Delayed Reproductive Death and ROS Levels in the Progeny of Irradiated Melanoma Cells

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The cell death and survival of proliferating (clonogenic) cells were investigated in two human melanoma cell lines to assess the optimal conditions for preparation of apoptotic bodies from melanoma cells. After 50 J/m^2 UVB+UVC the maximal levels of apoptotic cells assayed by Trypan blue staining, nucleosomal DNA fragmentation, MTT, and TUNEL tests were observed within 2–3 d of radiation. In 100 Gy gamma-irradiated cultures these apoptosis indicators were delayed for up to 3 weeks. In addition, clonogenic cells were observed only in exponentially growing cultures irradiated with UV at high cell density but not in gamma-irradiated cultures. The response of melanoma cultures after high UV radiation doses contrasted to the response in lethally gamma-irradiated cultures. UV-irradiated melanoma cultures were recovered within two weeks. Most of the clonogenic cells in the recovered colonies contained micronuclei. ROS levels determined by DCF fluorescence and a modified MTT test were also normalized obviously due to the extensive antioxidant defense system of melanoma cells.
UV radiation of tumor cells might be the preferential method for preparation of apoptotic bodies. The presence of clonogenic cells in the suspension of apoptotic bodies from melanoma cells used for pulsing of dendritic cells with tumor antigens might compromise this protocol for preparation of cell vaccines.

Key words: Radiation, Clonogenic Cells, ROS

Introduction

The effect of radiation on melanoma cells have been investigated by many authors (for review see Noonan *et al.*, 2003). Most of the investigations are concentrated on the effect of UVA and UVB, which penetrate the earth's atmosphere. The germicide effect of UV radiation is mainly contributed to UVC. UVC and gamma rays are used for preparation of apoptotic bodies from tumor cells. Killed tumor cells are an adequate source for antigen loading of dendritic cells (Chang *et al.*, 2000; Gregoire *et al.*, 2003; Zhou *et al.*, 2003).

The investigation of multi-log cell kill (90–100% of irradiated cells) includes an immediate and delayed biological effect on the progeny of irradiated cells, resulting in cell death or clonogenic cell survival. The biochemical and molecular biological mechanisms involved in the induction of different modes of cell death are not fully understood. One of the molecular mechanisms attributed to the biological effects of radiation is the generation of reactive oxygen species (ROS) and oxygen-derived free radicals directly or through endogenous photosensitization reactions. The important roles of ROS in cellular damage and cell death are being increasingly recognized. Overproduction of ROS as well as the oxidative stress is detected *in vivo* by using the ROS-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Bettega *et al.*, 2001; He *et al.*, 2002). Using a CCD camera system, Ogawa *et al.* (2003) found that ROS formation occurred immediately after irradiation, continued for several hours, and resulted in oxidative DNA damage, *i.e.* DCF fluorescence evaluated intracellular oxidative stress prior to apoptosis. The role of ROS in recovering of UV-damaged cells is not fully understood. He *et al.* (2002) found that DNA strand breaks and survival of cyanobacteria returned to their non-irradiated levels after 4–7 d of irradiation, whereas ROS levels decreased after 7 d of radiation.

In the course of investigation of multi-log delayed cell death in melanoma cell lines (Tsoncheva *et al.*, 2001) we have observed marked differences in apoptosis of gamma-irradiated or UV-irradiated cells. The normalization of ROS levels and mitochondrial dehydrogenases in melanoma cells after

11–15 d of high doses UVB+UVC radiation corresponded to the efficient recovering of these cells.

Materials and Methods

Cell lines and culture conditions

MelP and MelG melanoma cell lines were obtained as described earlier (Tsoncheva *et al.*, 2001) and cultured in RPMI-1640 medium with 10% fetal bovine serum.

For clonogenic cell assay 1×10^4 cells were placed in a 6-cm dish. After 24–48 h, the culture medium was replaced by 1 ml PBS for irradiation. Non-adherent cells were removed and counted for calculation of the plating efficiency. The cultures were exposed to various doses of radiation, and maintained in complete medium until the surviving cells formed colonies. The cultures were fixed in methanol and stained with Giemsa. The colonies of > 50 cells were counted.

For long-term survival assay 1×10^5 cells were placed in a 6-cm dish. After 24 h, the exponentially growing cultures were exposed to radiation, and maintained by medium change every 3 d. The recovered cultures were analyzed further.

Irradiation procedures

A commercial germicide 15 W lamp was used as a source of UV radiation. The culture medium from each 6-cm dish was replaced with 1 ml PBS. After irradiation for up to 20 s (corresponding to 100 J/m^2) the cultures were placed in new complete medium and incubated further.

Gamma rays irradiation of cell suspension (10^7 cells/ml PBS) was performed at a dose rate of 1.7 Gy/min as described (Tsoncheva *et al.*, 2001).

Agarose gel electrophoresis of DNA

Cells were harvested after trypsinization and washed with PBS. Approximately 10^6 cells in 50 μl suspension were lysed in 500 μl 100 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane-HCl (pH 7.5), 10 mM EDTA, 0.4% SDS and digested with 40 μl RNase at 37 °C for 1 h and with 100 μl proteinase K overnight. After phenol/chloroform deproteinization 0.1 volumes 3 M Na-acetate was added and DNA was precipitated with 2.5 volumes of ethanol. Dried DNA was dissolved in 50 μl 1 mM EDTA, 10 mM Tris-HCl (pH 7.5), then run on 1% agarose gel and stained with ethidium bromide.

Determination of reactive oxygen species (ROS)

Cells were harvested by trypsinization and suspended in PBS. 10 μl of the suspension were mixed with 10 μl 0.4% Trypan blue and counted. Aliquots containing 2×10^5 viable cells were adjusted to 0.9 ml end volume with PBS and 100 μl 0.5 mM DCFH-DA (Sigma) was added. The cell suspension was incubated for 1 h at 20 °C in the dark, then cooled on ice, and washed twice with PBS. The cells were lysed in 1 ml 10 mM Tris-HCl (pH 7.4), 0.5% Tween 20. After centrifugation at $10.000 \times g$ for 10 min the fluorescence of converted DCF in clear supernatants was measured with a spectrofluorimeter (RF-5000, Shimadzu) at room temperature, with an excitation wavelength of 485 nm and an emission band between 500 and 600 nm.

Aliquots of 2×10^5 viable cells from the same cell suspension were assayed for mitochondrial dehydrogenase activities by MTT [3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test. Cells were incubated in 2 cm²-well plates with 0.9 ml complete culture medium and 100 μl MTT stock solution (5 mg/ml, filtered through a 0.2 μm-filter). After incubation for 1 h at 37 °C in a humidified CO_2 incubator the cells were harvested and the converted MTT formazan dye was solubilized with isopropanol containing 0.04 N HCl.

Results and Discussion

DNA fragmentation of melanoma cells after gamma radiation and UV radiation

MelP and MelG cells were cultured after 100 Gy gamma radiation and DNA isolated after 18 h up to 21 d of radiation. A clear laddering pattern of DNA fragments after agarose gel electrophoresis was observed after 21 d of radiation (Fig. 1A). This result confirms our previous results (Tsoncheva *et al.*, 2001) and the hypothesis for inactivation of a pathway for immediate apoptotic DNA fragmentation by high doses of ionizing radiation (Gobbel and Chan, 2001).

On the contrary, the maximal effect of UV radiation on DNA laddering pattern was observed after 1–2 d of irradiation depending on radiation doses (Fig. 1B). The same results were obtained using Trypan blue staining and TUNEL test. The degradation of both, the cell wall and DNA, were dose-dependent.

Differences in response to UV and gamma radiation were reported earlier (Petrocelli *et al.*, 1996;
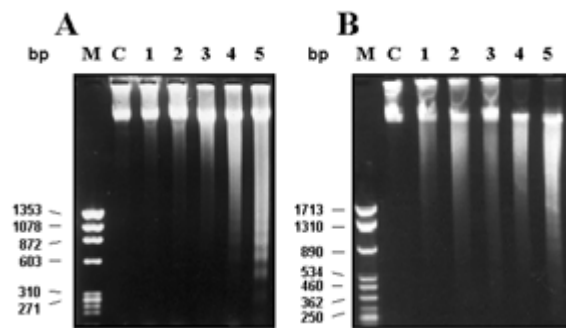
Fig. 1. Agarose gel electrophoresis of DNA from MelG cells. Panel (A): Cells were irradiated with 100 Gy gamma rays, DNA was extracted after 18 h of radiation (lane 1), 4 d of radiation (lane 2), 7 d of radiation (line 3), 14 d of radiation (line 4) and 21 d of radiation (lane 5); non-irradiated control cells (lane C); marker: ϕX174 RFDNA/HaeIII fragments (lane M). Panel (B): Cells were irradiated with 10 J/m^2 (lane 1 and 2), 15 J/m^2 (lane 3 and 4) and 50 J/m^2 (lane 5) UVB+UVC. DNA was extracted after 24 h of radiation (lane 1, lane 3 and lane 5) or 48 h of radiation (lane 2 and 4); non-irradiated control cells (lane C); marker: λDNAdv/HaeIII fragments (lane M).

Fig. 2. Clonogenic cell assay of low-dense cultures from UV-irradiated MelP cells, ($\bigcirc$) or 3T3 cells ($\blacksquare$).

Sheridan and West, 2001). Significant differences in gene expression profiles of logarithmically growing dermal diploid fibroblasts after gamma radiation (5 Gy), or UVC radiation (7.5 J/m^2) were recently reported by Heinloth *et al.* (2003). The stronger apoptotic response of melanoma cultures after high UV radiation doses contrasted to the slower apoptotic response in lethally gamma-irradiated cultures. In this aspect UV radiation is the preferential method for preparation of tumor cell apoptotic bodies. UV-irradiated tumor cells-pulsed dendritic cells stimulated T lymphocytes more efficiently than dendritic cells-pulsed with gamma-irradiated cells (Ishii *et al.*, 2003).

Survival fraction of UV-irradiated cells depends on culture condition

Low dense cell cultures (500 cells/cm^2) were assayed for clonogenic cells after 5–100 J/m^2 UV radiation. The clonogenic MelP and MelG cells decreased rapidly in 10–40 J/m^2 radiation interval, whereas non-melanoma cells seemed more radiation-resistant in the low dense culture condition (Fig. 2). At radiation doses > 40 J/m^2 by day 14 colonies were observed only in cultures of 3T3 cells used as controls in clonogenic assay. In addition, plating efficiency of melanoma cells was 25–30% by day 2 versus 35–50% for 3T3 cells by day
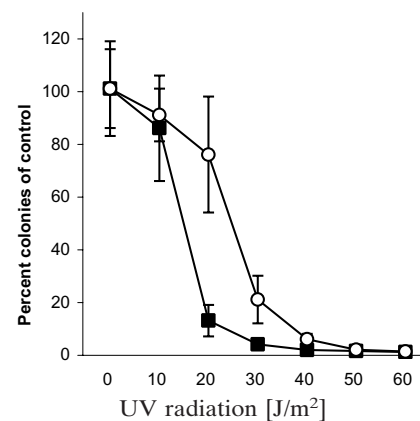
1. The longer pre-exponential growth phase and lower plating efficiency of low dense cultures of melanoma cells compared with 3T3 fibroblast are indicative of differences in growth conditions of these cultures in the moment of irradiation. The better growth conditions before irradiation might result in higher radiation-resistance of cultures.

To check this possibility the survival of melanoma cells irradiated in exponential growth phase was investigated. Exponentially growing cultures of melanoma cells were obtained as described in Materials and Methods and irradiated with 20–100 J/m^2 UVB+UVC. The growth arrest period (up to 5 d) and death cell number (up to 90% by day 3) were dose-dependent. These cultures were recovered after irradiation with doses up to 70–80 J/m^2. In one experiment a 100 J/m^2 irradiated culture was recovered. These results indicated that the clonogenic potential of UV-irradiated melanoma cells is dependent on both – radiation dose and culture conditions. Decreased growth rate and plating efficiency of low dense melanoma cell cultures significantly enhanced their radiation-sensitivity.

At high doses of UV radiation (50 J/m^2 UVB+UVC) when > 90% of the irradiated cells died within 5 d some melanoma cells retained their clonogenic potential. In contrast, after 100 Gy gamma radiation melanoma cells survived up to 3 weeks but clonogenic cells were not detected. Our results confirmed the statement that the programs of apoptosis have little or no effect on clonogenic survival after treatment with radiation in several

	Plating (%)	Trypan blue (%)	Colonies per cm^2
Non-irradiated cells	28 +/− 4	2.5 +/− 1.5	105 +/− 11
Day 5 post irradiation	3 +/− 2	80 +/− 5	9 +/− 3
Day 8	18 +/− 8	16 +/− 7.5	62 +/− 7
Day 10	25 +/− 10	7 +/− 2.5	101 +/− 15
Day 12	31 +/− 9	4 +/− 2	120 +/− 19
Day 14	23 +/− 11	4 +/− 2.5	104 +/− 20

Table I: Plating efficiency, Trypan blue positive cells and clonogenic cells of non-irradiated and 50 J/m^2 UV-irradiated MelP cell cultures.

tumor cell lines (Roninson *et al.*, 2001; Sheridan and West, 2001).

The presence of clonogenic cells in the suspension of irradiated apoptotic bodies used for pulsing of dendritic cells might compromise this protocol for preparation of cell vaccines (Chang *et al.*, 2000; Zhou *et al.*, 2002; Gregoire *et al.*, 2003). The case requires more detailed analysis.

Analysis of melanoma cells recovered after UVB+UVC radiation

After 50–70 J/m^2 UVB+UVC radiation MelP and MelG cells were seeded at low cell density to compare their plating efficiency and colony formation. The results are presented in Table I.

Melanoma cell cultures after 5 d of radiation contained < 15% viable cells with limited plating efficiency. After 12–14 d of radiation, melanoma cell cultures contained > 80% viable cells with clonogenic potential. Most of cells in the colonies contained micronuclei.

Exponentially growing cultures of MelP, MelG and 3T3 cells were irradiated with 40–60 J/m^2 UVB+UVC and their progeny were assayed for mitochondrial dehydrogenase activities and intracellular ROS in the course of recovering. The level of DCF fluorescence and MTT formazan dye absorbance in non-irradiated cell cultures depended on the metabolite activity of cells. It decreased rapidly in the lag-phase of cell growth. To distinguish UV induced ROS from the physiological redox processes in mitochondria DCF fluorescence arbitrary units were superimposed with absorbance of converted MTT dye. The most prominent discrepancy was observed in cultures after 5 d of radiation, where mitochondrial dehydrogenases are minimized due to the growth arrest and the DCF fluorescence peak reflected radiation-induced non-physiological ROS levels (Fig. 3). By day 12–14 after high dose UV radiation ROS levels in melanoma cells were normalized: DCF
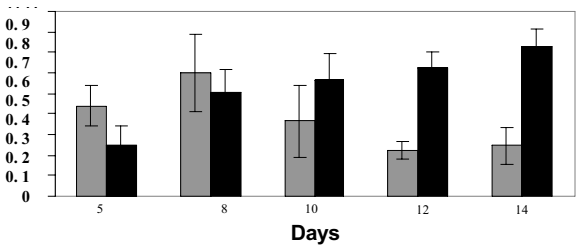

Fig. 3. Intracellular ROS level, and mitochondrial dehydrogenase activities in MelP cultures after 5 d, 8 d, 10 d, 12 d, and 14 d of 50 J/m^2 UV radiation; DCF fluorescence peak at 520 nm in arbitrary units $\times 10^{-3}$ (gray columns); formazan dye absorbance at 550 nm (black columns). Data are expressed as the mean +/− standard deviation of four determinations in two independent experiments.

fluorescence corresponded to mitochondrial dehydrogenases.

Bettega *et al.* (2001) observed an enhanced DCF fluorescence in the progeny of UVB-irradiated human hybrid cells. Our control experiments with 3T3 cells also showed enhanced DCF fluorescence up to 15 d of radiation. In contrast of other cells melanoma cells have obviously better antioxidant balance. Sander *et al.* (2003) found a significant over-expression of the antioxidant enzymes in human melanoma biopsies, when compared with surrounding non-tumor tissue, benign melanocytic naevi, and healthy volunteers. In non-melanoma tumors they observed a severely disturbed antioxidant balance with diminished antioxidant enzymes.

There are evidences for the role of melanin and melanotonin in survival of irradiated melanoma cells (Kowalczuk *et al.*, 2001; Park *et al.*, 2001; Ryoo *el al.*, 2001). In the present investigation we were unable to find reliable differences in experimental data obtained from MelP and MelG cells which differ in their melanin content before radiation. After irradiation melanin content in both lines increased rapidly.

Oxidative stress occurs in cells when the generation of ROS overwhelms the cellular natural antioxidant defenses. To combat excessive production of ROS melanoma cells are equipped with an extensive antioxidant defense system in which melanin might play an important role. Preparation of apoptotic bodies from melanoma tumor cells for pulsing of dendritic cells needs special attention for residual clonogenic tumor cells.

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