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Spontaneous apoptosis of melanotic and amelanotic melanoma cells in different phases of cell cycle: relation to tumor growth

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Abstract: Since the spontaneous alteration of native melanotic (Ma) into amelanotic (Ab) transplantable melanoma line it has been observed that this alteration is accompanied by the acceleration of growth of Ab line. The aim of the present study was to check and estimate spontaneous apoptosis of cells from cell cycle phases. Cytometric cell cycle analysis was performed by staining cells with propidium iodide (PI). Apoptosis estimated by the TUNEL method, alterations in the plasma membrane structure (annexin V staining), changes in the mitochondrial transmembrane potential - $\Delta\Psi m$ (JC-1 staining) showed that amelanotic melanoma cells have decreased ability to undergo spontaneous apoptosis. The obtained results showing that in the native melanotic line about 30% of cells are in S+G2/M phases and that 33% of these cells undergo apoptosis could lead to the conclusion that the slower growth of this melanoma line is the result of lower proliferation activity and higher rate of apoptosis of these tumor cells. The number of cells in S+G2/M phases in amelanotic melanoma line increases up to 40% and only 7% of them undergo apoptosis. This observation seems to suggest that the expansive growth of this melanoma line depends mainly on the decreased ability to undergo spontaneous apoptosis, especially in case of cells from S+G2/M phases. Moreover, the obtained results indicate that alteration of melanotic line into amelanotic one, accompanied by differences in many biological features also concerns basic cell processes such as cell cycle and cell death. (www.cm-uj.krakow.pl/FHC)

Key words: Melanoma - Spontaneous apoptosis - Cell cycle - Tumor growth

Introduction

Research on tumor development seems to indicate that the growth of tumor depends on cell proliferation and death, especially by programmed cell death - apoptosis [10, 11].

It is well known that tumor cells show higher ability to proliferate because of *e.g.* autocrine production of growth factors, higher expression of growth factor receptors and changes in the signaling pathways that are involved in the transduction of mitogenic stimuli into the cell [11, 41].

For over thirty years it has been known that different tumor cells [26], among them also melanoma [43] die by apoptosis, which has been considered the reason for the lower growth rate of some tumors [26]. However, investigations on the role of apoptosis in chemo-and radiotherapy [2, 48], clearly showed that tumor cells, also melanoma cells, have a decreased ability to undergo apoptosis [37, 42]. The molecular basis of this ability is far from being understood, despite growing evidence of the disturbances in the mechanism of melanoma cells apoptosis [18, 23, 46]. The decreased ability to undergo apoptosis and the cell cycle disturbances in different tumor cells are considered to be factors involved in tumor progression [11, 21].

In spite of growing evidence of links between apoptosis and cell cycle [15, 33, 50], the nature of these relationships have not been explained so far.

Our earlier comparative studies of two hamsters transplantable melanoma lines: melanotic (Ma) and amelanotic (Ab), except differences in the ultrastructure, changes in the plasma membrane glycoproteins and activity of some enzymes, showed also higher growth rate accompanied by the spontaneous alteration of melanotic melanoma line into amelanotic one [6, 8]. Our latest investigations [29, 30] indicated that the decreased ability to undergo apoptosis could favor the higher growth rate of the amelanotic melanoma.

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In spite of popular opinion that tumor growth depends not only on the dynamics of tumor clone cell proliferation but also on the ability of cells to die - especially by apoptosis [10], it is still not clear which of these phenomena predominates and decides for the individual tumor growth [17, 20, 25, 34]. We also do not know in what phases of cell cycle tumor cells die most easily.

The problems mentioned above also concern melanoma, where clear correlation between proliferation, apoptosis and tumor progression has not been found [44, 49]. On the other hand, the number of proliferating cells in melanoma is proposed to be a prognostic marker of patient's survival time [1].

Therefore, we were especially interested in checking to what extent the spontaneous alteration of melanotic line into amelanotic one - with higher growth rate and shorter animal survival - depends on the ability of cells in different cell cycle phases to undergo spontaneous apoptosis, especially in S+G2/M phases which can be used as indirect measure of proliferative activity of cells [3, 4, 9].

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. The experimental procedures were approved by the Animal Ethics Committee at Medical University of Gdańsk and conformed to the National Health and Medical Research Council's guide for the care and use of laboratory animals.

Transplantable melanomas. The melanotic melanoma line (Ma) was derived from a melanoma of the skin which had appeared spontaneously in a breed of golden hamsters in 1959 [6]. The amelanotic melanoma line (Ab) differing in many biological properties [6,8,27] originated from the melanotic form by a spontaneous alteration. Melanoma cells were isolated from solid tumors by a non-enzymatic method [7]. The suspension contained 95-98% of viable cells (estimated by trypan blue exclusion test). Cells after isolation were cultured for 4 hours in culture medium (RPMI, 10% FBS Gibco, antibiotics) [45].

Cell cycle analysis. Ethanol-fixed 1×10^6 melanoma cells were resuspended in 1 ml of staining solution (RNaseA 200 μg/ml and PI - propidium iodide, 5 μg/ml in PBS) [13]. Then cells were incubated for 30 min at 37˚C in the dark and the fluorescence was analyzed using a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA; Department of Pathophysiology of Medical University, Gdańsk). Control diploid cells were cells isolated from hamster spleens. 10000-20000 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). Cells in S and G2/M phases of cell cycle were analyzed together and used as an indirect measure of proliferative activity of cells [3,4,9].

Estimation of apoptosis by the TUNEL method. The APO-BRDU Kit (BD Bioscience Pharmingen) was used to determine apoptotic cells and cell cycle phases they come from. Cells were prepared following the manufacturer's protocol. Bivariate analysis of apoptosis (green fluorescence) and DNA content (PI) was performed using the flow cytometer.

Estimation of apoptosis by annexin V staining. Changes in plasma membrane structure - externalization of phosphatidyl serine - were assessed by staining cells with Annexin V and PI (Annexin V-Fluos staining kit, Roche) according to manufacturer's instructions. Among annexin-positive cells, we distinguished populations with low and high level of annexin binding (An^{LO} and An^{HI}, respectively). In association with various levels of staining with PI, the above allowed us to distinguish three populations of melanoma cells: (1) fully viable cells - low annexin binding and no PI incorporation (An^{LO},PI-), (2) viable, early apoptotic cells - high annexin binding and no PI staining (An^{HI},PI-), and (3) necrotic or late apoptotic cells - high annexin binding and strong PI staining (An^{HI},PI^{+}).

Estimation of changes in transmembrane mitochondrial potential ($\Delta\Psi$m). Changes of the $\Delta\Psi$m were analyzed using 5,5',6 ,6'-tetrachloro-1,1'3,3'-tetraethylbenzimidazolecarbocyanine iodide (JC-1; Molecular Probes, Inc.,Eugene, OR, USA). This assay detects changes in the integrity of the mitochondrial membrane, an early event in apoptosis [39]. Depending on the $\Delta\Psi$m JC-1 forms monomers (green fluorescence, low $\Delta\Psi$m) or aggregates (red fluorescence, high $\Delta\Psi$m) [39]. 1×10^6 cells/ml were incubated in PBS for 30 min at 37˚C with 10 μg/ml JC-1, washed twice with PBS and suspended at total volume of 500 μl.

Cells stained for TUNEL, annexin V and with JC-1 were analyzed using FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). After gating out small-sized (*e.g.* noncellular debris) objects, 20 000 events were collected for each experiment. Results were analyzed off-line using WinMDI 2.6 software (obtained from J. Trotter, The Scripps Institute, La Jolla, CA).

Analysis of apoptosis by detection of DNA fragmentation (laddering). DNA fragmentation was detected according to Bertrand *et al.* [5]. DNA from 2×10^6 cells was isolated and RNA removed by 2 mg/ml RNAse treatment (RNase A, Sigma-Aldrich, USA). Ethanol-precipitated DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4), resolved on 1.8% agarose gel and visualized by staining with ethidium bromide (0.5 μl/ml).

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

Results

Cell cycle analysis

The cytometric analysis of cell cycle showed that cells from S and G2/M phases accounted for about 30% of the melanotic melanoma cells and their number was significantly ($p<0.05$) lower in comparison to less differentiated, but faster growing amelanotic melanoma, where almost 40% of cells were in S and G2/M phases (Table 1, Fig. 1A). About 47% of cells, in slowly growing and more differentiated melanotic melanoma were in G0/G1 phases. Among amelanotic melanoma about 54% of cells were at that stage of cell cycle (Table 1).

In the histograms of cytometric analysis of DNA content there were also cells with lower DNA content - located below G0/G1 (<G0/G1), they included apoptotic cells (apoptotic bodies). In melanotic melanoma, about 23% of cells were located at sub G1 level but in amelanotic line only 6% (Fig. 1A).

Table 1. Cell cycle analysis and the ability to unergo spontaneous apoptosis of melanotic (Ma) and amelanotic (Ab) transplantable melanoma cells estimated by the TUNEL method

Melanoma cells	Cell cycle analysis		Spontaneous apoptosis			Cells dying by apoptosis	
	% of cells in		total % of apoptotic cells	% of apoptotic cells from		G0/G1	S + G2/M
	G0/G1	S and G2/M		G0/G1	S + G2/M		
melanotic (Ma)	47.1 ± 4.2	30.1 ± 4.2	21.1 ± 5.3	7.0 ± 2.0	9.5 ± 4.3	15%	32%
amelanotic (Ab)	54.3 ± 4.7	38.9 ± 5.3*	11.3 ± 3.1*	2.3 ± 0.4*	2.7 ± 0.4*	4%	7%

The values are the means ± SD of 5 and 6 experiments for Ma and Ab melanoma lines, respectively; *statistically significant 0.05>p>0.001

Table 2. The ability to undergo spontaneous apoptosis (estimated by: changes in the plasma membrane structure - externalization of phosphatidylserine, changes in the transmembrane mitochondrial potential- ΔΨm and TUNEL method) of melanotic (Ma) and amelanotic (Ab) transplantable melanoma cells.

Melanoma cells	Spontaneous apoptosis					
	Changes in the plasma membrane		% of cells with decreased ΔΨm	% of apoptotic cells (TUNEL method)		
	Mean % of cells An^{HI}/PI^{-} (early apoptosis)	Mean % of cells An^{HI}/PI^{+} (late apoptosis/ necrosis)		total	G0/G1	S/G2/M
melanotic (Ma)	36.9 ± 5.7	23.9 ± 4.1	5.7 ± 2.2	21.1 ± 5.3	7.0 ± 2.0	9.5 ± 4.3
amelanotic (Ab)	3.4 ± 1.0*	12.7 ± 3.9*	2.5 ± 1.0*	11.3 ± 3.1*	2.3 ± 0.4*	2.7 ± 0.6*

An^{HI}/PI^{-}: viable, early apoptotic cells - high annexin binding and no PI staining; An^{HI}/PI^{+}: necrotic or late apoptotic cells - high annexin binding and strong PI staining. The values are the means ± SD of: 12-14 experiments for estimation of changes in the plasma membrane, 4-7 experiments for estimation of changes in ΔΨm, 5-6 experiments for TUNEL method; *statistically significant 0.05>p>0.001

Spontaneous apoptosis of transplantable melanoma cells

Results of the TUNEL method showed that 21% of melanotic and only 11% of amelanotic melanoma cells were apoptotic and this difference was statistically significant (Table 2, $p<0.05$).

The apoptotic cells in both melanoma lines came from G0/G1 and S+G2/M phases. Among apoptotic melanotic melanoma cells, about 7% came from G0/G1 phase and about 10% from S+G2/M phases. In the amelanotic melanoma line, apoptotic cells from the abovementioned phases constituted 2% and almost 3%, respectively (Table 2). The comparison of these percentages to the proportion of cells from the particular cell cycle phases in each melanoma line reveal that in melanotic melanoma 32% of cells in S+G2/M phases and 15% of cells in G0/G1 underwent apoptosis, but among amelanotic cells these values were only about 7% and 4% (Table 1).

Binding of annexin V to cells of transplantable melanoma lines showed higher content of cells with phosphatidylserine externalization among cells of the native line, where about 37% cells belonged to the early apoptotic population $An^{HI}PI^{-}$. This proportion was significantly ($p<0.001$) higher than in the amelanotic melanoma line, where only 3% of cells expressed that phenotype (Fig. 1B, Table 2). As compared to amelanotic line, suspensions of melanotic melanoma cells typically contained also more necrotic cells. On the average, 24% of melanotic melanoma cells and only 13% of amelanotic melanoma cells were $An^{HI}PI^{+}$ (Fig. 1B, Table 2). Analysis of nucleosomal DNA fragmentation (laddering) showed a typical DNA ladder in the preparations of melanotic melanoma cell DNA, while DNA fragmentation in amelanotic melanoma line was very weak (Fig. 1B).

Spontaneous apoptosis of both melanoma line cells was accompanied by the decrease in mitochondrial transmembrane potential (ΔΨm), which was observed in about 6% of the native melanoma line and only in 2.5% of amelanotic cells (this difference was statistically significant $p<0.05$, Table 2, Fig. 1B) and suggesting that mitochondria participate in the spontaneous apoptosis especially in cells of the native line. Decrease in ΔΨm is an early event in apoptosis and this seemed to be confirmed in cells of amelanotic melanoma line where we found 2.5% of cells with decreased ΔΨm and 3.4% of cells with changed plasma membrane structure. Among cells of melanotic melanoma line, 6% had decreased ΔΨm, while 37% externalized PS (Table 2).

Discussion

Our results indicating that melanotic melanoma line, growing slowly, more differentiated, has lower content of cells in S+G2/M phases in comparison to faster growing, less differentiated amelanotic melanoma line are in agreement with authors showing that tumor growth depends on the content of proliferating cells [29] and the

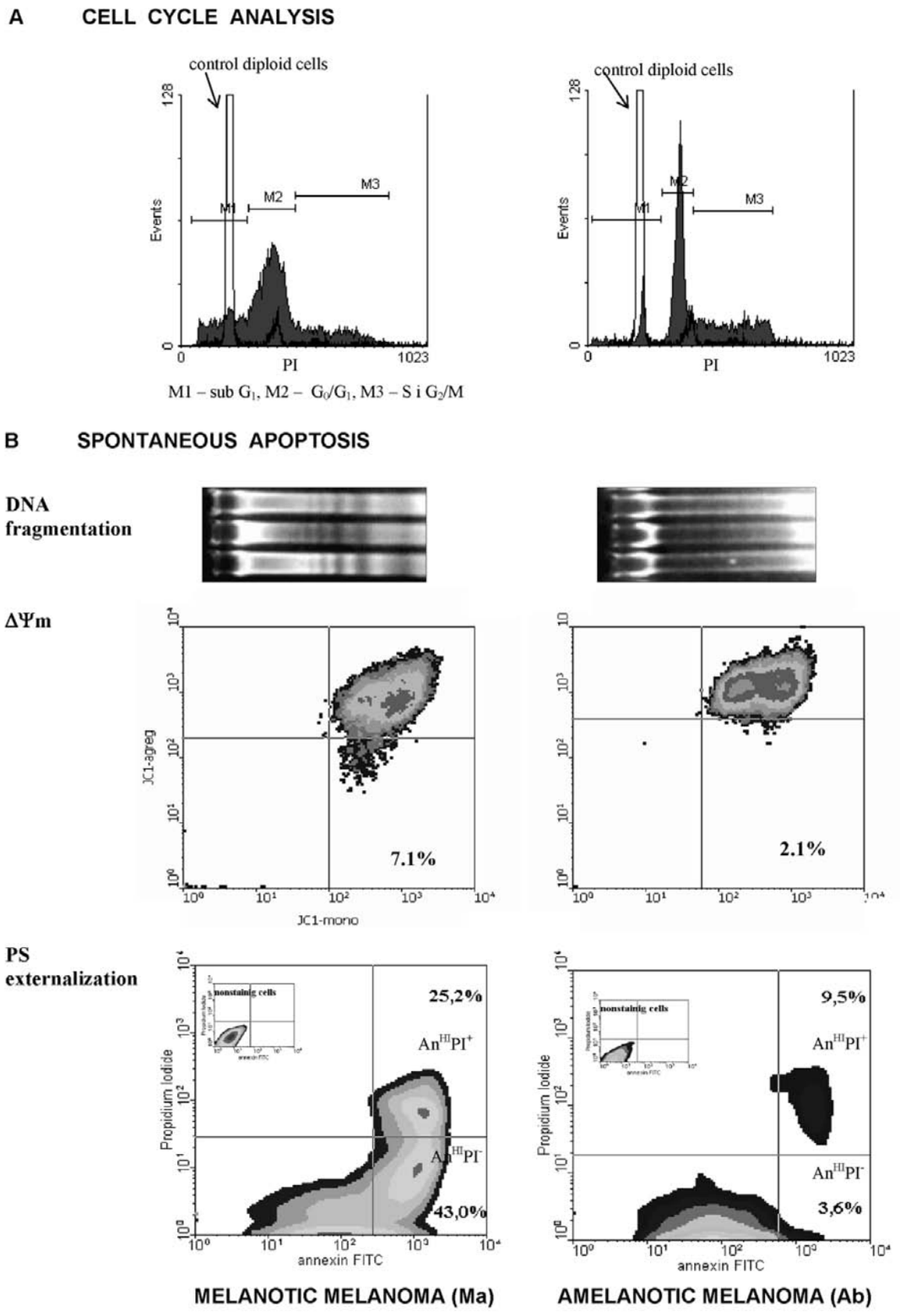

Fig. 1. Histograms of cell cycle analysis (**A**) and DNA fragmentation, changes in the transmembrane mitochondrial potential (ΔΨm) and in the plasma membrane structure (externalization of phosphatidylserine - PS) in spontaneous apoptosis (**B**) of melanotic (Ma) and amelanotic (Ab) melanoma cells. One of representative experiments.

growth rate is higher in less differentiated tumors [20, 34]. The higher proliferative activity of amelanotic melanoma cells reflects more aggressive phenotype in comparison to melanotic melanoma line, as observed also by others in human melanoma cells [3, 31].

The estimation of apoptotic cells by the TUNEL method indicates that melanotic melanoma cells have statistically higher ability to undergo programmed cell death in comparison to amelanotic ones. This was confirmed by our present results demonstrating decrease in $\Delta\Psi m$, changes in the plasma membrane structure and endonucleosomal DNA degradation (apoptotic ladder).

The phosphatidylserine externalization and apoptotic DNA fragmentation were more pronounced in cells from melanotic melanoma line than in the amelanotic one - growing faster. In amelanotic melanoma line, the percentage of cells with changed plasma membrane structure corresponds to the percentage of cells with decreased $\Delta\Psi m$. The higher percentage of cells with changes in the plasma membrane among cells of melanotic melanoma line, in comparison to cells with decreased $\Delta\Psi m$, could reflect not only apoptotic changes in the plasma membrane structure [47, 51].

In the native melanoma line, apoptotic cells came mainly from S+G2/M phases whereas in the amelanotic one similar proportions of all cell cycle phases were observed among apoptotic cells. Thus, above-mentioned results confirm our earlier suppositions, that cells of amelanotic melanoma line apart from the higher proportion of cells in S/G2/M phases have also decreased ability to undergo spontaneous apoptosis in all cell cycle phases.

We can not discuss our observation with those of other authors because we could not find any information in the literature from which cell cycle phases come melanoma cells undergoing spontaneous apoptosis.

The mechanism responsible for the decreased ability to undergo spontaneous apoptosis in transplantable amelanotic melanoma cells is difficult to explain so far. Some reports have indicated that melanoma cells show changes in the expression of antiapoptotic proteins from Bcl-2 and IAP (inhibitors of apoptosis proteins) families [19, 22, 40]. Among IAP proteins, a special role of survivin is stressed because of its involvement in cell proliferation [18].

Recently, a few studies showed that the expression of P-glycoprotein in neoplastic cells showed an antiapoptotic effect [24, 38]. This observation is in agreement with our earlier results [28], demonstrating higher P-glycoprotein expression in amelanotic melanoma cells - which have decreased ability to undergo apoptosis.

The results of other authors concerning the relationships between tumor growth and the ability of its cells to proliferate and undergo apoptosis are controversial; some authors showed that tumor growth depends mainly on cell proliferation [34], others indicated that both proliferation and apoptosis increases [17, 25], or that higher proliferation with decreased ability to undergo apoptosis support tumor progression [20, 25].

Observations of others that human melanoma cells have very low ability to undergo spontaneous apoptosis [22, 35, 41] and proliferating cells accounted for 20-40% of all cells [32, 36, 49] led them to the conclusion that the growth of human melanoma depends mainly on cell proliferative activity [35, 44].

In contrast, our present results may suggest that the expansive growth of the amelanotic melanoma line depends mainly on the decreased ability of cells in all cell cycle phases to undergo spontaneous apoptosis. According to the latest reports it seems that cell cycle regulation is an individual feature of each tumor type [12, 14, 16], similarly to the ability to undergo spontaneous apoptosis [37], which is in agreement with our present observations.

If decreased ability of cells to undergo spontaneous apoptosis also appears in human melanoma progression and in different melanoma lines not examined so far, such observation could be helpful in the therapy.

Finally, the obtained results indicate that alteration of melanotic line into amelanotic one, accompanied by changes in many biological features also concerns such fundamental cell processes as cell cycle and cell death.

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