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A comparative study on oncostatin M secretion by transplantable melanoma cells with regard to their biological properties and progression

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The authors have studied oncostatin M secretory activity of cells of two lines of transplantable melanoma with different biological properties. The level of OSM in melanoma cells supernatant was determined by ELISA test and OSM bioactivity was measured by using biotest line sensitive to OSM cytostatic activity — A 375 melanoma cells. OSM presence was confirmed in cellular lysates by western blot. It has been demonstrated that a spontaneous alteration of the transplantable melanotic melanoma line into an amelanotic line connected with tumour progression was accompanied by an increase of OSM release.

key words: transplantable melanoma progression, OSM secretion

INTRODUCTION

According to recent reports, Oncostatin M (OSM) is a glycoprotein cytokine playing, among a multitude of biological functions, a role in the inhibition of melanoma cell growth, and the effect of the inhibition seems to depend on the melanoma cell phenotype [3,6,8,9,20].

Simultaneously, many authors have indicated that the sensitivity of human melanoma cells to OSM activity depends on the rate of growth and degree of melanoma progression. The same melanoma cells are able to synthesise and secrete OSM [9,13,16,18,23].

According to contemporary views the secretion by tumour cells of autocrine factors regulating their growth and interactions between the tumour and the host is a very interesting problem in tumour biology, but still far from resolution.

Therefore, in this work (a continuation of our study on the immunobiology of two transplantable melanoma lines of common origin but differing in many biological features) we attempted to find out if there exist any differences in OSM secretion by the native melanoma cells and the amelanotic melanoma line cells which originated from the melanotic form by a spontaneous alteration in which the loss of pigment was accompanied by an acceleration of growth, a lower degree of differentiation and higher immunogenicity [1,11].

MATERIAL 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 standard diet and tap water *ad libitum*. The experimental procedures were approved by the Animal Ethics Committee at the Medical University of Gdańsk and conformed to the National Health and Medical Research Council's guide for the care and use of laboratory animals.

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Transplantable melanomas

The tumours were transplantable melanotic and amelanotic melanomas.

The melanotic melanoma line derived from a spontaneous melanoma of the skin, which had appeared spontaneously in a breed of golden hamsters in 1959 and was described by Bomirski [1] in 1977 as Ma line.

The amelanotic melanoma line originated from the melanotic form by a spontaneous alteration described in 1977 as Ab line [1].

These melanoma lines differ in many biological features.

Isolation of melanotic and amelanotic melanoma cells

Melanoma cells were isolated from solid tumours by a non-enzymatic method, described previously, to obtain single-cell suspensions [2]. The suspension consisted 95–98% of viable cells (estimated by test with trypan blue).

Preparation of supernatants of melanoma cells culture

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

A 375 cells (human malignant melanoma, kindly provided by Prof. J. Georgiades, Amarillo Cell Culture, USA) were used as control of other melanoma cells; 5×10^5/ml A 375 cells were incubated in DMEM (Dulbecco's Modified Eagle Medium) (Biomed Lublin) with 10% FCS (foetal calf serum; GIBCO) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) for 6 and 24 h in 6-well plates (Corning) in 5% CO_2 at 37°C. After that time supernatants were harvested and stored at –70°C until later use.

Cytokines determination by biological assay and ELISA test

OSM bioactivity in melanoma cells supernatants was determined for its ability to inhibit proliferation of A 375 melanoma cells (biotest line sensitive to OSM cytostatic activity) by a modification of the procedure described by Linsley et al. [14] in 96-well plate (Corning) using a MTT dye-binding assay (3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide; 5 mg/ml PBS; SIGMA). Control sample were A 375 cells (1 x 10^5/ml) incubated in medium without supernatants. Absorbance at 570 nm was determined on a microplate reader (BioRad). Samples were assayed in triplicate.

Units of OSM biological activity were defined as the volume of supernatant required to cause a 50% inhibition of the growth of A 375 melanoma cells.

Recombinant mouse OSM (R & D, Minneapolis MN, USA) was used as standard with a specific activity 2–9 ng/ml.

Level of OSM in melanoma cells supernatants was determined by the Quantikine human OSM immunoassays (Research and Diagnostic Systems, Minneapolis, 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). The concentration of cytokines in the supernatants was determined by comparing the optical density of the samples to the standard curve (2-1500 pg/ml OSM). Sensitivity limits of the ELISA for OSM was 2.1 pg/ml. Samples were assayed in triplicate.

Western blot detection of OSM

Fresh isolated melanoma cells (2 x 10^6) were lysated in hypotonic Tris/Nonidet 40/Tween-100 buffer with protease inhibitor cocktail (aprotinin 100 μg/ml, leupeptin μg/ml, iodoacetamide 1.8 mg/ml, phenylmethyl sulphonyl fluoride (PMSF) at 1 mM) for 1 hour on ice, spun for 5 minutes at 10,000 rpm, supernatants were collected and stored at –70°C until further processing. Proteins contained in the lysates were resolved by standard polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel [12]. Separated proteins were then electrophoretically transferred onto nitrocellulose (60 V, 2.5 h). Nitrocellulose sheets (Schleicher & Schuel) with transferred protein were then processed for OSM detection, i.e. blocked with 3% BSA (bovine serum albumine) in PBS, washed and exposed to the biotinylated anti-mouse OSM (R & D, Minneapolis MN, USA) for 2 h at room temperature. After washing out the unbound antibody, antibody that was bound to the membrane was colorimetrically detected using avidin-alkaline phosphatase solution (BioRad) and BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt)/NBT (p-nitro blue tetrazolium chloride; BioRad) solution.

Recombinant mouse OSM (R & D, Minneapolis MN, USA) was applied as standard protein. Prestained SDS-PAGE Standards (BioRad) was used as molecular weight marker.

Protein estimation

Total protein content in 1, 6, 24, 48 h cultured melanoma cells supernatants was determined by the method of Lowry et al. [15], using bovine albumin fraction V (Sigma) as standard. In all experiments protein content in wells containing medium without melanoma cells was also measured and subtracted from experimental values. Samples were assayed in triplicate.

Statistical evaluation

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

RESULTS

The content and biological activity of OSM

The results obtained are presented in Figure 1. They showed that both transplantable melanoma line cells were able to secrete OSM already after 1h incubation. Quantitative estimations by ELISA test indicated that melanotic melanoma cells secreted 5.2 pg/ml and amelanotic melanoma cells 5.7 pg/ml after that time.

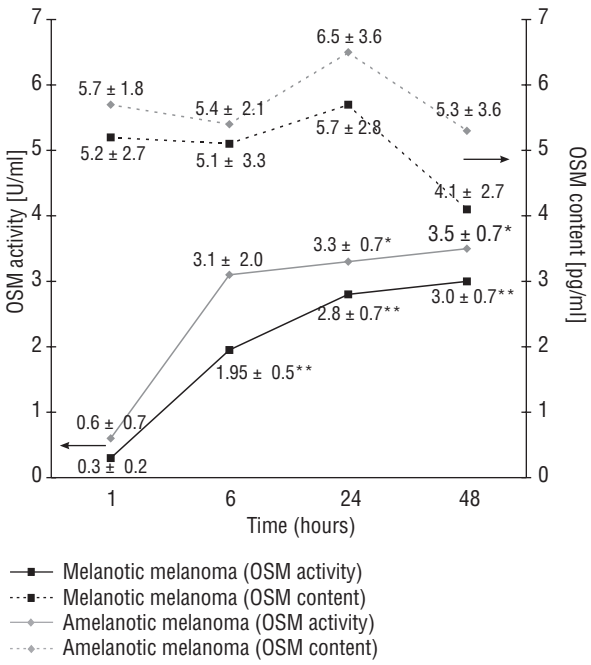

Figure 1. OSM secretion by transplantable melanoma cells estimated by ELISA test and by biological activity test. The values are the means ± SD of 4–12 experiments. Experiments done in triplicate. P values were calculated using U Mann-Whitney test. * — statistically significant ($p < 0.01$) increase of Ab melanoma cells OSM activity after 24, 48 h in comparison with 1 h culture; ** — statistically significant ($0.001 < p < 0.05$) increase of Ma melanoma cells OSM activity after 6, 24, 48 h culture in comparison with 1 h culture

During 24 h incubation there were no marked differences in OSM secretion by those two melanoma cells. After that time both melanoma cells secreted only about 10% more OSM in comparison with the OSM level after 1h.

After another 24 h the OSM content in the supernatants decreased by about 28% in the melanotic melanoma cells (in comparison with highest value after 6 h) and by about 18% in the amelanotic melanoma cells.

The biological activity of OSM, presented in Figure 1, showed that during 48 h this activity changed, but the observed changes were not correlated with the above-mentioned OSM content estimated by ELISA test.

After 1 h incubation the content of OSM in the supernatants of both melanoma cells was over 5 pg/ml while the biological activity of this cytokine was at the detectability level. Only after 6 h did we observe an increase in the biological activity of OSM: 6-fold in melanoma and 5-fold in amelanotic melanoma cells, while the content of OSM did not change.

The biological activity of OSM showed a tendency to increase during a further incubation time up to 48 h even when its content in the supernatants decreased. When comparing the ability of both melanoma lines to secrete OSM it should be borne in mind that the amelanotic melanoma line cells were more active in OSM secretion, and the OSM biological activity was also higher than in the melanotic melanoma cells.

Changes observed during 48 h were more stable in the amelanotic melanoma cells.

Both transplantable melanoma cells secreted less OSM than A 375 cells (after 6 h 7.0 ± 2.5 pg/ml, 24 h 8.4 ± 1.7 pg/ml) used as control of OSM secretion by other than transplantable melanoma cells.

OSM presence was confirmed in cellular lysates by western blot (Fig. 2).

Total proteins content and calculation of OSM content per 1 mg of the protein secreted

The results obtained regarding the quantitative estimations of the dynamic secretion of total proteins by both transplantable melanoma cells are presented in Figure 3.

The results showed that the profile of dynamic secretion of protein was different from OSM secretion.

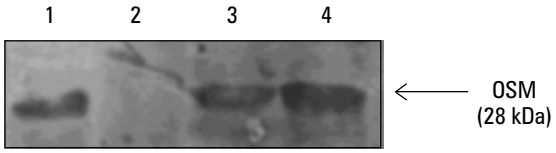

Figure 2. Western blot detection of oncostatin M (28 kDa) in cellular lysates of transplantable melanoma tumors. Lane 1 is for positve control of recombinant mouse OSM; lane 2 for negative control of culture medium without cells; lanes 3 and 4 for lysates of melanotic and amelanotic melanoma cells, respectively. Results from one of three separate experiments with similar results are presented.

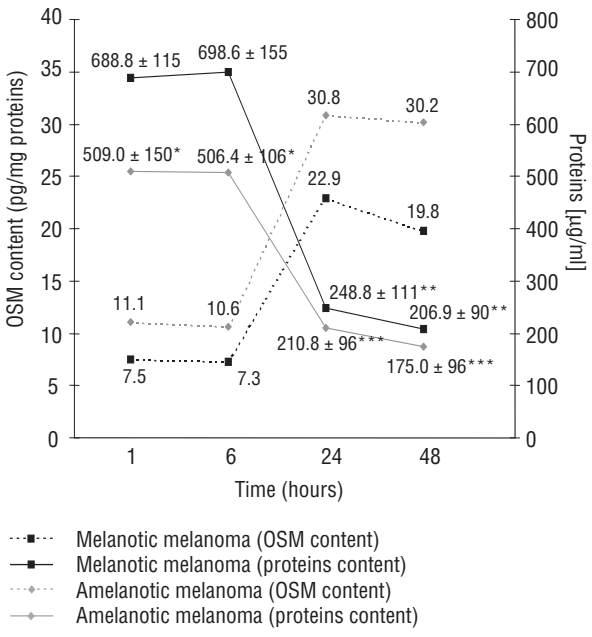

Figure 3. Dynamics of proteins and OSM (in 1 mg secreted proteins) secretion by transplantable melanoma cells. The values are the means ± SD of 6–12 experiments. Experiments done in triplicate. P values were calculated using U Mann-Whitney test.
* — statistically significant ($p < 0.05$) decrease of protein secretion by Ab melanoma cells in comparison with Ma melanoma cells after 1, 6 h culture;
** — statistically significant ($p < 0.001$) decrease of protein secretion by Ma melanoma cells after 24, 48 h culture in comparison with 1, 6 h culture;
*** — statistically significant ($p < 0.001$) decrease of protein secretion by Ab melanoma cells after 24, 48h culture in comparison with 1, 6 h culture

The secretory activity of both transplantable melanoma cells was higher during 6 h incubation; later on we observed a decrease in protein secretion: by about 64% in the melanotic melanoma cells and by over 58% in the amelanotic melanoma cells.

This decreasing tendency stayed during the next 24 h observation. Moreover, it was noticed that cells of the native melanoma line secreted proteins more actively than the amelanotic melanoma cells, during the whole incubation time; for 1 h and 6 h incubations these differences were statistically significant.

In that situation the calculation of the OSM content in 1 mg of total protein secreted showed that after 24 h there was 34.5% more OSM in the supernatant of amelanotic melanoma cells than in the supernatant of native line cells, and after 48 h this value increased to 52%. It is worth noticing that after that time the protein secretion decreased in both melanoma cells to a similar degree, by about 17%.

DISCUSSION

Our comparative study regarding the secretion of OSM by cells of two lines of transplantable melanomas showed that a spontaneous alteration of a native melanotic line into an amelanotic line with a more aggressive tumorigenic phenotype is accompanied by an increase in vitro of the OSM secretory activity; this alteration is also connected with the progression of this melanoma but it does not concern the conspicuous changes in the dynamics of OSM secretion or in its biological activity.

In the results obtained it is worth noticing that the OSM secreted during a 6-hour culture does not show any biological activity, and that OSM activity in the supernatants was highest after a 48-hour culture, while the content of cytokine in the supernatant decreased.

What is interesting is the fact that the amelanotic line of melanoma tumour with a higher rate of growth secreted more OSM (the decrease of OSM secretion after 48 hours of culture is smaller than in the line growing more slowly) than the native melanotic line characterised by a slower rate of growth.

Thus, these results seem to suggest that the OSM secreted by the cells of the amelanotic melanoma line does not affect these cells as an autocrine factor inhibiting the growth of melanoma. It is necessary to take into account also Montero et al. [19] who report that OSM increased the releasing of soluble sgp 130 by melanoma cell lines. This tumour-derived sgp 130 binding OSM in supernatants inhibits the growth-inhibiting activity of cytokine.

Our results may also suggest that the spontaneous alteration of a melanotic melanoma line into an amelanotic form accompanied by tumour progression is connected with a diminished susceptibility of this line of melanoma to the antiproliferative action of OSM.

This finding is consistent with the concept of acquired "multicytokine resistance" during melanoma progression, underlined by some authors [9,16,23].

On the other hand, this observation agrees with Gibbs et al. [5] showing a lack of regularity of different melanoma lines in the reactivity to the antiproliferative action of OSM.

However, considering the reports that OSM is a multifunctional cytokine, it is difficult in the case of transplantable melanoma to connect the secretion of OSM only with the function of an autocrine factor regulating melanoma growth.

Studies by Giavazzi [4] and Heyman et al. [7] demonstrated the ability of OSM to modulate the expression of adhesion molecules on the melanoma cell surface, suggesting a potential role of this cytokine in the modulation of tumour progression.

Investigations indicating OSM's ability to modify the activity of tissue inhibitor of metaloproteinase explain the changed conditions in the connective tissue surrounding the tumour, and the potential role of this cytokine in neoplastic metastases [17,22].

It is possible to assume that the OSM secreted by melanoma cells changes the local environment in another way, by influencing the cells involved in the immune response and modifying their function.

Some authors think that OSM is important in the melanoma progression, inducing tumour cells to release other cytokines [18].

Our earlier study showed that the progression of transplantable melanoma is associated with a decreased secretion of IL-6 and TNF-α [24], whereas in the present work OSM secretion increases; this seems to indicate that in the biology of transplantable melanoma OSM plays a different role than the above-mentioned cytokines.

Perhaps the OSM secreted by melanoma cells influences the cells of the immunological system, inducing cytotoxicity of the effector cells but only for a melanoma with a lower immunogenicity, according to Revel's [21] suggestion, and for this reason the rate of growth of the melanoma form with a higher immunogenicity does not decrease.

Our previous findings also suggest that the amelanotic melanoma line with a higher immunogenicity, inducing macrophage secretion of different cytokines, including OSM, was able to suppress macrophages and evoke conditions of tumour escape from immunosurveillance [10].

Furthermore, the fact that it is in a 48 h culture that the secretion of total protein by melanoma cells decreases but OSM secretion and its biological activity are highest, also seems to show that in the biology of transplantable melanoma this cytokine may perform a special function, not connected directly with the regulation of the growth rate. This seems possible particularly when we take into account the above-mentioned observation showing that in vitro secretion of such cytokines as TNF-α and IL-6 in 48-hour cultures decreased [24].

Thus, on the basis of the results obtained, we may suppose that the elevated levels of OSM secreted by the cells of a melanoma with a higher immunogenicity and higher growth rate are to a lesser degree an autocrine factor regulating the growth of melanoma, but may to a greater extent be involved in the immunomodulatory function, changing the tumour-host interaction during melanoma progression.

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