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Expression of CD44 on two lines of transplantable melanoma cells – relationship with cytokine secretion and tumor progression

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Abstract: In the present work it was investigated if a spontaneous alteration of the native melanotic transplantable melanoma form into amelanotic form, connected with the tumor progression, is accompanied by changes of CD44 surface glycoprotein expression. We also tried to find out if there exists any correlation between changes in CD44 expression and IL-6, TNF-α, and IL-10 secretion. Cells of two hamster transplantable melanoma lines: melanotic and amelanotic were used. The levels of TNF-α, IL-6, IL-10 in supernatants were determined by the ELISA test. For the detection of CD44 expression by flow cytometry, isolated melanoma cells were stained with the rat anti-mouse CD44 monoclonal antibody. The stained cells were also examined using a fluorescence microscope and a confocal microscopy system. The obtained results indicate that a spontaneous alteration of the native melanotic form into amelanotic form and the associated tumor progression was accompanied by a decrease in CD44 glycoprotein expression on the cell surface and a decrease in IL-6, TNF-α and especially IL-10 secretion by amelanotic melanoma cells. Our observations suggest a relationship between CD44 expression and locally secreted cytokines in the course of transplantable melanoma progression.

Key words: CD44 - Transplantable melanomas - Cytokines - IL-6 - IL-10 - TNF-α

Introduction

Many investigations show that changes in the expression of highly glycosylated proteins from CD44 family - the main receptors for hyaluronic acid - and changes in the ligand binding to this receptor on malignant tumor cells (among them in melanoma cell) are associated with the increase in tumor proliferation and metastatic abilities [1, 5, 6, 13, 32, 33, 34]. There are also other observations, suggesting that the role of CD44 glycoproteins in melanoma biology has not been fully explained so far [25, 28, 29, 30]. Besides that, there is another very interesting and unexplained problem: the role of cytokines locally secreted by tumor cells in the growth and progression of melanomas, especially in the light of observations that cytokines play the regulatory role in CD44 and hyaluronic acid binding [9, 20, 23, 26, 30].

Our previous study revealed that a spontaneous alteration of the native melanotic transplantable melanoma form into amelanotic form (with higher growth rate and shorter survival time of animals) is associated with multiple qualitative and quantitative changes of membrane glycoproteins [14, 19, 31]. In this study, as a continuation of our investigations on the diversity of cell surface glycoproteins of two lines of transplantable melanomas (of common origin but differing in many biological features [2, 4, 18]), we address the question if these changes concern also glycoproteins from CD44 family.

Our previous observations of the differences in secretory activity of melanoma cells between both lines concerning IL-6, TNF-α and IL-10 [15, 17], associated with a spontaneous alteration of transplantable melanomas, allow us to look for a correlation between changes in CD44 expression on cells of both melanoma forms and changes in the secretory activity of these cells concerning the examined cytokines.

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Materials 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.

Transplantable melanomas. The tumors were transplantable melanotic and amelanotic melanomas. The melanotic melanoma line derived from a spontaneous melanoma of the skin which had appeared in a breed of golden hamsters in 1959 and was described by Bomirski in 1977 as Ma line [4]. The amelanotic melanoma line originated from the melanotic form by a spontaneous alteration and was described in 1977 as Ab line [4] which grows faster, causes animal death within a shorter time [2], and shows changed antigenicity and immunogenicity [18].

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

CT6 cell line. CT6 (murine cytolytic T cells clone CT6) cell line (obtained from the Nencki Institute, Warsaw, Poland) was used as a positive control for CD44 expression.

Preparation of supernatants of melanoma cell culture. Isolated melanoma cells at the concentration of 5×10^5/ml were incubated in RPMI 1640 (Biomed Lublin) with 10% FCS (fetal calf serum; GIBCO) and antibiotics for 48 h in 6-well plates.

TNF-α, IL-6, IL-10 determination by ELISA test. The level of TNF-α, IL-6, IL-10 in melanoma cell supernatants was determined by the Quantikine mouse ELISA assays (Research and Diagnostic Systems, Mineapolis, MN, USA).

Detection of CD44 expression by flow cytometry and confocal microscopy. Isolated melanoma cells were cultured in RPMI 1640 with 10% FCS and antibiotics for 24 h. After that time cells were harvested, washed with PBS and immunostained with rat anti-mouse CD44 monoclonal antibody [PE-conjugated anti-CD44 (Pgp-1) mAb, IM7; Pharmingen, San Diego, CA, USA; 1 μg/10^6 cells]. The background control of nonspecific binding to the cells was PE-labeled anti-rat IgG2b (Pharmingen, San Diego, CA, USA). Cells were incubated for 45 min at 4°C, washed with PBS three times and analyzed for fluorescence with the FACS Calibur (Becton Dickinson, USA; Department of Hematology, Medical University, Gdańsk).

Fluorescence data were acquired from 20 000 cells per sample. Off-line analysis was performed using WinMDI 2.6 software (obtained from J.Trotter, Scripps).

Immunostained (as for cytometry) cells were examined also using the fluorescent microscope DMLB (Leica, Germany), and a confocal microscopy system MicroRadiance (CLMS; Bio-Rad, UK), equipped with an argon ion laser and mounted on the light microscope Eclipse 600 (Nikon, Japan), using the LaserSharp 2000 software (Bio-Rad, UK). The stacks of serial optical sections 0.6 μm apart were obtained with a CLSM (× 40 and × 60) under objective lenses of NA=1.3 and 1.4, respectively.

CT6 cells stained in the same manner as melanoma cells were used as a positive control of the CD44 expression.

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

Results

CD44 expression on the transplantable melanoma cells

The results of CD44 antibody binding by hamster transplantable melanoma cells obtained by flow cyto-

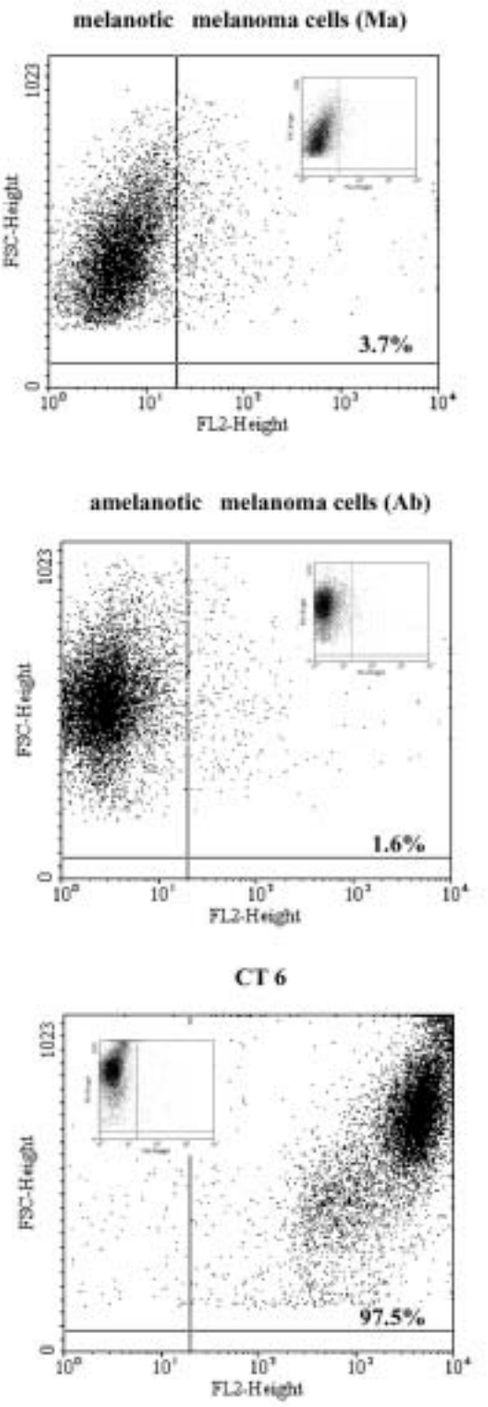

Fig. 1. Expression of glycoprotein CD44 on transplantable melanotic (Ma) and amelanotic (Ab) melanoma cells and CT6 cells as positive control. The plots show results of one representative experiment of flow cytometric detection of CD44-positive cells (the upper right quadrants of each plot). Inserts show fluorescence levels of isotype control. The percentages in the corner of each plot represent the positive cells among the population of cells after elimination of isotype control bacground.

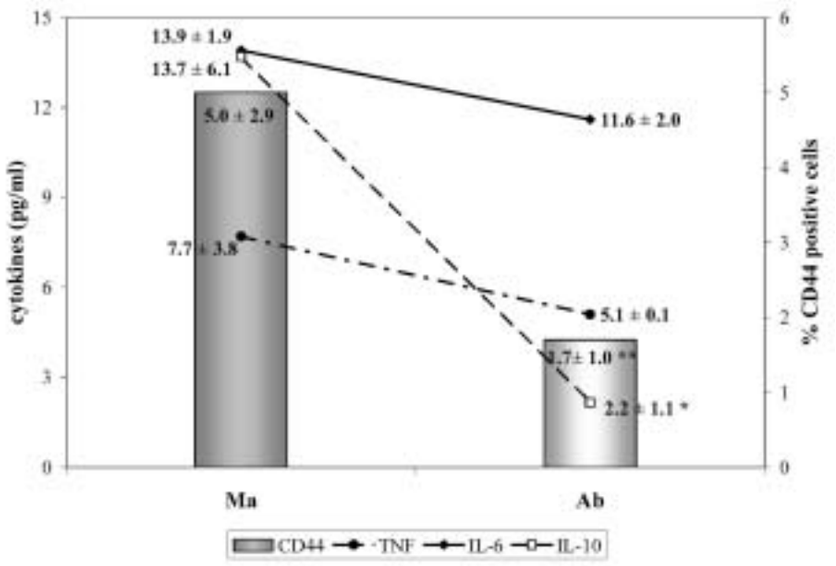

Fig. 2. The content of TNF-α, IL-6 and IL-10 in supernatants of cultured transplantable melanoma cells in comparison to percentage of CD44-positive cells.The values are means ± SD of 5-15 experiments for estimations of cytokine secretion done in triplicate and 5-12 experiments for CD44 expression. For each assessment of CD44 expression, 20 000 cells were analysed in a flow cytometer. *Statistically significant decrease in IL-10 secretion by amelanotic melanoma cells in comparison to native line; 0.001<p<0.01; **statistically significant decrease in the percentage of CD44-positive cells among amelanotic melanoma cells in comparison to native line; 0.001<p<0.01

metry are presented in Figures 1 and 2 and illustrated in Figure 3 as images from a confocal microscopy system. Only few melanotic and amelanotic melanoma cells bound anti-CD44 antibody in the comparison with CT6 cells (positive control), among which 97 ± 2.6% were CD44-positive (Fig. 1).

In spite of low percentage of CD44-positive melanoma cells, we observed significant differences between the melanotic line and the more aggressive amelanotic line.

About 5% of the native melanotic melanoma cells bound anti-CD44 antibody, while among amelanotic melanoma cells only 1.7% showed CD44 antigen expression (Figs. 1, 2). Decrease in the content of CD44-positive cells among amelanotic melanoma line which has a more aggressive phenotype is statistically significant ($p<0.01$) in the comparison with native melanotic melanoma line.

Images from confocal microscope seem to confirm the results of the cytometric analysis and indicate that only single cells of both transplantable melanomas in the cell suspension were CD44-positive. The analysis of serial optical sections of CD44-positive cells shows that CD44 antigen localizes on the transplantable melanoma cell surface, *i.e.* plasma membrane and is unequally distributed (Fig. 3).

Estimation of cytokine (TNF-α, IL-6, IL-10) content in transplantable melanoma cell supernatants after 48 hrs culture

The results obtained from a quantitative assessment of TNF-α, IL-6, and L-10 content in melanoma cell supernatants are presented in Figure 2. They indicate that amelanotic melanoma cells - the more aggressive line, in the comparison with the native melanotic line of this tumor, show decreased secretory activity concerning all examined cytokines.

We observed over 6 times lower content of IL-10 (statistically significant $0.001<p<0.01$) in the supernatants of amelanotic melanoma cells in comparison to melanotic melanoma line.

These cells secreted also about 16% less IL-6 and about 34% less TNF-α than cells of the native line, but these differences were statistically insignificant.

Discussion

The obtained results indicate that a spontaneous alteration of native melanotic form into amelanotic one with higher tumorogenicity, was accompanied by a decrease in CD44 glycoprotein expression. They are in accordance with our earlier observations that phenotypic changes in amelanotic melanoma cells are associated with the decrease in the content of glycoproteins on the cell surface and changes in their connections in plasma membrane [14, 16, 19].

According to Karjalainen *et al.* [13], the reduction of CD44 expression on melanoma cell surface is connected with poor prognosis. But other authors indicated that the increase in CD44 expression was accompanied by an increase in tumor aggressiveness [1, 5, 33].

Thus, the decrease in CD44 expression on cells of amelanotic melanoma with a higher growth rate and shorter survival time of animals in the comparison with native melanotic line, does not agree with the findings that during melanoma progression CD44 expression increases and that CD44, as the main receptor for hyalu-

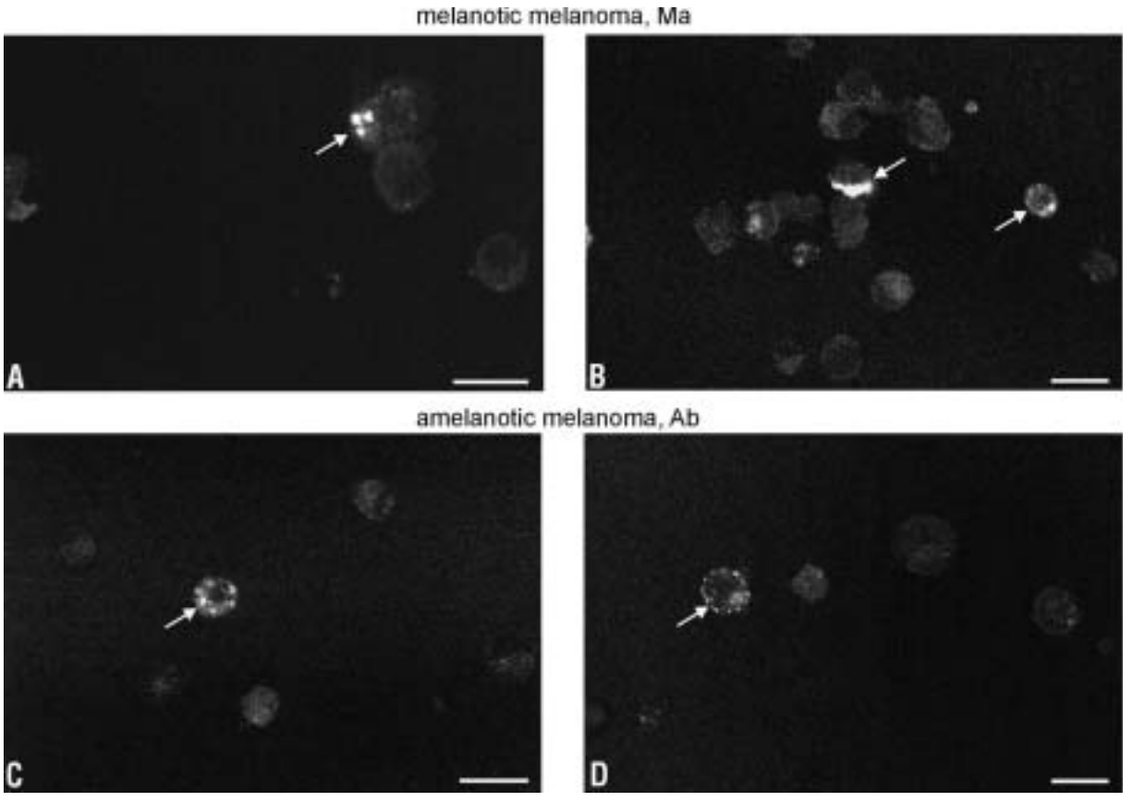

Fig. 3. CLMS images of CD44-positive cells among isolated melanotic (A,B) and amelanotic (C,D) melanoma cells. Micrographs show CD44-positive cells among isolated melanoma cells, immunostained with anti-CD44 antibody-PE. Scale bar = 25 μm.

ronic acid, simultaneously induces tumor cell proliferation which can depend on CD44-hyaluronic acid interactions [1, 5, 33].

The decrease in CD44 expression on cells of amelanotic melanoma line may not be the manifestation of the reduction of these surface molecules but may result from the appearance of a new isoform which can be shed from cell surface. The observations of other authors pointed to the CD44 glycoprotein shedding from the melanoma cell surface [1, 24].

It is also worth to notice that alteration of CD44 glycosylation process can affect hyaluronic acid binding ability [11] and according to English *et al.* [7] sialic acid may be a major regulator of the hyaluronic acid binding function. Our earlier investigations showed the decrease in sialic acid content on the surface of amelanotic melanoma cells [14].

Looking for the correlation between CD44 expression and cytokine secretion by cells of both melanoma lines, we observed that amelanotic melanoma cells had about 66% lower CD44 expression and secreted less TNF-α, IL-6, and IL-10 [15, 17]. This observation suggests connections between CD44 and locally secreted cytokines.

Our observations referring to IL-6 and CD44 agree with those of Garcia de Galdeano *et al.* [9] who found the increase in CD44 expression on B16 melanoma cells in the presence of IL-6. Osada *et al.* [27] showed that TNF-α significantly up-regulated the expression of CD44 on the epidermal Langerhans cells. Other authors found that TNF-α converts CD44 from its inactive form to the active one [26]. Levesque *et al.* [22] described that ligation of CD44 on cells of the immune system induced the production of IL-1 and TNF-α.

Thus, the 34% decrease in TNF-α secretion by amelanotic melanoma cells may be one of the reasons for the observed decrease in CD44 expression on their surface.

So far in the literature we have not found any information about relations between endogenously secreted IL-10 by melanoma cells and CD44 expression, but studies on cells of the immune system indicated that IL-10 induced CD44-hyaluronic acid interactions [23]. Levesque *et al.* [22] observed that binding of hyaluronic acid to CD44 induced TNF-α secretion by monocytes but IL-10 inhibited cytokine-induced hyaluronic acid binding to monocyte CD44 [23].

It is unlikely that 84% decrease in IL-10 secretion by amelanotic melanoma cells in comparison to native line observed in our investigations could also be the reason for the decrease in CD44 expression on these cells. Haegel-Kronenberger *et al.* [10] suggested that only

great number of CD44 molecules on the cell surface triggered, among other cytokines, IL-10 secretion.

Although there is substantial information about the participation of CD44 molecule [5, 12, 33] and the examined cytokines [8, 21] in melanoma growth and progression, because of scanty information about the correlation between CD44 expression and cytokine secretion by melanoma cells, it is difficult to compare our results with others and look for the biological sense of these connections. The present observation seems to be important for further investigations concerning the above problem.

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