Mitochondrial transmembrane potential in spontaneous and camptothecin-induced apoptosis of melanotic and amelanotic melanoma cells.

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Received March 16, 2006

In this work we tried to estimate the role of mitochondria in the ability of cells of two: melanotic and amelanotic transplantable melanoma lines to undergo spontaneous and camptothecin-induced apoptosis.

We measured mitochondrial transmembrane potential ($\Delta\Psi$) changes during culture without (spontaneous apoptosis) and with camptothecin (induced apoptosis) by using JC-1 staining and flow cytometry analysis. Cytochrome c release and PARP cleavage as the biological effects of $\Delta\Psi$m changes belonging to the phenomena observed during apoptosis were estimated by Western blotting.

The results of our investigations showed in both transplantable melanoma cells the features indicating apoptosis: $\Delta\Psi$ changes, cytochrome c release and PARP cleavage, but the degree of observed changes depended on the phenotype of melanoma cells examined. After camptothecin treatment the changes were more pronounced in the amelanotic melanoma cells- the more aggressive line.

Key words: mitochondrial transmembrane potential, transplantable melanomas, apoptosis, camptothecin.

Tumor treatment methods such as the use of antineoplastic drugs, radiation and immunotherapy all include the initiation of apoptosis as a part of their action

It has been postulated that tumor progression is not only the result of uncontrolled tumor cell proliferation but also a decreased ability to undergo programmed cell death. This problem seems to be very important in tumor biology, especially in chemotherapy-resistant tumors [1,2,3] of which melanoma is an example. Because of melanoma chemoresistance its therapy is a serious clinical problem [2,3]. Although there have been many publications concerning melanoma cells programmed death and efforts to explain the molecular mechanism of this phenomenon, the problem of apoptotic changes during melanoma progression is still obscure [2,3,4].

As a model of our study we used two transplantable melanoma lines of common origin but differing in the rate of growth [5], antigenicity, immunogenicity and changes in the plasma membrane structure [6,7]. The first-melanotic melanoma line (Ma) has a lower growth rate and lower immunogenicity, the second-amelanotic one (Ab), which spontaneously originated from the melanotic melanoma line is characterized by a faster growth rate, higher tumorogenicity and shorter animal survival time [5].

These two forms of melanoma cells: melanotic and amelanotic occur also in human melanoma, and the appearance of amelanotic cells accompanies the progression of pigmented melanoma [8].

In our preliminary studies we estimated spontaneous and camptothecin-induced apoptosis of Ma and Ab melanoma lines by: changes in the plasma membrane structure (Annexin V/PI test), DNA fragmentation ("apoptotic ladder"), proportion of cells in a hypodiploid fraction (cell cycle analysis by propidium iodide-PI staining) and caspase 3 activity (colorometric test with Ac-DEVD-pNA). Results of the above-mentioned studies clearly showed that melanotic melanoma cells had higher ability to undergo spontaneous apoptosis and amelanotic melanoma cells were more susceptible to camptothecin-induced apoptosis but the mechanism of these changes has not been explained and needs further investigation [9, 10].

In the present work we wanted to explain how the mechanisms of apoptosis changed with the progression of this tumor.

Mitochondria are now thought to play the central role in the regulation of apoptosis during which a number of pro-

Abbreviations: CPT; camptothecin; $\Delta\Psi$m; mitochondrial transmembrane potential, JC-1;5,5',6,6'-tetrachloro-1,1'3,3'tetraethylbenzimidazolecarbocyanine iodide; PARP; Poly (ADP-ribose) polymerase

teins are released from mitochondrial intermembrane space to cytosol [11,12,13,14,15].

The inner mitochondrial membrane is characterized by a transmembrane potential ($\Delta\Psi$) – a proton gradient established by the electron transport chain and required for the synthesis of ATP. Changes in $\Delta\Psi$ and the mitochondrial membrane Permeabilization leading to cytochrome c efflux are the first signals of apoptosis [13,16]. The efflux of cytochrome c to cytosol plays an essential role in the cascade activation of factors causing cell death e.g. caspase 3 which cleaves Poly (ADP-ribose) polymerase (PARP) to a 89-kDa fragment which is a known marker of apoptosis [11,14,15].

The relationship between $\Delta\Psi$ decrease and mitochondrial membrane permeabilization is very complicated and not yet well understood [11,15,17,18]. $\Delta\Psi$ may be used on its own as an indicator of the energization state of mitochondria. Maintenance of $\Delta\Psi$ is fundamental for the normal performance and survival of cells.

Camptothecin – an inhibitor of topoisomerase I induces apoptosis in some tumor cells [19,20] including melanoma cells [21,22].

In this work, a continuation of our study on the abilities of cells of two transplantable melanoma lines to undergo apoptosis we would like to estimate the role of mitochondria in this process by estimating the $\Delta\Psi$ changes and the biological effects of these changes: cytochrome c release and caspases activation measured by PARP cleavage.

Material and methods

Animals and transplantable melanomas. 2-3 months old male Syrian (golden) hamsters Mesocricetus auratus Waterhouse were used. The original transplantable melanotic melanoma derived from a spontaneous melanoma of the skin and was described by Bomirski as Ma line [5]. The amelanotic melanoma line originated from the Ma form by a spontaneous alteration of tumor phenotype described as Ab line [5] in which loss of pigment was accompanied by changes in many biological features [9]. Once established, these melanomas possessed a considerable degree of phenotypic stability over decades of passaging [5]. Since their discovery, each melanoma line is maintained in vivo by consecutive, subcutaneous transplantations of tumor material every 21 (Ma) or 11 (Ab) days.

Isolation of single, viable melanoma cells and their culture. Melanoma cells were isolated for each experiment from solid tumors (removed from animals at the schedule time of transplantation as above) by a non-enzymatic method described in [23]. The cell suspensions that were used in experiments consisted from 95 – 98% of viable cells as estimated by trypan blue exclusion assay.

Isolated cells at the concentration of $1x10^6$ cells/ml were incubated in the culture medium (RPMI 1640, 10% FBS (Gibco), antibiotics) without (spontaneous apoptosis) or with (induced apoptosis) 4 μg/ml camptothecin (CPT) (Sigma-Aldrich St Louis, USA) for 4 and 24 hours at 37°C .

Cytofluorimetric analysis of $\Delta\Psi m$. Changes of the $\Delta\Psi m$ were analyzed using 5,5',6,6'-tetrachloro-1,1'3,3' tetraethylbenzimidazole -carbocyanine iodide (JC-1; Molecular Probes,Inc., Eugene, OR, USA) This assay detects the collapse of $\Delta\Psi m$, an early event in apoptosis [24]. JC-1 forms monomers in mitochondria with a low negative $\Delta\Psi m$ (green fluorescence) or aggregates in mitochondria with a high negative $\Delta\Psi m$ (red fluorescence). Thus, the fluorescence of dye changes from red to green as the mitochondrial membrane becomes depolarized towards low $\Delta\Psi m$.

According to this assay we can estimate the percentage of cells with depolarized mitochondria, which are moving to the lower right corner on images from flow cytometer analysis, because these cells lose red fluorescence [24].

This method allows us also to calculate the FL2/FL1 ratio and use this value as the indicator of mitochondrial energetic state of all cells [25]. The FL2/FL1 ratio was calculated dividing the mean fluorescence intensity values of FL2 (red fluorescence- corresponding to high mitochondrial membrane potential in high-polarized, high energy mitochondria) by those of FL1 (green fluorescence- corresponding to low mitochondrial membrane potential in low-polarized, low energy mitochondria). According to the author of this method, decreasing value of FL2/FL1 reflects $\Delta\Psi m$ decrease [25].

As a positive control for reduction of $\Delta\Psi m$ the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP; 100 μM; Sigma-Aldrich) was added to cells 15 min before staining with JC-1. After 4 and 24 hours culture cells were harvested and $1x10^6$ cells/ml were incubated in PBS for 30 min at 37°C with 10μg/ml JC-1,washed twice with PBS, suspended at total volume of 500μl and analyzed on a FACScan 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. Bivariate plots of FL2 versus FL1 were used to analyze $\Delta\Psi$. Results were analyzed off-line using WinMDI 2.6 software (obtained from J. Trotter, The Scripps Institute, La Jolla, CA).

The content of mitochondria in melanoma cells. The mass of mitochondria in melanotic and amelanotic melanoma cells has been done by 10-N-nonyl acridine orange (NAO; Molecular Probes Inc., Eugene, OR, USA) that binds mitochondria and whose fluorescence was detectable in FL1 [26]. Cells were incubated at the concentration of $2x10^6$ cells/ml PBS with 2μM of NAO for 10min at 37°C, washed twice and analyzed on a FACScan flow cytometer as desribed ealier.

Cytochrome c release. Cytochrome c release was estimated by Cytochrome c Release Apoptosis Assay Kit (Oncogene Research Product). To detect cytochrome c, cytosolic fraction from $10x10^6$ cells was prepared according to the manufacturer protocol. 60μg of cytosolic proteins were separated on 15% SDS-PAGE gel, then electrophoretically transferred onto nitrocellulose membrane for 2 hrs at 60 V constant voltage and after overnight blocking probed with a primary mouse mono-

clonal anti-cytochrome c antibody (Oncogene Research Product) at a dilution 0.5 μg/ml. Horseradisch peroxidase-conjugated anti mouse IgG (Sigma-Aldrich, St Louis, USA) was used as a secondary antibody at a 1: 10000 dilution. Specific for cytochrome c bands were visualized by a chemiluminescence detection kit (Pierce SuperSignal West Pico, Pierce Biotechnology, Rockford, IL, USA).

The blots were reprobed with an antibody against β-actin (Sigma-Aldrich, St Louis, USA) at a dilution 1: 6000 to confirm equal amounts of protein in the lanes. Band intensity was semiquantified using Quantity One software (BioRad,UK) by GelDoc2000 system (BioRad,UK) and was shown on blots as a ratio to actin.

PARP cleavage as a marker of caspase activation. $2x10^6$ cells were lysated in hypotonic Tris/Nonidet 40/ Tween-100 buffer with protease inhibitor cocktail (aprotinin 100 μg/ml, leupeptin 100 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 10000 rpm, supernatants were collected and stored at -70°C until further processing. The lysates were resolved by standard polyacrylamide gel electrophoresis (SDS-PAGE) on 8 % gel and proceeded with standard Western blotting procedure as described above for cytochrome c detection. Primary mouse monoclonal anti-PARP antibody (Oncogene Research Product) was used at a dilution 0.5 μg/ml.

Statistical analysis. Statistical analysis was performed using the Mann-Whitney U test; the value of p less than 0.05 was regarded as significant.

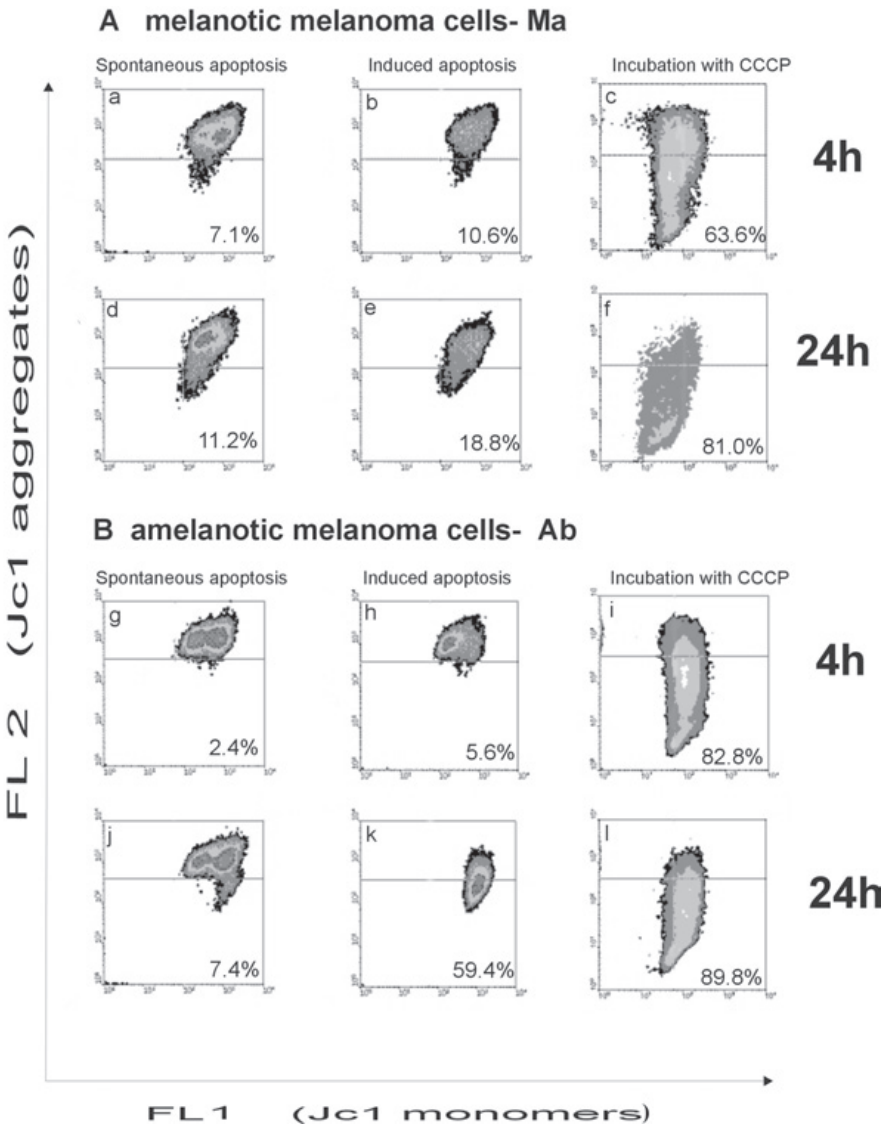

Fig. 1. Changes in the percentage of cells with low ΔΨm –measured as decrease of FL2 fluorescence (lower part of each figure) during spontaneous (without camptothecin) and camptothecin-induced apoptosis after 4h and 24h incubation of melanotic –Ma (A) and amelanotic-Ab (B) melanoma cells. As a positive control for reduction of ΔΨm protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP; 100μM) was used.
Representative results.

Results

ΔΨm changes in spontaneous and camptothecin-induced apoptosis.

After 4h incubation about 6% of the native-Ma melanoma line (Table 1; Fig.1a) and only 2.5% of the amelanotic-Ab cells (Table1; Fig.1g) had a decreased ΔΨm (Table 1, $p<0.05$). When the cells were cultured with camptothecin, the percentage of cells with decreased ΔΨm increased to about 10% for the Ma line cells (Fig.1b, Table 1, $p<0.05$) and to 5% for the Ab melanoma line cells (Fig. 1h, Table 1, $p<0.05$).

The native-Ma melanoma cells seemed to have mitochondria at a lower energetic state because the FL2/FL1 fluorescence ratio was 1.2, while in the amelanotic melanoma cells this value was almost 4 for all cells (Fig. 2B; $p<0.05$). Except cells with only green fluorescence we observed two populations of cells, among amelanotic melanoma cells. The FL2/FL1 for these populations were 6.6±1.8 and 2.2±0.4. Both

Table 1. The percentage of cells with low mitochondrial transmembrane potential (ΔΨm) and the FL2/FL1 fluorescensce of all cells of two transplantable melanoma line : melanotic (Ma) and amelanotic (Ab) during spontaneous (incubation without camptothecin) and camptothecin-induced apoptosis.

Transplantable melanoma line		Spontaneous apoptosis		Camptothecin-induced apoptosis	
		4h	24h	4h	24h
Melanotic **Ma**	Percentage of cells with low ÷Φm	5.7±2.2	12.0±3.5	10.5±3.4**	20.1±2.9**
	FL2/FL1 fluorescence ratio of all cells	1.21±0.1	1.63±0.4	1.55±0.3	1.24±0.3***
amelanotic **Ab**	Percentage of cells with low ÷Φm	2.5±1.0*	7.5±2.3*	5.0±1.2*,**	58.8±11.0*,**
	FL2/FL1 fluorescence ratio of all cells	3.83±1.3	1.77±0.2	3.92±1.1	0.32±0.1***

Results are mean ± SD of 4 for Ma and 7 for Ab line experiments.
Statistical analysis by Mann-Whitney U-test:
* statistically significant 0.001<p<0.05 differences in the percentage of cells with low ΔΨm between Ma and Ab line
** statistically significant 0.001<p<0.05 increase of cells with low ΔΨm after induction of apoptosis by camptothecin
*** statistically significant (0.01<p<0.05) changes of the FL2/FL1 ratio in comparison to spontaneous apoptosis

values were higher than FL2/FL1 value for all melanotic melanoma cells so we treat all amelanotic cells as one group. The mean fluorescence of JC-1 aggregates (FL2) indicated that at basal conditions the amelanotic melanoma cells had a higher ΔΨm than the melanotic melanoma cells (Fig. 2A).

After 24h incubation without CPT there were about 12% of cells with decreased ΔΨm among the melanotic melanoma cells (Table 1; Fig. 1d) and after the induction of apoptosis by CPT their proportion increased significantly to 20% (Table 1; Fig.1e). This was accompanied by a decrease in the of FL2/FL/1 fluorescence ratio, but its value was still over 1 (Fig. 2B).

After the same time there were only 7.5% of cells with a low ΔΨm among the amelanotic melanoma cells (Table 1; Fig. 1j), but the FL2/FL1 ratio decreased significantly (p<0.01) from 3.8 to about 1.8 (Fig. 2B). After incubation with CPT the proportion of cells with lower ΔΨm increased to almost 60% (Table 1; Fig. 1k; p<0.001), and the FL2/FL1 ratio decreased from 1.8 to 0.3 (Table 1; Fig. 2B; p<0.01). Observed in a probe without camptothecin, two populations of amelanotic melanoma cells had a high red fluorescence (Fig 1g) , and after 24h incubation with this inducer of apoptosis they changed into one population with a lower FL2 and higher FL1- the whole population of amelanotic cells moved to the right (Fig. 1k; Fig. 2A).

The exposure of cells to the uncoupler agent CCCP, which has been used as a positive control, dissipates ΔΨm in both the melanoma lines examined (Fig.1c and f for Ma; i and l for Ab).

Mass of mitochondria in hamster melanoma cells.

Estimated by NAO staining the mass of mitochondria was lower in melanotic melanoma cells (mean fluorescence 1890±307) than in amelanotic melanoma cells (mean fluorescence 3635±189). There was only one population of cells among amelanotic melanoma line but two populations of cells among melanotic melanoma cells: one with similar fluorescence intensity as in amelanotic line and the second one with lower fluorescence intensity- about 700 relative fluorescence units (Fig. 3). Over 40% of melanotic melanoma cells belonged to the population with the lower mass of mitochondria.

Cytochrome c release and PARP cleavage

Immunoblotting detection of cytochrome c in the cytosolic fraction of the cells examined showed the presence of this molecule in both melanotic and amelanotic melanoma lines during spontaneous and camptothecin-induced apoptosis. However after a prolonged time of incubation with camptothecin the increase in the amount of cytochrome c in the cytosolic fraction was more pronounced in the amelanotic melanoma cells (Fig 4A).

During camptothecin-induced apoptosis the percentage of melanotic melanoma cells with a low ΔΨm increased slowly during prolonged incubation, amounting to about 5% after 4h and 8% after 24h, in comparison with spontaneous apoptosis (Table 1). Cytochrome c release seemed not to have changed much (Fig. 4A)

Meanwhile in the amelanotic melanoma CPT induced during 4h a decrease of ΔΨm in 3% and during 24h in 51% more cells than during spontaneous apoptosis (Table 1), with an increased amount of released cytochrome c (Fig. 4A)

PARP cleavage as an indicator of caspase 3 activation was also more pronounced in the amelanotic melanoma cells (Fig. 4B). After 4h incubation with CPT we observed an increase in 89-kDa fragment content in the amelanotic melanoma cells and after additonal 20h it was the only fragment of PARP left, the 115 kDa fragment was not present after 24 hrs. In the melanotic melanoma cells changes in the content of 89 kDa fragment after induction of apoptosis by CPT were less visible, and after 24h there was still some 115 kDa fragment present (Fig. 4B).

Discussion

In this work we demonstrated that the higher percentage of cells with a lower ΔΨm among melanotic melanoma cells than among amelanotic cells is in agreement with our previous results. We have shown before that melanotic melanoma cells have a higher ability to undergo spontaneous apoptosis than amelanotic line [10].

The lower FL2/FL1 ratio of melanotic melanoma cells value (1.2) than that of amelanotic melanoma cells (3.8) indicated that native melanoma cells have lower basal ΔΨ. Some au-

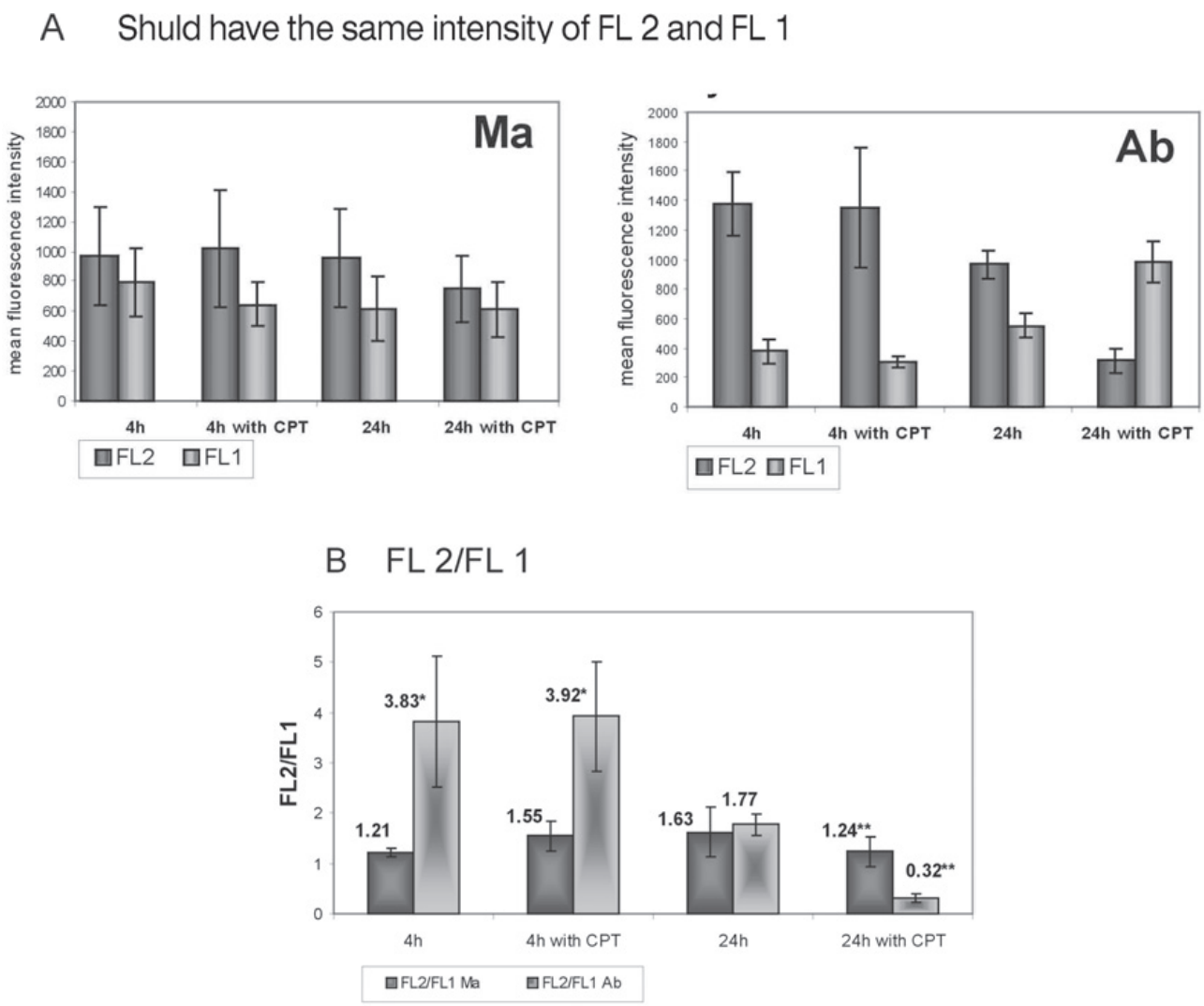

Figure 2. Changes of red (FL2) and green (FL1) mean fluorescence intensity (A) and the ratio of FL2/FL1 (B) during spontaneous and camptothecin (CPT) induced apoptosis among melanotic (Ma) and amelanotic (Ab) melanoma cells.
The green fluorescence (FL1) refers to the JC-1 monomers and the red fluorescence (FL2) correspondence to the formation of JC-1 aggregates. The ratio of red/green fluorescence of JC-1 is depicted as FL2/FL1.
Results are mean ± SD of 4 for Ma and 7 for Ab line experiments.Statistical analysis by Mann-Whitney U-test:
*** statistically significant higher FL2/FL1 ratio of amelanotic melanoma cells to native-Ma line; p<0.01**
**** statistically significant decrease of the FL2/FL1 ratio in comparison with spontaneous apoptosis; p<0.01**

thors pointed out that the value of basal $\Delta\Psi$ in different cells could vary and that it is the individual property of cells [27,28] which depends on the production of cellular ATP and reactive oxygen species (ROS) [29,30]. The results of earlier work by Scislowski showing that amelanotic melanoma cells have a higher rate of aerobic and anaerobic glycolysis could partly explain higher basal $\Delta\Psi$, in comparison to the native-Ma line [31].

Follstad observed that cells with a low $\Delta\Psi$ are more sensitive to the induction of apoptosis [28]. But in the available literature there is no information about the ability of cells to undergo spontaneous apoptosis in relation to their basal value of $\Delta\Psi$. The role of the ATP content in cells during induction of apoptosis is stressed by some authors [14,15].

Among melanotic melanoma cells there were two populations of cells according to the mass of mitochondria but only one population of cells, when we analyzed $\Delta\Psi$. In amelanotic melanoma cells there was only one population of cells with high mitochondrial mass but two populations of cells during $\Delta\Psi$ measurement. Thus it seemed that observed basal differ-

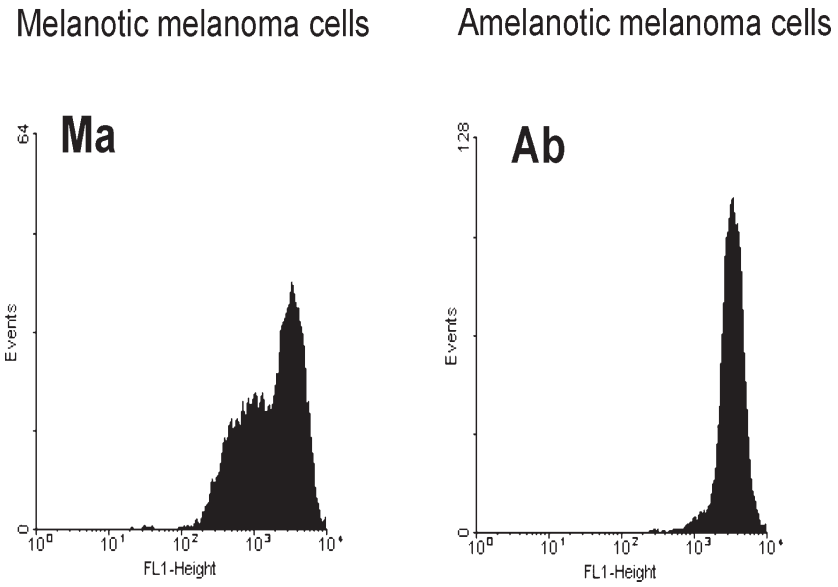

Figure 3. Mitochondrial mass in melanotic (Ma) and amelanotic (Ab) melanoma cells estimated by NAO staining.
Representative results of four experiments.

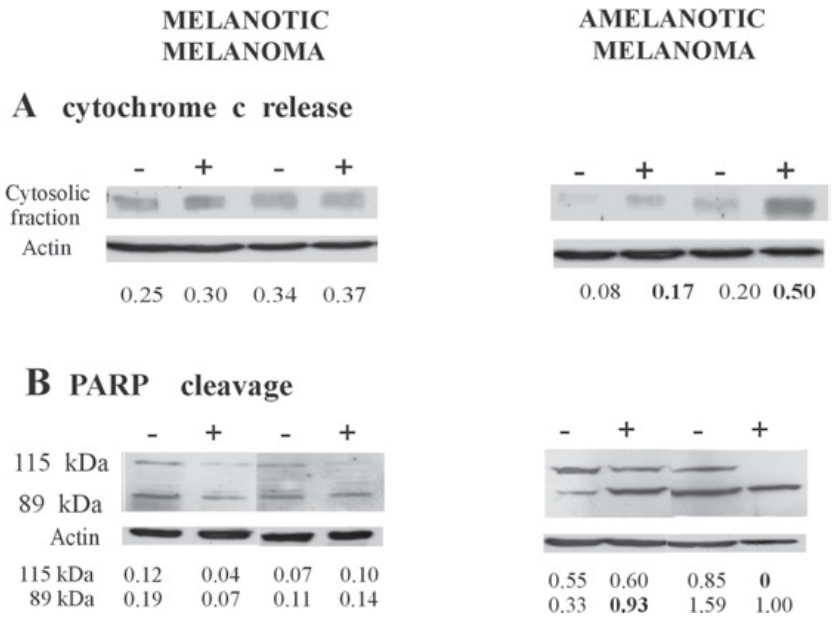

Figure 4. Cytochrome c content in the cytosolic fraction of melanotic (Ma) and amelanotic (Ab) melanoma cells (A) and PARP cleavage (B) after 4h and 24 h incubation without (-) and with (+) CPT.

A. Cytosolic fraction of cells has been prepared as described in Material and Methods and 60 μg of proteins per lane have been used to determine cytochrome c by Western blotting.

B. Lysates from $2x10^6$ cells have been prepared as described in Material and Methods and 20 μl of lysates have been used to determine 115 kDa fragment (non cleaved) and 89 kDa fragment which is cleaved from PARP by caspase during apoptosis.

Bands intensity of digitalized images were semiquantified using Quantity One software (BioRad) and shown on blots as a ratio to actin.

Representative results of three experiments.

ences at FL2/FL1 value did not depend on the mitochondrial mass in cells of examined melanoma cells, which is in agreement with other authors observation [32].

Observed diversity of mitochondrial mass among melanotic melanoma cells seemed to correlate with more heterogenous DNA content, in comparison to amelanotic cells observed in our earlier work [33].

The FL2/FL1 ratio almost unchanged, with little changes in the cytochrome c release and PARP cleavage showed that the native-Ma melanoma cells followed the mitochondrial way of apoptosis but to a lesser extent than the amelanotic melanoma cells.

Induction of apoptosis by CPT showed that amelanotic melanoma cells- more tumorogenic cells, growing faster, are extremely sensitive to the action of this substance. After 24hrs almost 60% of cells had a low $\Delta\Psi$ and the FL2/FL1 fluorescence ratio fell below 1. Camptothecin- induced apoptosis of amelanotic melanoma cells was accompanied by the decrease of $\Delta\Psi$, cytochrome c release and PARP cleavage, which indicated mitochondrial involvement in the apoptotic process of these cells.

Dividing cells are very sensitive to CPT action especially in the S-phase of cell cycle [20,21,22]. It could partly explain the higher susceptibility to CPT of amelanotic melanoma line- growing faster; having more proliferating cells [34].

It is worth to notice that the native-melanotic melanoma cells were less sensitive to CPT.

There are some known mechanisms of CPT resistance such as: active efflux by P glycoprotein, reduced level of topoisomerase I expression, alterations in the structure of topoisomerase I due to different mutations [35].

Our melanotic melanoma cells showed P-glycoprotein expression but with a low activity and only a small fraction of amelanotic melanoma had P-glycoprotein with higher activity

[36,37]. Thus it seemed that glycoprotein P is not the only factor responsible for differences at sensitivity to CPT action in our model.

The efflux of cytochrome c from the intermembrane space of the mitochondria to cytosol plays an essential role in the cascade activation of factors causing cell death e.g. caspases [11,12,13,14,15]. Activated caspase-3 reversible acts on the permeabilized mitochondria to damage mitochondrial function[38].

Our earlier work showed that during spontaneous and camptothecin induced apoptosis caspase-3 activity was higher in the amelanotic melanoma cells [10], in which we now observed increased content of cells with a low $\Delta\Psi$, significant decrease in the ratio FL2/FL1, cytochrome c release and PARP cleavage.

These observations indicated that in the apoptotic death of amelanotic melanoma cells mitochondria play a more important role than in the native-melanotic melanoma cells.

To conclude, the spontaneous alteration of melanotic melanoma line into amelanotic one was also connected with more pronounced role of the intrinsic pathway in amelanotic melanoma cells apoptosis.

So apoptosis and apoptosis disturbances in melanoma cells should be analyzed in a more detailed manner, to understand the biology of this tumor, having some future therapeutic success in mind.

Reference

[1] REED J. Mechanism of apoptosis avoidance in cancer. Cur Opin Oncol 1999; 11: 68-75

[2] HELMBACH H, SINHA P, SCHADENDORF D. Human melanoma: drug resistance. Recent Results Cancer Res 2003; 16: 93-110

[3] DEMIERRE M, SONDAK V. Cutaneous melanoma: pathogenesis and rationale chemoprevention. Critical Rev Oncol Hematol 2005; 53: 225-39

[4] HUSSEIN R, HAEMEL A, WOOD G. Apoptosis and melanoma: molecular mechanism. J Pathol 2003; 199: 275-88

[5] BOMIRSKI A, SŁOMIŃSKI A, BIGDA J. The natural history of a family of transplantable melanomas in hamsters. Cancer Metastasis Rev 1988; 7: 95-118

[6] KOZŁOWSKA K, ŻURAWSKA – CZUPA B, MIERZEWSKI P, KOSTULAK A. Use of macrophages migration inhibition test to evaluate antigenic differences in golden hamster transplantable melanomas. Int J Cancer 1980; 26: 211-5.

[7] KOZŁOWSKA K,. ŻURAWSKA–CZUPA B. Heterogeneity of the surface material in isolated cells of transplantable hamster melanomas. Cancer Res 1983; 43: 1168-71

[8] GUPTA R, LALLU S. Cytodiagnosis of amelanotic metastatic malignant melanoma: An imunocytochemical study. Diagn Cytopathol 1997; 16: 238-41

[9] KOZŁOWSKA K, ZARZECZNA M, CICHOREK M. Sensitivity of transplantable melanoma cells to cytokines with regard to their spontaneous apoptosis. Pathobiol 2001; 69: 249-57

[10] KOZŁOWSKA K, CICHOREK M, ZARZECZNA M, BROŻEK J, WITKOWSKI JM. Heterogeneous susceptibility to spontaneous and induced apoptosis characterizes two rrelated transplantable melanomas with different biological properties. Pigment Cell Res 2002; 15: 233-8

[11] RAVAGNAN L, ROUMIER T, KROEMER G. Mitochondria, the killer organelles and their weapons. J Cell Physiol 2002; 192: 131-7

[12] ORRENIUS S. Mitochondrial regulation of apoptotic cell death. Toxicol Lett 2004; 149: 19-23

[13] MARCHETTI P, CASTEDO M, SUSIN SA, ZAMZANI N, HIRSCH et al. Mitochondrial permeability transition is a central coordinating events of apoptosis. J Exp Med 1996; 184: 1155-60

[14] GREEN D, REED J. Mitochondria and apoptosis. Science 1998; 281: 1309-12

[15] DESAGHER S, MARTINOU J. Mitochondria as the central control point of apoptosis. Trends in Cell Biol 2000; 10: 369-77

[16] ZAMZANI N , MARCHETTI M, CASTEDO D, ZANIN J ,VAYSSIERE P et al. Reduction in mitochondrial potential consitutes an early irreversible step of programmed lymphocyte death in vivo. J Exp Med 1995; 181: 1661-72

[17] HENRY-MOVAT J, DIVE C, MARTINOU J, JAMES D. Role of mitochondrial membrane permeabilization in apoptosis and cancer. Oncogene 2004; 23: 2850-60

[18] GOGVADZE V, ROBERTSON J, ENOKSSON M, ZHIVOTOVSKY B, ORRENIUS S. Mitochondrial cytochrome c release may occur by volume-dependent mechanism not involving permeability transition. Biochem J 2004; 378: 213-17

[19] LI T, LIU L. Tumor cell death induced by topoisomerase-targeting drugs. Annu Rev Pharmacol Toxicol 2001; 41: 53-77

[20] BAILLY C. Homocamptothecins: potent topoisomerase I inhibitors and promising anticancer drugs. Crit Rev Oncol Hematol 2003; 45: 91-108

[21] PANTAZIS P, EARLY J, MENDOZA J, DEJESUS A, GIVANELLA B. Cytotoxic efficacy of 9-nitrocamptothecin in the treatment of human malignant melanoma cells in vitro. Cancer Res 1994; 54: 771-76

[22] LI G, BUSH J, Ho V. p53-dependent apoptosis in melanoma cells after treatment with camptothecin. J Invest Dermatol 2000; 114: 514-19

[23] BOMIRSKI A, KOZŁOWSKA K, ŻURAWSKA – CZUPA B. An attempt to prepare nonenzymatically a single cell suspension from solid transplantable melanomas. Proceedings of the IX th International Conference of Pigment Cell. Houston. Texas, 1975; 5-6

[24] SALVIOLI S, DOBRUCKI J, MORETTI L, TROIANO L, FERNANDEZ M et al. Mitochondrial heterogeneity during staurosporine-induced apoptosis in HL60 cells analysis at single cell and single organelle level. Cytometry 2000; 40: 189-97

[25] COSARRIZA A, CECCARELLI D, MASINI A. Functional heterogeneity of an isolated mitochondrial population revealed by cytofluorimetric analysis at a single organelle level. Exp Cell Res 1996; 22: 84-94

[26] RATINAUD M, LEPRAT P, JULIEN R. In situ flow cytometric analysis of nonyl acridine orange- stained mitochondria from splenocytes. Cytometry 1988; 9: 206-212

[27] DIAZ G, SETZU D, ZUCCA A, ISOLA R, DIANA A et al. Subcellular heterogeneity of mitochodrial membrane potential: relationship with organelle distribution and intercellular contacts in normal, hypoxic and apoptotic cells. J Cell Sci 1999; 112: 1077-84

[28] FOLLSTAD B, WANG D, STEPHANOPOULOS G. Mitochondrial membrane potential differentiates cells resistant to apoptosis in hybridoma cultures. Eur J Biochem 2000; 267: 6534-40

[29] KALBACOVA M, VRBACKY M, DRAHPTA Z, MELKOVA Z. Comparison of the effect of mitochondrial inhibitors on mitochondrial membrane potential in two different cell line using flow cytometry and spectrofluorometry. Cytometry 2003; 52A: 110-16

[30] KADENBACH B, ARNOLD S, LEE I, HUTTEMANN M. The possible role of cytochrome c oxidase in stress-induced apoptosis and degenerative diseases. Biochem Biophys Acta 2004; 1655: 400-8

[31] SCISŁOWSKI P, SŁOMIŃSKI A, BOMIRSKI A. Biochemical characterization of three hamster melanoma variants. II. Glycolysis and oxygen consumption. Int J Biochem 1984; 16: 327-31

[32] KLUZA J, LANSIAUX A, WATTEZ N, HILDEBRAND M, LEONCE S et al. Induction of apoptosis in HL-60 and B16 melanoma cells by the acronycine derivative S23906-1. Biochem Pharmacol 2002; 63:1443-1452

[33] WACHULSKA M, KOZŁOWSKA K, CICHOREK M. The DNA ploidy and proliferation activity of transplantable melanoma cells in regard to their function. Neoplasma 2005; 52:280-285

[34] CICHOREK M, KOZŁOWSKA K, WACHULSKA M, ZIELIŃSKA K. Spontaneous apoptosis of melanotic and amelanotic melanoma cells in different cell cycle: relation to tumor growth. Folia Histochem Cytobiol 2006; 44:31-36.

[35] XU Y, VILLALONA-CALERO M. Irinotecan: mechanism of tumor resistance and novel strategies for modulating its activity. Annal Oncol 2002; 13: 1841-51

[36] KOZŁOWSKA K, WITKOWSKI JM, ZARZECZNA M, CICHOREK M. Diversity of plasma membrane properties of transplantable hamster melanomas with regard to the expression of P–glycoprotein. Folia Histochem Cytobiol 1999; 37: 173-7

[37] WITKOWSKI JM, KOZŁOWSKA K, ZARZECZNA M. Expression and activity of P-glycoprotein in transplantable hamster melanomas. Arch Dermatol Res 2000; 292: 354-61

[38] RICCI J, GOTTLIEB R, GREEN D. Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis. J Cell Biol 2003; 160: 65-75