

# Evaluation of Delayed Apoptotic Response in Lethally Irradiated Human Melanoma Cell Lines

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To assess the lethal doses of gamma radiation and corresponding apoptotic response in new established human melanoma cell lines we exposed exponentially growing cultures to 8–100 Gy gamma radiation. The apoptosis and cell survival were determined by trypan blue exclusion, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction, agarose gel electrophoresis, colony forming assay, and long-term survival assay. The maximal DNA fragmentation 3 days after irradiation was observed in cultures irradiated with 20 Gy (36.9% TUNEL positive cells). The cultures irradiated with 50 and 100 Gy contained 18.7% and 16.4% TUNEL positive cells, respectively. Cultures exposed to 8 and 20 Gy gamma radiation recovered by week 3–4. Lethally irradiated (50 and 100 Gy) cultures which contained less apoptotic cells by day 3 died by week 5. A detectable increase in melanoma cell pigmentation after irradiation was also observed. The survival of human melanoma cell cultures after exposure to gamma radiation does not correlate with the level of apoptotic cells by day 3. At high radiation doses (> 50 Gy) when the radiation induced cell pigmentation is not inhibited the processes of apoptotic DNA fragmentation might be preferentially inactivated.

## Introduction

The significance of apoptosis in the response of cancer cells to gamma radiation is a major concern. The overall response of the cells to ionizing radiation is dependent on radiation doses. Low doses (up to 2 Gy per fraction) has been applied for therapy of various carcinomas (Kokawa *et al.*, 1999). Intermediate doses (10–12 Gy) has been applied for preparation of feeder layers for cultivation of hematopoietic cells (Verfaillie *et al.*, 1990). High doses (100–150 Gy) has been applied for preparation of tumor cell vaccines (Dillman *et al.*, 1993; Osanto *et al.*, 2000). The apoptotic response of different cancer cells to therapeutic radiation doses *in vivo* and *in vitro* is well documented. DNA is considered as important target for radiation-induced apoptosis (Ayene *et al.*, 2000). DNA lesions produced by ionizing radiation triggers apoptosis by a kinetically slow process (Cregan *et al.*, 1999; Belyakov *et al.*, 1999; Mendonca *et al.*, 1999). The apoptotic response varied widely in individual cases and different cells (Barber *et al.*, 2000; Meijer *et al.*, 1999; Uberti *et al.*, 1999). The

variation in apoptotic pattern does not correlate with the p53 status of the cells due to the existence of different apoptotic pathways (Cregan *et al.*, 1999; Ogawa *et al.*, 2000; Mothersill *et al.*, 1999).

The information concerning apoptotic response after high radiation doses is poor. Co-ordinate activation of DNA binding proteins (Sp1, NF-kappa B, p53) after high radiation doses may lead to divergent alteration in cell cycle checkpoints and gene expression (Yang *et al.*, 2000; Bulavin *et al.*, 1998; DeSimone *et al.*, 2000). Cell death of progeny cells is a specific effect of ionizing radiation (Mendonca *et al.*, 1999). Even at high doses of gamma radiation, a dose-dependent cell fraction is capable of continued cycling, i.e. loss of colony-forming ability is not coupled to apoptosis (Petit-Frere *et al.*, 2000). In this respect specific forms of radiation induced programmed cell death as “delayed reproductive death” (Belyakov *et al.*, 1999) or “clonogenic” cell death (Ayene *et al.*, 2000) are conceivable.

In the course of preparation of cell vaccines from two new established human melanoma cell lines we used lethal doses of gamma radiation

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> 50Gy. The existence of clonogenic cells was detected by long-term survival assay even after 20 Gy gamma radiation where the apoptotic response measured as DNA-strand breaks was maximal.

## Materials and Methods

### *Cell lines and culture conditions*

Melanoma cell lines were established from patients with metastases of malignant melanoma using minor modifications of the method described by Dillman *et al.* (1993). MelP originates from a lymph node and MelG from a tumor node. The nodes were placed in 6 cm petri dishes containing 5 ml RPMI-1640 medium and minced with a scissors. Cells were suspended using wide-mouthed pipette and transferred in a T-25 tissue culture flask containing 1 ml fetal bovine serum (FBS). The procedure was performed within 2 h of tumor harvest. The non-adherent cells were transferred to a second T-25 flask 1 h later. The cultures were maintained further in medium with 10% FBS. The non-adherent cells obtained by medium change were cultured in reduced volume at minimal cell density  $5 \times 10^5/\text{ml}$ . The adherent cells of the confluent cultures were treated with 1 mM EDTA in phosphate buffered saline (PBS) and 0.1% trypsin. The cells were suspended by gently pipetting and seeded at minimal cell density of  $2 \times 10^5/\text{ml}$ . By week 8 of culture the population doubling time was 2–3 days.

Cisplatin solution (0.1 mg/ml) was prepared *ex tempore* in sterile redistilled water. Cells were exposed for 4 h to the drug then washed and subcultured in  $2 \text{ cm}^2$  wells in medium without cisplatin.

### *Irradiation procedures*

Exponentially growing melanoma cells were collected and resuspended in fresh culture medium in sterile 15-ml plastic tubes. Each tube was positioned co-axially in a cylindrical tissue equivalent phantom to provide secondary particle equilibrium and irradiated with  $^{60}\text{Co}$  gamma rays at the dose rate of 1.7 Gy/min. Separate samples were irradiated to 8, 20, 50, and 100 Gy, respectively.

### *Trypan blue exclusion test*

Cells were harvested in a 15 ml tube and centrifuged 5 min at 500 g. Ten  $\mu\text{l}$  of the cell suspension

were mixed with 10  $\mu\text{l}$  0.4% Trypan blue on a piece of parafilm and transferred to hemocytometer chamber. The cells in the 1-mm center square were counted keeping a separate count of the stained non-viable cells.

### *Long-term survival assay*

Cells exposed to cisplatin or gamma rays were collected in sterile 15-ml tubes, centrifuged for 5 min at 500 g and resuspended in sterile RPMI-1640, 15% FBS. Aliquots of  $10^5$  cells were cultured in  $2 \text{ cm}^2$  culture wells. The cultures maintained 2 to 5 weeks were feeded once weekly and the cells removed by medium change were scored. The well cultures were harvested with trypsin-EDTA. The viable and dead cells in four parallel samples were counted as described above. To calculate the total cell number per well the number of cells removed by medium change was added.

### *DNA strand breaks labeling by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction*

Cells ( $3\text{--}5 \cdot 10^3$ ) were fixed in 4% neutral buffered formaldehyde solution at room temperature for 15 minutes, then resuspended in phosphate buffered saline (PBS), and spotted onto organosilane (3-aminopropyltriethoxysilane) pretreated slides. Superfluous fluid was removed after 10 minutes. The spots were air-dried under a laminar hood and dehydrated with ethanol. After rehydration followed by quenching of the endogenous peroxidase with 0.6% hydrogen peroxide for 15 min the preparations were pre-incubated in TT buffer (150 mM potassium cacodylate, 25 mM Tris-HCl (Tris [hydroxymethyl] aminomethane hydrochloride), 0.25 mg/ml bovine serum albumin, 2.5 mM  $\text{CoCl}_2$ , pH 6.8) for 15 min at  $37^\circ\text{C}$ . TT mix was prepared *ex tempore* from 20  $\mu\text{l}$   $5 \times$  TT buffer, 10  $\mu\text{l}$  25 mM  $\text{CoCl}_2$ , 5  $\mu\text{l}$  1 mM dATP, 1  $\mu\text{l}$  1 mM biotin-16-dUTP, 1  $\mu\text{l}$  (10–50U) terminal transferase (Boehringer Mannheim) and 63  $\mu\text{l}$  redistilled water in a microcentrifuge tube on ice. Each spot sample was covered with TT mix and parafilm. The end-labeling was carried out for 2 h at  $37^\circ\text{C}$  in a humidified chamber. The slides were rinsed gently with PBS from a wash bottle and placed in PBS wash bath for 5 min. Biotinylated avidin-peroxidase (Amersham) diluted 1:500 was applied

to each spot and incubated for 60 min. in humidified chamber. The slides were rinsed and washed with PBS and allowed to drain. Freshly prepared DAB solution (0.02% 3,3'-diaminobenzidine, 0.04%  $\text{NiCl}_2$ , 0.01% peroxide in PBS) was applied to cover the slides and the reaction was monitored with the microscope. Slides were immersed in water. Microphotographs were prepared and 200–400 cells were counted for calculation of the percentage of the cells with dark nuclei.

#### Agarose gel electrophoresis of DNA

Cells ( $7 \times 10^5$ ) were lysed in 20  $\mu\text{l}$  of 10 mM EDTA and 50 mM Tris-HCl (pH 8) buffer containing 0.5% sodium dodecyl sulfate (SDS) and 0.5 mg/ml proteinase K. After incubation 2 h at 50 °C, 5  $\mu\text{l}$  of 1 mg/ml RNase A were added. Samples were incubated overnight at 37 °C, heated to 70 °C, and 10  $\mu\text{l}$  loading buffer containing 10 mM EDTA, 1% low melting agarose, 0.04% bromphenol blue, 40% sucrose were added (Palayoor *et al.*, 1998). Electrophoresis was carried out in 2% agarose gel at 70 V.

## Results and Discussion

#### Morphological characterization of melanoma cells:

The human melanoma cell lines MelP and MelG were established after a 10–12 week of culture. They keep morphological heterogeneity of the population for up to 18 months continuous growth (Fig. 1a). The ratio adherent/non-adherent cells was higher in freshly-passaged cultures. Large round cells with limited proliferative potential predominated in the aged cultures. Approximately

30% of MelP cells were positively stained for melanin by the ferrosulfate/ferricyanide method (Lillie, 1957). The melanin-containing cells sedimented more rapidly and formed a brownish colored lower part of the cell pellet. MelG cultures were more strongly adherent and contain less melanin-positive cells. After irradiation a considerable part of MelG cells adhere again but large round non-adherent cells predominated in MelP cultures (Fig. 1b). The morphological characteristics of irradiated melanoma cells indicated accumulation of polyploid cells with lobulated nuclei and micronuclei due to continued DNA replication on G2-arrested cells (Bulavin *et al.*, 1998).

Non-irradiated MelG cell lysates ( $2 \times 10^7$  cell/ml) were colorless but a dense brown pigmentation was observed in the cell lysates obtained 24–72 h after irradiation. The pigmentation of melanoma cells in aged cultures is considered to indicate their maturation. The accelerated pigmentation of gamma irradiated immature MelG cells requires further investigations.

#### Apoptotic effect of gamma radiation and cisplatin

Gamma irradiated (8–100 Gy) MelP or MelG cells were cultured in three independent experiments at density  $2 \times 10^5$  cells/ml. By day 3 cells were harvested and counted (Table I). Aliquots of  $4 \times 10^5$  cells were processed for the TUNEL reaction, and aliquots of  $6 \times 10^5$  cells for agarose gel electrophoresis of DNA. The percentage of cells with DNA breaks reached a maximum in 20-Gy irradiated cultures and did not increase after 50- and 100-Gy radiation doses (Table I). Typical microphotographs of the TUNEL reaction are presented by Fig. 2. The comparative analysis of DNA degrada-

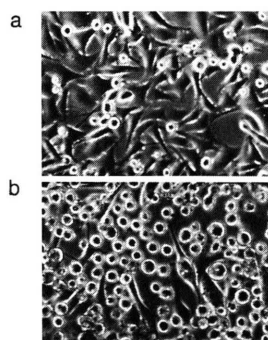


Fig. 1. Phase-contrast micrographs of MelP cells by week 18 of culture, a; by day 7 after irradiation, b. ( $\times 120$ ).

Table I. Apoptotic effect 3 days after exposure to cisplatin or gamma radiation.

	Trypan blue positive cells mean $\pm$ SE (%) <sup>a</sup>		TUNEL positive cells (%) <sup>b</sup>	
	MelP	MelG	MelP	MelG
No damage	5.2 $\pm$ 1.2	4.2 $\pm$ 0.9	3.6	3.8
Cisplatin 3 $\mu\text{g/ml}$	53.3 $\pm$ 5.0	42.7 $\pm$ 3.9	16.0	12.4
Cisplatin 10 $\mu\text{g/ml}$	93.0 $\pm$ 2.1	90.5 $\pm$ 3.2	34.2	n.d.
Irradiation 8 Gy	n.d.	28.5 $\pm$ 1.8	n.d.	21.5
Irradiation 20 Gy	42.7 $\pm$ 3.7	44.9 $\pm$ 3.3	31.8	36.9
Irradiation 50 Gy	n.d.	50.8 $\pm$ 4.2	n.d.	18.7
Irradiation 100 Gy	41.5 $\pm$ 2.9	54.0 $\pm$ 5.7	16.8	20.1

<sup>a</sup> Calculated from 3 separate experiments.

<sup>b</sup> Data from two typical experiments.

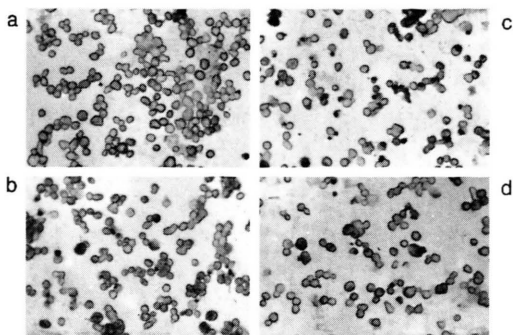


Fig. 2. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction for DNA strand-breaks in non-irradiated MelG cells, a; or 3 days after gamma irradiation with 8-Gy, b; 20-Gy, c; 50-Gy, d. ( $\times 120$ ).

tion by agarose gel electrophoresis was based on an equal cell number in each sample. All irradiated samples (8, 20, and 50 Gy) showed a similar level of DNA degradation (Fig. 3). A discrete fraction corresponding to a 500-bp DNA fragment was reproducibly observed in the samples obtained from non-irradiated melanoma cells. This fraction was still visible in 8-Gy irradiated samples. It was not detected in DNA samples obtained by the standard phenol deproteinization procedure.

The appearance of DNA fragments is a typical feature of apoptosis and the labeling of breaks by the TUNEL reaction showed good overall correlation and reproducibility (Barber *et al.*, 2000). The level of DNA fragmentation by day 3 after irradiation was maximal in 20-Gy irradiated cultures with a decrease in 50- and 100-Gy irradiated cultures. A non-linear DNA fragmentation response to

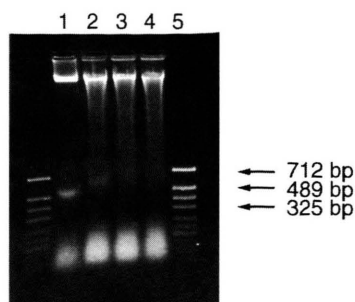


Fig. 3. Agarose gel electrophoresis of DNA from non-irradiated MelP cells, line 1; or 3 days after irradiation with 8-Gy, line 2; 20-Gy, line 3; 50-Gy, line 4; pBS/MspI marker, line 5.

increased radiation doses was reported by Kokawa *et al.* (1999) in patients with cervical carcinoma during a fractionated radiotherapy and Mothersill *et al.* (1999) in primary explant cultures of human urothelium. Our results could not be simply explained with the reduction of cycling cells. In the absence of cycling cells after 20-Gy irradiation the DNA fragmentation was maximal. The lower DNA fragmentation by day 3 after 50- and 100-Gy irradiation could be a consequence of inactivation of apoptotic pathways at these radiation doses.

To compare the apoptotic effect after gamma radiation with the effect of another DNA damaging agent exponentially growing MelP and MelG cells were exposed to 3 or 10  $\mu\text{g/ml}$  cisplatin. The apoptotic effect increased linearly up to day 3. In cultures exposed to 3  $\mu\text{g/ml}$  cisplatin the apoptotic effect was similar as in irradiated cultures (Table I). The cultures exposed to 10  $\mu\text{g/ml}$  cisplatin contained approximately 90% trypan blue positive cells by day 3. These cultures died by day 5.

Lack of detectable DNA strand breaks in a considerable part of gamma-irradiated or cisplatin treated human melanoma cells with damaged cell membranes suggests that DNA degradation could be partly compensated by DNA repair. The following experiments support this assumption.

#### *Survival of melanoma cells exposed to cisplatin or gamma radiation*

Irradiated melanoma cells (8 Gy, 20 Gy, 50 Gy, and 100 Gy) were sub-cultured at a density of  $2 \times 10^5$  cells/ml with one medium change per week. The cell fractions removed by medium change were scored for the end calculation of the total cell number of the cultures as described in Methods. These fractions contained 90–95% trypan blue positive cells. A considerable part of the adherent cells in 8- and 20-Gy irradiated cultures remained trypan-blue negative and these cultures recovered after 3 weeks (Fig. 4b and 4c). 20-Gy gamma irradiated MelP and MelG cells did not produce colonies in cultures initiated at 100 cells/cm<sup>2</sup> for colony-forming assay but some of them survived in cultures initiated at  $10^5$  cells/cm<sup>2</sup> for long-term survival assay. 50-Gy or 100-Gy irradiated cultures contained less viable cells. Their number decreased gradually up to week 4



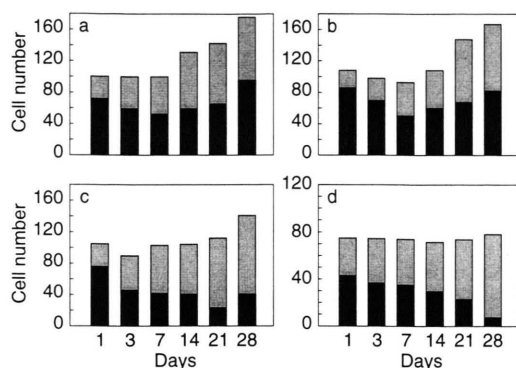


Fig. 4. MelG cells were exposed to cisplatin 3 µg/ml, a; or gamma radiation 8-Gy, b; 20-Gy, c; 50-Gy, d. Total number of the viable cells (black columns) and trypan blue positive cells (grey columns) per well-culture by day 1 to 28. Each column represent average cell numbers  $\times 10^{-3}$  from four parallel samples.

(Fig. 4d). These cultures died by week 5. To compare the clonogenic potential of cisplatin exposed MelP and MelG cells were used conditions resulting similar apoptotic effects as gamma irradiation by day 3. The survival patterns of cisplatin-treated cultures exposed to 3 µg/ml cisplatin (Fig. 4a) and 8-Gy irradiated cultures (Fig. 4b) were similar.

A considerable part of irradiated cells avoid immediate apoptosis because of a G2 arrest in the cell cycle (DeSimone *et al.*, 2000; Tamamoto *et al.*,

1999). This consideration remains valid in our experiments with radiation doses up to two orders of magnitude higher than "therapeutic" doses. On the other hand a three-fold increase of cisplatin concentration irreversibly led to cell death.

DNA-damaging agents trigger cell-cycle arrest with different consequences: DNA repair or programmed cell death. These consequences may be different in distinct cells from a cell population depending on several factors: nature and dose of the damaging agent, cell type, and cell cycle position. Apoptosis as a single cell event can not predict or exclude the existence of clonogenic cells in the population. The modification of the clonogenic survival assay described here as long-term survival assay is a sensitive method to detect clonogenic cells especially in cell lines which are not able to grow at limiting dilution.

Gamma radiation doses of 100-Gy are optimal for preparation of cancer cell vaccines by two reasons: absence of clonogenic cells and good survival ensure save and prolonged exposition of cancer cells to the host immune system.

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