Characterization of interleukin 10 and NO release by transplantable melanoma cell lines with regard to their progression

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The results from our investigations concerning the secretory activity of interleukin 10 (IL-10) by two transplantable melanoma cells showed that a spontaneous alteration of the native-melanotic line into an amelanotic form and the tumor progression connected with it, were accompanied by a 6-fold decrease of the IL-10 content in the supernatant of these melanoma line cell culture.

Simultaneously, the intracellular content of IL-10 indicated that there was only a small population of IL-10 positive cells, numerically similar in both melanoma cell lines.

A comparison of the IL-10 and nitric oxide (NO) secreted by both melanoma lines does not show any correlation between the changes in the content of these substances, which seems to indicate that NO does not act as an autocrine regulator of IL-10 secretion.

Key words: IL-10, NO, transplantable melanoma, tumor progression.

It is known that an immune system can inhibit tumor development [23]. On the other hand, the immune system of tumor-bearing patients shows a lot of functional changes caused by the biological activities of tumors e.g. cytokine secretion [21].

Although cytokines secreted in a tumor microenvironment are more and more often regarded as factors able to modify the tumor – host interaction [20, 23], the mechanism of changes in the immunologic response is still not fully understood.

Obscure also is the influence of the tumor biological properties on the host immunological response, despite the known fact that factors released by melanoma cells can modify this response [13].

According to Giovalleri et al [12] the kind of cytokine released by a tumor seems to define the type of immunological response, whereas differences in the concentration of locally secreted cytokines make possible a modification of the primary antitumor reaction.

For this reason, it seems particularly interesting to estimate the secretory activity of cytokines released locally by melanoma cells in relation to the tumor biological properties and determine possible relationships between the cytokines secreted.

At present, the role of IL-10 in melanoma growth and metastasizing is discussed but opinions on this topic are still controversial [1, 5, 7]. Some authors now hold the opinion that IL-10 is an immunosupressive factor facilitating the escape of a tumor from immunosurveillance and its progression [7, 9, 14], others consider IL-10 as a factor decreasing melanoma tumorogenicity and ability to metastasize [1, 11, 18].

Furthermore, attention is paid to the production of NO influenced by IL-10. This substance is strongly involved in the immunobiology of melanoma and influences its development by a cytotoxic activity [10] or by affecting the tumor cell apoptosis [25].

For this reason, in continuation of our comparative study of the secretory activity of two transplantable melanoma lines of common origin which spontaneously changed their levels of differentiation, growth rates and immunogenicity, 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 IL-10 and establish if these changes are in any way connected with changes in the nitric oxide secretion previously described [16].

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 reared at the Department's animal facility and fed standard diet and tap water *ad libitum*. The experiments procedures were approved by the Animal Ethics Committee at the Medical University of Gdańsk and confirmed to the National Health and Medical Research Council 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. The amelanotic melanoma line (Ab) originated from the melanotic form by a spontaneous alteration differing in many biological properties [2, 4, 15].

Isolation of melanotic and amelanotic melanoma cells. Melanoma cells were isolated from solid tumors by a nonenzymatic method [3]. The suspension consisted 95–98% of viable cells (estimated by trypan blue test).

Isolation of peritoneal macrophages. Peritoneal exudate cells were induced by injecting animals with 10 ml of 2.98% thioglycolate medium (Gibco), and five days later were washed out of the peritoneal cavity by means of 0.9% NaCl. Then they were isolated by the method described previously [30]. Macrophages were used as a positive control of IL-10 content for cytometric analysis and Western blotting.

Preparation of supernatants of melanoma cells culture. Isolated melanoma cells at the concentration 3 x 10^6/ml were incubated in RPMI 1640 (culture medium) (Biomed Lublin) with 10% FCS (GIBCO) and antibiotics (100 U/ml penicilin, 100 μg/ml streptomycin) for 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.

Assay of NO concentration. Nitric oxide (NO), quantified by the accumulation of nitrite (NO_2^-) (as a stable end product) in the 48 h melanoma cell supernatants, was measured by a microplate assay method according to Ding et al [6].

Briefly, 50 μl of each supernatant per well was transferred to a 96-well microassay plate (Nunc) and incubated with an equal volume of modified Griess reagent (Sigma) at room temperature for 10 min. The samples were done in triplicate. The absorbance at 540 nm was determined in a BioRad microplate reader.

NO concentration was calculated from a sodium nitrite ($NaNO_2$, Sigma) standard curve. In all experiments nitrite contents in wells containing medium without melanoma cells was also measured and substracted from experimental values. Data are expressed as μM nitrite/3x10^6 cells.

IL-10 determination by ELISA test. Level of IL-10 in melanoma cells supernatants was determined by the Quantikine mouse IL-10 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-10 was 4.0 pg/ml. Samples were assayed in triplicate.

Western blot detection of IL-10. Fresh isolated melanoma cells or fresh isolated macrophages (2x10^6) 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, phenyl-methyl 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 [19]. Separated proteins were then electrophoretically transferred onto nitrocellulose (60 V, 2.5 h). Nitrocellulose sheets (Schleicher & Schuel) with transferred protein were then processed for IL-10 detection, i.e., blocked with 3% BSA in PBS, washed and exposed to the biotinylated anti-mouse IL-10 (R & D, Minneapolis MN, USA) for 2 h at room temperature. After washing out the unbound antibody, that antibody bound to the membrane was detected by colorimetry 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. Prestained SDS-PAGE Standards (BioRad) were used as molecular weight marker.

Detection of intracellular IL-10 by flow cytometric analysis and confocal microscopy system. The technique was originally described by Sander et al [26], but has been modified in these studies. Briefly, isolated melanoma cells after 24 h culture in RPMI 1640 (Biomed, Lublin) with 10% (FCS, GIBCO) and antibiotics (100 U/ml penicilin, 100 μg/ml streptomycin) were incubated in fresh medium with GolgiStop as desribed by the manufacturer (BD Pharmingen).

Melanoma cells were then washed twice in PBS with 1% FCS, fixed for 20 min in PBS containing 4% paraformaldehyde (SIGMA) and permeabilized for 20 min using PBS with 0.2% saponin (SIGMA), all at room temperature. This was followed by incubation 5x10^5 cells with 50 μl of PE-labeled IgG_{2b} anti-mouse IL-10 (BD Pharmingen) or PE-labeled isotype control IgG_{2b} (BD Pharmingen) (4 μg/ml), for 30 min in the dark at room temperature, in PBS with 1% FCS and 0.2% saponin.

Cells were washed in PBS with 0.2% saponin, resus-

pended in PBS with 2% paraformaldehyde and analysed on a FACSCalibur flow cytometer (Becton Dickinson, USA, Department of Hematology of Medical University, Gdańsk). Fluorescence data were acquired from 10,000 cells per sample. Offline analysis was performed using WinMDI 2.6 software (obtained from J. Trotter, Scripps).

Immunofluorescently stained (as to cytometry) slides were examined also using a fluorescent microscope DMLB (Leica, Germany), and a confocal microscopy system MicroRadiance (Bio-Rad, UK), equipped with an Argon ion laser and mounted on a light microscope Eclipse 600 (Nikon, Japan), using the software LaserSharp2000 (Bio-Rad, UK). The confocal microscopy images were obtained using 40x and 60x oil immersion objective lenses of N.A.=1.3 and 1.4, respectively.

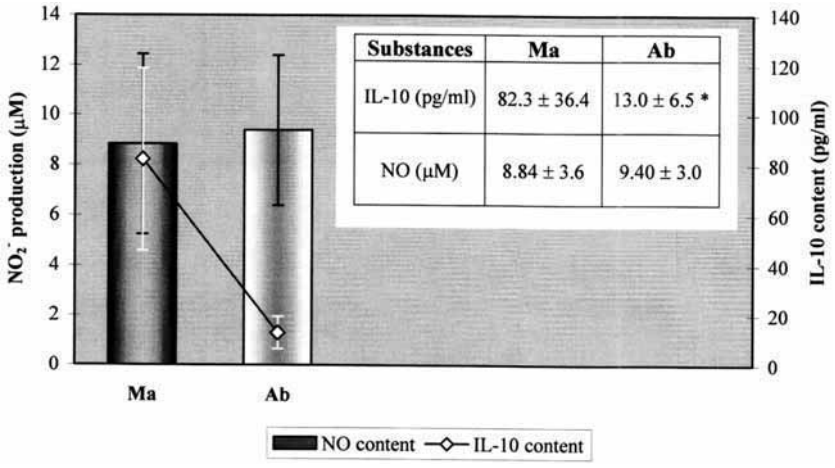

Figure 1. IL-10 and nitric oxide (NO) content in supernatants of 48 h culture of transplantable melanotic (Ma) and amelanotic (Ab) melanoma cells ($3x10^6$/ml).
The values are the means ±S.D. for 6 (IL-10) and 11–15 (NO) experiments.
***Statistical analysis by nonparametric tests U Mann-Whitney: statistically significant ($0.001<p<0.01$) decrease of IL-10 secretion by amelanotic melanoma cells in comparison with melanotic melanoma cells.**

Isolated peritoneal macrophages stained in the same manner as melanoma cells were used as a positive control of intracellular IL-10 content.

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

Results

Estimation of IL-10 and NO content in melanoma cell supernatants after 48 hrs culture. The results obtained from a quantitative study of IL-10 and NO content in melanoma cell supernatants are presented in Figure 1. They indicate that both transplantable melanoma cells were able to secrete IL-10 and NO, but their secretory activity was different, especially for IL-10.

The native-melanotic melanoma cells secreted 6 times more IL-10 than the amelanotic melanoma cells. Simultaneously, the results concerning NO production showed that a spontaneous alteration of a melanotic line into an amelanotic line of melanoma with a higher rate of growth is accompanied by a tendency to increase NO secretion. However, the observed increase was 6% and was not statistically significant.

Estimation of intracellular content of IL-10 in transplantable melanoma cells by flow cytometry and confocal microscopy systems. The results obtained from the estimation of intracellular IL-10 production are presented in Table 1 and illustrated in Figure 2 as dotplots after a cytometric analysis and images performed by a confocal microscopy system. They indicate that in a population of isolated hamster transplantable melanoma cells only a small percentage of cells have intracellular IL-10.

It was found that slightly above 2% of the melanotic melanoma cells and almost a half less cells, the second type of tumor had this cytokine inside the cell. These values were highly statistically significant, lower for the amelanotic melanoma than for the native line ($p<0.01$; Tab. 1).

It is also necessary to add that in the same experimental conditions 20% of isolated peritoneal macrophages, used as a positive control of intracellular IL-10 content, bound IL-10 antibody (Fig. 2C).

It is worth to notice that the images performed by a confocal microscopy system shown in Figure 2, seem to confirm the results of a cytometric analysis and indicate that only a few of both transplantable melanoma cells present in the

Table 1. Flow cytometric detection of intracellular IL-10 production among transplantable melanoma cells

Cells	Percentages (%) of cells with intracellular IL-10 content
Melanotic melanoma	2.5 ± 0.4
Amelanotic melanoma	1.4 ± 0.6*

The values are the percentages of positive cells among of population of transplantable melanoma cells stained anti-IL-10 antibody-PE, after background of isotype control elimination. The values are the means ±SD of 6 experiments. *Statistical analysis by nonparametric U Mann-Whitney test – statistically significant decrease of the percentage of positive cells among of population of amelanotic melanoma cells stained anti-IL-10 antibody-PE in comparison to melanotic melanoma; $p<0.01$.

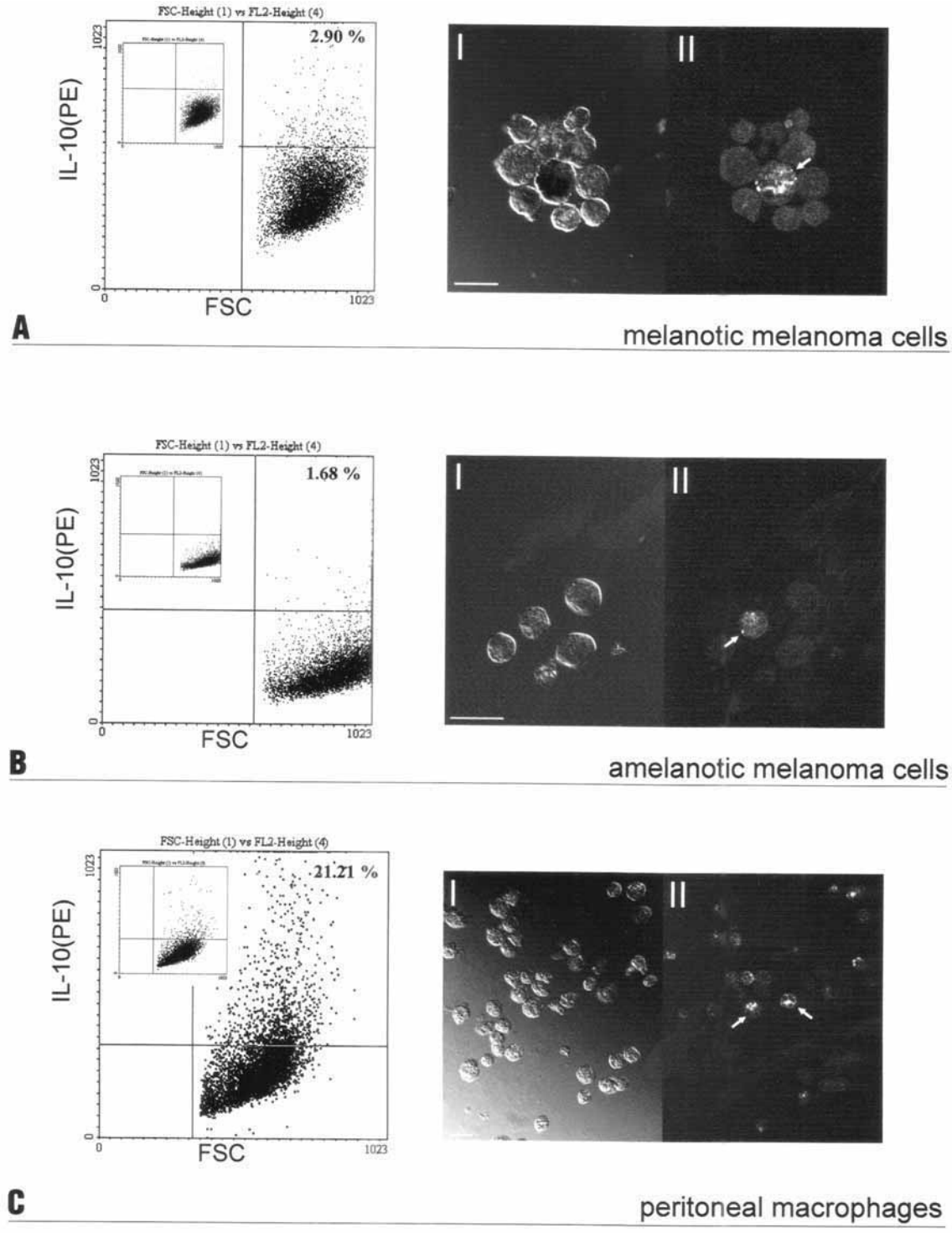

Figure 2. Detection of intracellular IL-10 content in isolated melanoma cells (A, B) and peritoneal macrophages (C) by flow cytometric analysis and confocal microscopy system.

The plots show results of representative experiments flow cytometric detection of IL-10-producting cells. The positive cells there are in the upper right quadrants of each plot. The quadrants were appointed on the grounds of the range of fluorescence of isotype control (small plots in upper left of great plots). The precentages in the corner of each plot represent the positive cells among the population of cells stained anti-IL-10 antibody-PE, after background of isotype control elimination.

Photomicrographes maded using confocal microscopy system from the same optical section show intracellular localization of IL-10 in cells. I – image of slide obtained using transsmission light; II – images of cells immunofluorescently stained anti-IL-10 antibody-PE; Arrow indicate positive cells. Scale bar m=25 μm.

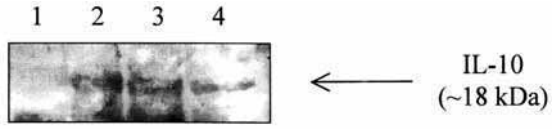

Figure 3. Western blot detection of interleukin 10 (~18 kDa) in cellular lysates of transplantable melanoma tumors. Lane 1 is for negative control of culture medium without cells; lane 2 for positive control of macrophages lysates; lanes 3 and 4 for lysates of melanotic and amelanotic melanoma cells, respectively.

suspension prepared for staining bound antibody against IL-10; therefore they indicated intracellular location of this cytokine.

Western blot detection of IL-10 in cellular lysates. The presence of IL-10 was confirmed in the cellular lysates of melanoma cells by western blot (Fig. 3). Lysates obtained from hamster peritoneal macrophages were used as a positive control of the content of IL-10.

Discussion

The results from the present studies show that a spontaneous alteration of the native-melanotic melanoma line into the amelanotic form, less differentiated but with a higher growth rate and with a higher immunogenicity was accompanied by a dramatic decrease of IL-10 secretion, so we could say that there was a reverse correlation between the rate of growth and the amount of IL-10 secreted.

It is worth to notice that although the melanoma phenotype change was connected with changes in the secretory activity of these cells regarding total proteins or other cytokines (as mentioned in our earlier works) [17], this change was not so strongly marked as in the case of IL-10 secretion. Maybe IL-10 plays a special role in the biology of transplantable melanomas which reflect its role in the modulating influence on the tumor phenotype and thereby on the changed tumor-host interaction.

The significance of IL-10 in the biology of melanoma is strongly emphasized by many authors although the reported results are still controversial. Some of these results indicate an antitumor activity of IL-10 [14, 18].

In the animal model IL-10 gene transfection into melanoma cells caused a decrease of tumorogenicity and longer survival time of animals [11, 28].

These observations seem to be in agreement with our results indicating a 6-fold decrease of IL-10 secretion in amelanotic melanoma cells with a lower degree of differentiation, higher growth rate and shorter time of survival in comparison to the native melanoma line.

But the results from other investigations concerning the immunobiology of melanoma show that IL-10 is a suppressor factor for the host antitumor response [7]. The evidence for this has been e.g. the fact that a considerable percentage of patients with melanoma had a higher level of IL-10 in the serum. It has also been shown that after IL-10 gene transfection B16 cell proliferation was faster than in untransfected B16 cells [9]. Also, an injection of exogenous IL-10 into tumor caused a B16 melanoma progression [27].

The above-mentioned observations show that the in vivo role of Il-10 in tumor development, and especially in melanomas, is far from clarification.

It is interesting to notice that the earlier, uniform view on IL-10 role has been clearly modified because nowadays this cytokine is no longer treated as a cytokine playing a role only in the immunosuppressive response against tumor development [1].

Also other observations indicating that not all melanoma cells secrete endogenous IL-10 in culture make it difficult to create a uniform conception of IL-10 role in melanoma immunobiology [8, 14, 22].

The results of our immunoblotting showed the presence of this cytokine in melanotic and amelanotic melanoma cells.

Analysis of the intracellular location of this cytokine showed, however, that only a small percentage of cells contained IL-10. This seems to suggest that not all, but only some clones of melanoma cells are able to produce this cytokine.

On the basis of these results it is difficult to say whether only this small part of cells was able to synthesize IL-10 or whether this cytokine was actively secreted from cells to the environment.

While searching for a correlation between the role of IL-10 and NO secretion by normal [24, 29], and tumor cells [12] as postulated by others, we observed that during a 48 hrs medium culture of amelanotic melanoma cells the content of NO was a little higher than in melanotic cells. But it is difficult to say if IL-10 plays the role of an autocrine suppressor of NO secretion because changes in the amount of IL-10 secretion do not correlate with the changes of NO secretion.

Our results mentioned-above are not in agreement with KUNDU et al [18] observations which indicate that IL-10 induced the NO level in tumor cells. They are also different from observations which showed that IL-10 suppressed NO production by macrophages [12, 29].

Both our earlier investigations concerning the correlation between IL-6, TNF-α, OSM secretion and NO production [16, 17] and the present studies of IL-10 and NO secretion by transplantable melanoma cells seem to indicate that NO and cytokines are produced independently by the melanoma cells examined without any distinct interactions. This observation corresponds with PETER et al [22] report which underlines that cells of each melanoma line have their own immunological and secretory profile.

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