Disseminated malignant melanoma is a chemoresistant cancer. Percent of partial remissions amounts to 20 – 25 and a median survival time during remission is from 6 to 8 months (1-4). To gain a success with chemotherapy it is needed to choose such chemotherapeutic drugs or their combination, which have a higher ability to destroy tumor cells.

Due to difficulties in carrying out studies on biology of malignant melanoma in humans, there are studies carried out on malignant melanoma in animals (mice, rats, hamster and fish) (5-8).

Melanin is described as a free radical itself and on the other hand as an antioxidant with the capability to bind radical-forming agents. Melanin is a stable free radical and was the first free radical found in biological systems. Free oxygen radicals are generated during cell metabolism or as a result of external factors, e.g. drugs (9). Free radicals are eliminated from cells with the antioxidant enzymatic systems which include: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Chemical compounds, like: glutathione, vitamins A, C, E and melanin exhibit antioxidating properties (10). Melanin quenches excited states and binds radical-forming agents. All likely contribute to its putative role in antioxidant defense. On the other hand, the ability of melanin to binding toxic radical-gener-
ating agents may sometimes be detrimental (e.g., cytostatic drugs resistance).

Radically mediated drug toxicity can occur by production of activated drug metabolites (mainly by the microsomal oxidation system) or by production of active species of oxygen. Examples include cis-platinum and the aminoglycoside antibiotics, such as adriamycin.

Melanin synthesis is still an obscure process. Cytostatic drugs used in the treatment of malignant melanoma may modulate activity of tyrosinase and increase semiquinones production during melanogenesis (11, 12). Semiquinones may damage cells with the following induction of apoptosis.

Recently oncologists have been increasingly interested in apoptosis, which is morphologically, biochemically and topographically different from necrosis. The process of apoptosis is associated with resistance or sensitivity of malignant cells to cytostatic and cytotoxic drugs.

Apoptotic cells lose phospholipid asymmetry of plasma membrane. Phosphatidylserine (PS) is translocated to the outer layer of the membrane. This PS exposure is a hallmark of early apoptosis. Early apoptotic cells can be detected using Annexin V which has affinity to phosphatidylserine (13-16).

The influence of drugs used in treatment of disseminated malignant melanoma (dacarbazine, cis-platin, vinca-alkaloids) as well as actinomycin D and cytosine arabinoside on melanin contents in B16 and Cloudman S91 melanoma cells and cell cycle in vitro was assessed. An attempt was made to determine dependence between melanogenesis and apoptosis processes in melanoma cells after exposure to the tested drugs in vitro.

EXPERIMENTAL

Culture and passage of melanoma cells in vitro

Parental of B16 mouse melanoma cell line was obtained from the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences in Wroclaw in 1985. The cell line was maintained through intraperitoneal transplantation and passage on C57Bl/6J mice. B16 mouse melanoma cells were obtained from nodules in exudative form of mouse melanoma C57Bl/6J.

Cloudman mouse melanoma (Cloudman S91) was obtained from the Laboratory of Cytobiochemistry of the Institute of Physiological Chemistry of Philipp University in Marburg (Germany). Since 1996 they have been continuously maintained in the Tissue Culture Laboratory of the Department of Medical Biology (Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz).

Both mouse melanoma – B16 and Cloudman S91 cells – were cultured in Dulbecco medium (modification of Eagle’s medium GIBCO, UK) supplemented with 4.5 g/L of glucose and 10% fetal bovine serum (Fetal Bovine Serum, Sigma, USA), penicillin at 100 U/mL (Polfa Tarchomin S.A., Poland), streptomycin at 100 µg/mL (Polfa Tarchomin S.A., Poland), gentamicin at 20 µg/mL (Krka, Slovenia) and diflucan at 0.125 µg/mL (Pfizer, Germany).

Both established mouse melanoma cell lines (B16 and Cloudman S91) feature monolayer growth on the culture flask bottom surface. The B16 melanoma cells grow slower and make clusters. Adhesion of the Cloudman S91 cells was diminished in comparison to the B16 cells.

Considering various growth rates of B16 and Cloudman S91 mouse melanoma cells the medium was renewed every 3 days for B16 cells and every 2 days for Cloudman S91 cells, according to the recommendations of American Type Cell Culture (B16 – ATCC: CRL – 6322, Cl S91 – ATCC: CCL – 53.1; Rockville, USA). A subcultivation ratio was 1:10.

Conditions of cell culture maintaining, detaching of melanoma cells from the culture flasks and preparation of cell suspension for assays was previously described (15, 16). 10^5 cells were suspended in culture flasks (25 cm² of cell growth surface, Grainer, USA) filled with 5 mL of culture medium. Cultures were routinely maintained at 37°C with 5% CO₂, 98% humidity (IG150 incubator, Jouan, France). Growth and morphology of cells were controlled under a phase-contrast microscope with reverse optics (Nikon TMS, Japan) before each passage.

The cells growing as monolayer were detached from the bottom of the culture vessel with 0.04% EDTA (ethylenediaminetetraacetic acid, Sigma, USA) in PBS without Ca²⁺ and Mg²⁺ ions (Manufacturers of Serum and Vaccines, Lublin, Poland). After adding EDTA solution, cells were observed until cell layer was dispersed (usually within 3-5 min). The detached cells were centrifuged for 5 min at 500 × g and washed twice using 37°C PBS. Then the cells were suspended in 1 mL culture medium and counted in Neubauer chamber using the trypan blue test (Bio Whitaker, USA). 10^5 cells/1 mL medium suspensions with the viability of 90 ± 2% were used for further tests.

Preparation of chemotherapeutic agent solutions

The drugs used in human disseminated melanoma: actinomycin D (Act-D, Cosmegen,
Merk & Co., Inc, USA), cisplatin (Cis-Pt, Blastolem, Lemery, Poland), dacarbazine (DTIC, Lachema, Slovenia), vincristine (VCR, Gedeon Richter, Hungary), and drugs administered in leukemias and lymphomas treatment: adriblastin (ADR, Doxorubicin, Farmitalia, Italy) and cytosine arabinoside (Ara-C, Cytostar, Upjohn, USA) were tested in vitro. All drugs were dissolved in the sterile solution of PBS.

Influence of chemotherapeutic agents on B16 and Cloudman S91 melanoma cells viability

Viability of B16 and Cloudman S91 cells in vitro (determination of drug cytotoxicity) after addition of cytostatics to the culture medium was investigated by counting the cells fixed with analytically pure 70% alcohol under a phase-contrast microscope. All experiments were performed three times.

Melanoma cells in amount of $3 \times 10^5$ cell/well in 2.8 mL of medium were cultured in 12 well culture plates with flat bottom (Costar). Cells were incubated for 24 h without any agent and with 0.2 mL of an appropriate chemotherapeutic solution for 24 h. After this period the cells were rinsed three times in cold PBS and three times in cold analytically pure ethanol (POCH, Gliwice). Plates were then dried and cells were counted under a phase-contrast microscope. Cells were counted following the formula:

$$X = \frac{S \times n}{s}$$

where:
- $X$ – number of melanoma cells in one well of a culture plate,
- $S$ – surface of cell growth in a culture plate well,
- $s$ – surface of mesh in the eyepiece of microscope,
- $n$ – number of cells in the field of one mesh (eyepiece of the microscope).

Drug doses for the apoptotic assays and cell cycle estimations were selected on the base of previously received EC_{50} concentrations (Table 1). EC_{50} concentration was evaluated for each tested drug.

The cytotoxic and cytostatic effect of tested drugs was presented as a number of alive B16 and Cloudman S91 cells in comparison to the control. Viability of cells was counted by applying following equation:

$$P = \frac{E \times 100\%}{K}$$

where:
- $P$ – viability of cells after cytostatic drug adding, presented in %,
- $E$ – mean cells number in examined well of a culture plate,
- $K$ – mean cells number in examined well of a culture plate.

Apoptosis studies

$10^5$ of B16 and Cloudman S91 cells were cultured in a polystyrene 25 cm² flasks (Grainer) for 24 h, before chemotherapeutic agents were added. Cells were incubated with cytostatics for 24 h, and then detached from the growth surface using 0.04% EDTA solution. Cell suspensions in EDTA were centrifuged for 4 min at $500 \times g$. Cells were suspended in $490 \mu$L of cold binding buffer (Immunotech, USA). Tubes were kept on ice.

Apoptotic cells were stained with annexin V-FITC (Immunotech, USA) and propidium iodide (Immunotech, USA).

Stock solution of annexin V-FITC was tenfold diluted with binding buffer. 250 μg of propidium iodide were dissolved in 1 mL of diluted binding buffer. All procedures were carried out at 4°C.

To test tubes containing $490 \mu$L suspensions of B16 and Cloudman S91 cells, 5 μL of annexin V-FITC solution and 5 μL of propidium iodide were added. Then the tubes were mixed for 5 s using an automatic stirrer (Coulter vortex).

After 10 min of incubation in darkness, cytometric measurements were taken with the use of EPICS XL (Coulter) equipped with System II™ Software, Version 1.0 (Phoenix Flow System Inc., San Diego, USA).

DNA content and evaluation of cell cycle

$10^5$ of B16 and Cloudman S91 cells were incubated with tested drugs at concentrations presented in Table 1.

The received cell suspensions in EDTA/complete medium were centrifuged 4 min at $500 \times g$. Cell pellets were rinsed with PBS and centrifuged again under similar conditions. Supernatants were rejected and cell sediments were suspended in $100 \mu$L of PBS. For each silicon test tube containing $50 \mu$L of cell suspensions, $50 \mu$L of DNA-Prep LPR (Coulter, Immunotech, USA) reagent permeabilising cell membranes was added. Test samples contents were centrifuged with an automatic stirrer. After 8 s of incubation, $1000 \mu$L of propidium iodide (DNA-Prep Stain; Coulter, Immunotech, USA) were added. Tubes were mixed again and left for 20 min in darkness at a room temperature.

Analysis was carried out using flow cytometer EPICS XL, equipped with Multicycle AV Software (Phoenix Flow System Inc., San Diego, USA).
Contents of melanin

Contents of melanin in mouse melanoma cell suspensions exposed to cytostatic drugs were determined by colorimetric method using 1M NaOH, as previously described by Drewa and Schachtschabel (17). Cell suspensions of B16 and Cloudman S91 served as control groups. Melanin content is presented as a %/mg of protein.

Statistical analysis

Melanoma cell viability was estimated using the regression curve. Regression curve equation was used to calculate EC50 values. Signification of correlation coefficient was checked by Student’s t-test.

RESULTS AND DISCUSSION

Flow cytometry was able to distinguish four populations of cells: alive, apoptotic (during the process of apoptosis), secondary necrotic (late apoptosis or dead cells as a result of apoptosis) and necrotic. In both control samples percentage of alive cells was high, approx. 98%.

Adriblastin, actinomycin D, cisplatin and vincristine induced apoptosis in B16 and Cloudman

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Figure 1. Melanin contents [%/mg of protein] and percentage of apoptotic CIS91 melanoma cells after exposure to cytostatic drugs at EC50 concentration.

Figure 2. Melanin contents [%/mg of protein] and percentage of apoptotic B16 melanoma cells after exposure to cytostatic drugs at EC50 concentration.
S91 cells. The highest percentages of apoptotic cells after exposure to drugs at EC<sub>50</sub> concentrations were observed in B16 and Cloudman S91 melanoma cells after adriblastin, and in Cloudman S91 cells after vincristine and actinomycin D (Table 1).

Cloudman S91 cells were characterized by higher sensitivity to apoptosis and more intensified melanogenesis than B16. Increasing melanin contents in melanoma cells was observed after exposure to cytostatic drugs (Figures 1 and 2). The highest melanin increase was noted in B16 cells after exposure to actinomycin D (111%/mg of protein). A similar melanin increase was observed in Cloudman S91 cells after exposure to actinomycin D, cisplatin, cytosine arabinoside, vincristine and adriblastin (Figure 3). A decrease of melanin content in B16 cells was caused by cisplatin and by dacarbazine in both tested lines.

The analysis of cell cycle of peripheral blood lymphocytes (C57Bl/6J mouse) was carried out. This measurement is based on propidium iodide ability to combining double-stranded DNA. Stained DNA emits the fluorescence which is directly proportional to DNA content in tested sample. Cell ploidy and cell cycle were calculated from DNA content.

B16 and Cloudman S91 cells were previously established as hypertetraploids when comparing them with C57Bl/6J lymphocytes (Figure 4). Phases of cell cycle (G0/G1, S, G2/M) of B16 and Cloudman S91 cells after exposition to drugs are shown in Table 2.

Adriblastin, actinomycin D, dacarbazine arrested B16 and Cloudman S91 cells in G2/M and S cell cycle phase (Figures 5, 6, and 9). Cytosine arabinoside caused absence of cells of both lines in G2/M phase with following G0/G1 and S accumulation (Figure 7). Cisplatin stopped B16 cells in G2/M phase, and Cloudman S91 cells in S phase (Figure 8). The addition of vincristine to B16 culture stopped cells in G2/M phase. Hypertetraploidal and aneuploidal cell cycles with the following G2/M and S accumulation of Cloudman S91 cells after incubation with vincristine were observed (Figure 10).

The cytotoxic effect of cytosine arabinoside on both lines was not observed. A percentage of apoptotic and necrotic B16 and Cloudman S91 cells was low after incubation with cytosine arabinoside at EC<sub>50</sub>. The cells of both tested lines were sensitive to dacarbazine in concentration of 500 µg/mL, and died as a result of intensive necrosis. After exposure of Cloudman S91 cells to vincristine, significant changes were observed in the course of apoptosis in comparison to B16 cells (Table 1).

Adriblastin and actinomycin D caused increased melanogenesis in both tested lines with following apoptosis at EC<sub>50</sub> concentrations. Dacarbazine at EC<sub>50</sub> presented an adverse effect on melanin content. At this concentration only a few apoptotic cells were observed, which represented the level in control group. Vincristine seemed to influence on melanogenesis and apoptotic occurrence in the similar manner to adriblastin and actinomycin D at EC<sub>50</sub> concentrations.
Cisplatin is the only one drug which acts differently on B16 or Cloudman S91 cells. Cisplatin at EC₅₀ caused a substantial decrease of melanin in B16 cells. At this concentration it was found only 11% of apoptotic cells. When Cloudman S91 line was treated with cisplatin at EC₅₀, increasing melanogenesis was observed with slightly rising apoptotic cells population.

Cytostatic drugs testing is able to reflect effectiveness in future treatment because cells death is the only reason of culture diminishing (18). Anticancer drug can induce both, apoptosis and necrosis, depending on concentration, cell type and experimental conditions (19). Apoptosis often takes place after exposure to cytostatic drug (20). The apoptotic cells are quickly removed by
phagocytosis (21). The quantitative evaluation of apoptosis process in vivo is difficult due to its asynchrony and short time of half-life of apoptotic cells (22).

The management of human disseminated melanoma is based on dacarbazine and vincristine. The response rate for such treatment is approx. 20% with no difference between one or multi drug therapy (23-29). In this study also adriblastin, actinomycin D, cytosine arabinoside and cisplatin have been included. Most cytostatic drugs caused an increase of melanin contents in both tested lines, except for cisplatin and dacarbazine in B16 cells and dacarbazine in Cloudman S91 cells.

Adriblastin is used to treat solid tumors as well as hematopoietic neoplasms. The drug penetrates cells by way of passive diffusion and combines to DNA base pairs. Adriblastin inhibits topoisomerase II activity with the subsequent DNA synthesis slow down and errors generation in replication and transcription (30). An increase of cells in S and G2/M phases was observed in both B16 and Cloudman S91 after exposure to adriblastin. The reduction of adriblastin anthracycline ring caused generation of free radicals. Free radicals are responsible for breaks in DNA double strain and they damage membranes and proteins (31). Melanins are biopolymers containing high concentrations of oxidizing (o-quinone) and reducing (o-hydroquinone) groups. Melanins and melanosomes may suppress activity of free radicals in two ways: by donation (reduced form of melanin) or by electrons’ capture (oxidized...
Adriablastin induced apoptosis in both tested lines but apoptotic activity was higher in Cloudman S91 than in B16 cells. However, increased production of melamins in mouse melanoma cells of both tested lines was noted. Induction of apoptosis in different types of mouse cells, e.g. thymocytes and lymphoid cells after adriblastin treatment was previously described (33, 34).

Actinomycin D is an inhibitor of DNA-dependent RNA synthesis. The drug combines the residue of deoxyguanine and also modulates activity of topoisomerase I and II (35). The investigation of cell cycle showed an arrest of B16 and Cloudman S91 cells in S and G2/M phases, as in the case of adriblastin. B16 and Cloudman S91 melanoma cells were resistant to actinomycin D at the concentrations tested. Similar results were obtained by Lasek et al. (36). Therefore, it could be stated that this antibiotic only acts cytostatically on B16 and Cloudman S91 melanoma cells.

Melanosomes are target to various cytotoxic agents (37). Melanin antioxidative precursors increase in the case of free oxygen radicals (ROS) a leak from damaged melanosomes. Necrosis of melanoma cells can be triggered off by indirect products of melanogenesis released from damaged melanosomes (38, 39). The highest melanin concentrations occurred after exposure of B16 and Cloudman S91 cells to actinomycin D, although the percentage of apoptotic cells was lower than after incubation with adriblastin. This confirmed participation of melanin precursors in sweeping off free radicals. Abdel-Malek et al. (40) and Hirobe (41) had observed that this cytostatic drug inhibits melanogenesis.
Both dividing and non-dividing cells. Melanoma cells resistance to alkylating drugs is an effect of intracellular drug detoxication, glutathione S transferase system (GTS) activation and modulation of anti and proapoptotic proteins (45-47). Cisplatin induced apoptosis in Cloudman S91 cells more intensely than in B16 cells. Melanogenesis was inhibited in B16 cells whereas in Cloudman S91 cells melanin content considerably increased. Based on those facts we found no connections between melanogenesis and apoptotic activity after cisplatin.

The most frequently used chemotherapeutic drug in disseminated melanoma treatment is dacarbazine (DTIC). Apoptosis at the level similar to control group was observed at EC50 concentration. This drug does not affect melanogenesis in B16 cells and decreased melanin content in Cloudman S91 cell line. DTIC caused stop in S and G2/M phases in both tested lines.

The cytostatic action of vincristine is connected with microtubular proteins gathered in mitotic spindle. Vincristine may indirectly generate production of free radicals (48). B16 cells were more resistant to vincristine. A slight increase of melanin content in the cells was observed, which can be a cell answer to free radicals generation.

Adriblastin, actinomycin D and vincristine are all supposed to increase free radical oxygen level within the cells. These drugs also have influence on melanogenesis rate with following apoptosis occurrence. This observation confirms the connection between triggering apoptosis and melanin contents of cells. This information could be important in planning of management of disseminated melanoma diseases.

Dynamic development of apoptosis research will allow to look forward and introduce new programs of chemotherapy.

REFERENCES


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