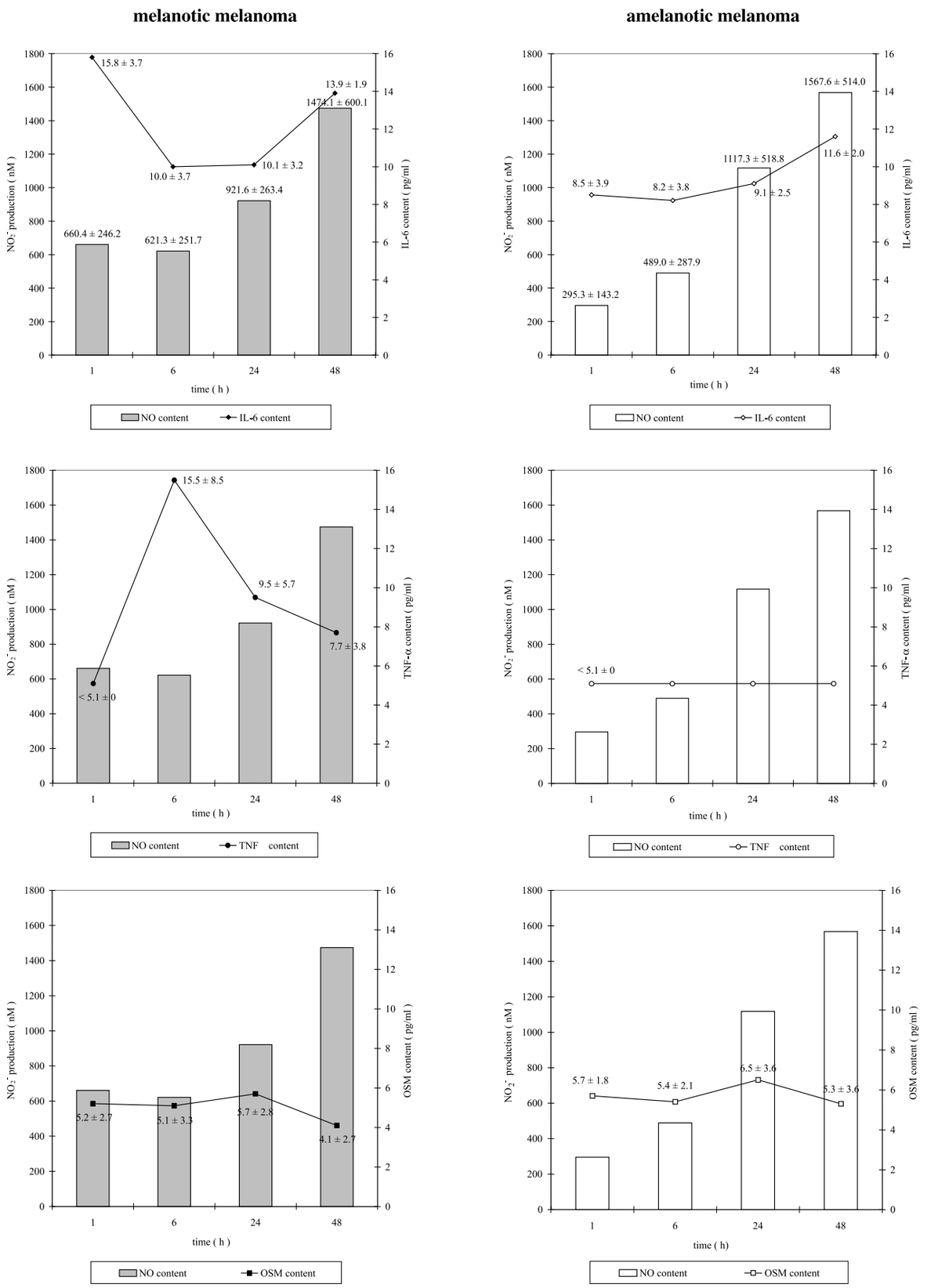

Fig. 3. NO and cytokine release by transplantable melanoma cell lines

higher (but not statistically significant) after 6, 24, 48 h in the amelanotic line than in the native one (Fig. 1). Although in the melanotic melanoma line the inhibition effect showed a tendency to decrease with extended incubation to 6, 24, 48 h, in the amelanotic melanoma cells the inhibition of NO secretion remained the same, regardless of the time of incubation with the NO synthase inhibitor (Fig. 1).

It must be added that the NO secretion by the cells of both melanoma lines was independent of the overall secretory activity of these cells measured by the total protein secreted, which decreased during incubation time from 1 h to 48 h; the maximum observed decrease occurred between 6 and 24 h, i.e. during the highest increase in NO production (Fig. 1 and 2).

As regards the characteristics of cytokine secretion, the profile of this secretion varies and depends on the kind of cytokines and the tumor line (Fig. 3). Only during OSM secretion could we see that the dynamics of the secretion of this cytokine by both melanoma cells was similar.

Only qualitative differences in OSM secretion were observable; the amelanotic line, less differentiated, secreted more OSM, but in comparison with the native form this difference was on the border line of statistical significance (Fig. 3). On the other hand, the dynamics of IL-6 and TNF-α secretion by both melanoma forms was completely different (Fig. 3). The native melanoma cells, more differentiated, secreted more IL-6 and TNF-α than the amelanotic melanoma cells. With extended incubation time, both melanoma cell lines secreted increasing amounts of IL-6 and, similarly to NO, this did not depend on the dynamics of total protein secretion. TNF-α secretion by melanotic melanoma cells grew rapidly and after 6 h was 3 times higher, but after 48 h it returned to the initial value; the amelanotic melanoma cells, less differentiated, secreted a little less TNF-α, but during the whole incubation time (from 1 to 48 h) its content was at the same level (Fig. 3).

Discussion

The results for the secretory activity of cells of two transplantable melanoma lines of the same origin but differing in biological features, analyzed comparatively, showed that a spontaneous alteration of the native form into an amelanotic line, with a higher tumorigenicity and lower differentiation, was accompanied by changes in the profiles of IL-6 and TNF-α secretion *in vitro* during 48 h of culture, but a very similar profile of OSM secretion, while the dynamics of NO secretion by the cells of these two melanoma lines was similar, differing only quantitatively. Besides this, we have demonstrated that the nature of endogenous cytokine release, apart from IL-6, was not correlated with NO release by the cells of these two melanoma lines. Therefore our observations show that endogenously released cytokines such as TNF-α and OSM are not associated with any modification of NO production by melanoma cells; while the level of OSM was similar during the 48 h of cell culture, the level of NO in this culture increased continuously, and after 48 h it was at its highest.

We also observed a similar lack of correlation between the release of TNF-α and NO. It is also noteworthy that the nature of TNF-α release by these two lines was quite different; while the cells of the melanotic melanoma secreted changeable amounts of TNF-α over 48 h, the cells of the amelanotic melanoma secreted similar amounts of this cytokine, and at the same time the secretion of NO by the cells of these two melanomas increased.

It should be underlined that the increase of NO secretion showed an inverse correlation with the secretion of total protein, the content of which may be considered as the exponent of the melanoma cell secretory function.

The content of total protein decreased with increasing culture time (while the content of NO increased) and was also lower in the culture of amelanotic melanoma cells, which seems to indicate that a spontaneous alteration of a melanotic line into an amelanotic one is accompanied by a global change of the secretory activity. It is difficult to compare these results with those reported by another, who claimed that tumor cells secrete more proteins than normal cells[20].

The most pronounced differences between the two lines of melanoma cells considered could be seen in the nature of their IL-6 secretion, but as regards the correlation of IL-6 and NO secretion, only in the case of the amelanotic line did we notice a similarity between the secretion of this cytokine and that of NO. However, it is difficult to consider on the basis of our results, the increase of IL-6 content in the supernatants as an autocrine inducer stimulating NO production by amelanotic melanoma cells, because this observation refers only to one melanoma line, and in the literature we cannot find any information about the influence of endogenous IL-6 on NO production by melanoma cells.

As mentioned above, some authors have demonstrated that cytokines are able to modify NO secretion by normal and neoplastic cells, but these investigations concern the influence of exogenous cytokines[4, 10, 15, 19]. In their observation of macrophages, FUNATOGAWA et

al.[13] found that NO production in LPS-activated macrophages was correlated with TNF-α and IL-6, but these cytokines did not play any role as autocrine regulators of nitric oxide synthase.

SHIJO et al.[25] described an inverse correlation between the inhibition of nitric oxide synthase activity and the production of TNF-α by neutrophiles.

Thus, the problem of autocrine regulation of NO production by cytokines in neoplastic cells, including melanoma cells, has not been explained, but many authors underline the importance of tumor-derived cytokines and NO in the progression of melanoma. XIE et al.[27] and DONG et al.[11] described the influence of exogenous cytokines on NO production in metastatic melanoma lines and a lack of this modifying influence on NO production in nonmetastatic lines.

Such observations indicate that exogenous cytokines may influence NO production in connection with the biological features of melanoma. GERECITANO et al.[14] found an inverse correlation between the inducible nitric oxide synthase gene expression and the ability of murine melanoma to metastasize. JOSHI[17] showed that the nitric oxide synthase gene was amplified in a tumorigenic melanoma line but not in a nontumorigenic cell line, and NO production increased during the vascular stage of melanoma. It may be assumed that NO production may depend on both the biological properties of the melanoma and the stage of its development.

Our observations seem to agree with the above reports because they indicate that the melanoma cell line with the faster growth rate and lower cell differentiation produced more NO than the more differentiated native line. FAST et al.[12] have found that, although NO secretion by tumor cells did not depend on endogenous cytokines, NO production by tumor cells in response to exogenous TNF-α was directly correlated with their level of sensitivity to TNF-mediated cytotoxicity. RODECK et al.[24] supposed that although melanoma cells, in contrast to melanocytes, release multiple cytokines, they do not act as autocrine factors. Not all authors agree with this; for example ARMSTRONG et al.[2] found that IL-6 released by melanoma cells inhibited in an appreciable way the growth and progression of melanoma.

This problem is difficult to investigate because, as underlined by some authors, the cytokine secretion by different melanoma cell lines shows a great variability and heterogeneity[2, 3, 9, 22].

BERGENWALD et al.[4] indicated even that the production of TNF-α *in vitro* by primary and metastatic melanoma was variable, and although it is known that the secretion of NO and cytokines and their mutual interactions in the biology of melanoma are very important, this problem is still difficult to explain.

To sum up, the results from our present investigation demonstrate that endogenous cytokines do not modify NO release, but that the dynamics of cytokine secretion depends on the biological properties of melanoma cell lines and the nature of the changes that accompany a spontaneous alteration of a melanotic melanoma into an amelanotic line allows us to presume that these changes may modulate the tumor phenotype and, thereby, change tumor-host interaction.

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