Radiosensitivity of Human Melanoma Cell Lines

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Abstract. Cutaneous melanoma is a skin cancer resulting from the malign transformation of skin-pigmented cells, the melanocytes. The radiotherapy, alone or in combination with other treatments, is an important therapy for this disease. The objective of this paper was to determine *in vitro* the radiosensitivity of two human melanoma cell lines with different metastatic capability: WM35 and M1/15, and to study the effect of drugs on radiobiological parameters. The Survival Curves were adjusted to the mathematical Linear-quadratic model using GraphPad Prism software. Cells were seeded in RPMI medium (3000-3500 cells/flask), in triplicate and irradiated 24 h later. The irradiation was performed using an IBL 437C H Type equipment (189 TBq, 7.7 Gy/min) calibrated with a TLD 700 dosimeter. The range of Doses covered from 0 to 10 Gy and the colonies formed were counted at day 7th post-irradiation. Results obtained were: for WM35, α=0.37±0.07 Gy^{-1} and β=0.06±0.02 Gy^{-2}; for M1/15, α=0.47±0.03 Gy^{-1} and β=0.06±0.01 Gy^{-2}. The α/β values WM35: α/β = 6.07 Gy and M1/15: α/β = 7.33 Gy were similar, independently of their metastatic capability and indicate that both lines exhibit high radioresintance. Microscopic observation of irradiated cells showed multinuclear cells with few morphologic changes non-compatible with apoptosis. By means of specific fluorescent dyes and flow cytometry analysis we determined the intracellular levels of the radicals superoxide and hydrogen peroxide and their modulation in response to ionizing radiation. The results showed a marked decreased in H_2O_2 intracellular levels with a simultaneous increase in superoxide that will be part of a mechanism responsible for induction of cell radioresistance. This response triggered by irradiated cells could not be abrogated by different treatments like histamine or the combination of oligoelements plus phospholipase (O-LM).

1. Introduction

Cutaneous melanoma is a cancer type that in many places has attained epidemic proportions and whose incidence is exponentially increasing [1]. It is know that this cancer is the result of a malignant transformation of pigment producing skin cells, melanocytes, [2] and is characterized by its high capability of local and distant invasion [2]. Even if the treatment selection depends on first place of the illness stage, radiotherapy alone or in combination with other treatments, is an important therapeutic alternative for this affection [3].

One of the possibilities to study melanoma is the use of *in vitro* models, which allows the study of the radiosensitivity of these malignant cells as well as the analysis of anti-proliferative effect of different drugs. The effects of ionizing radiation on cell lines are analyzed in vitro by means of culture in adequate conditions and results are analyzed by Survival Curves. Those Curves are graphic representations of the proportion of survival cells as a function of Absorbed Dose, expressed in Gray (Gy), which are adjusted to an adequate mathematical model [4, 5]. The Curves adjustment allows determining mathematically the radiobiological parameters [4, 5].

Reactive oxygen species (ROS) are involved in causing ionizing radiation-induced damage. One of the potential bases of radiosensitivity is the difference in tissue levels of ROS [6]. The enzymes Superoxide dismutases (SODs) catalyze the dismutation of Superoxide (SO_2) into O_2 and Hydrogen peroxide (H_2O_2), and the peroxide can be destroyed by catalase (CAT) or by glutathione peroxidase (GSH-Px). After irradiation, the accumulation of these radicals may activate different pathways that led to the protection of cells from irradiation damage and death. In general, cancer cells contain lowered MnSOD as compared with the normal cell type from which the tumor arose. Many studies have focussed on the tumor suppressive effect of MnSOD [7]. The Ras protein induces cellular pathways conducing to the production of superoxide, thus

SO_2 and ROS derived from it play an essential role in mitogenic signals and malignant transformation [8].

The development of drugs that radiosensitize the malignant cell is yet a challenge for oncologists and radiobiologists. We have demonstrated previously that the treatment of melanoma cells with the combination of Selenium (Se), Zinc (Zn) and Manganese (Mn) (4 μg/kg each) plus Lachesis *muta* (4 ng/kg) (O-LM), produces a significant inhibition on malignant cell growth. In addition, we have reported the protective effect of O-LM against high doses of chemotherapy [9, 10]. Also, the role of Histamine (Hi) in normal and cancer cell proliferation has been extensively investigated. In previous studies we have demonstrated that histamine behaves as an autocrine growth factor in different cells lines derived from human neoplasias and the treatment with histamine at high doses or with specific agonists exerts a significant inhibition on cell proliferation [11, 12].

The objective of this paper was: a) to determine the radiobiological parameters in two melanoma cell lines with different metastasic capability: WM35 and M1/15; b) to study the effect of different drugs with possible radiosensitivity action (O-LM and Hi), and c) to analyze the modulation of ROS intracellular levels in response to irradiation.

2. Materials and Methods

2.1. Cell lines.

Two cell lines from human melanoma were employed: WM35, derived from primary lesions of early stage without invasion on surrounding tissue, and M1/15 derived from liver metastasis developed in immunosupressed mice inoculated with a human melanoma cell line. Cells were gently supplied by Dr. Andras Falus, Department of Genetics, Cell and Immunobiology, Semnelweiss University, School of Medicine, Hungary.

2.1.1. Cell culture.

Cell lines were kept in culture in RPMI 1640 medium (Gibco BRL, Grand Island Biological Co., NY) supplemented with calf serum 10% (Gibco BRL), glutamine 0.3 g/l (Sigma Co, St Louis, USA) and gentamicine 0.04 mg/l. To carry on radiosensitivity studies 3300 cells were seeded in plastic flasks and after 24 hrs were irradiated with doses ranging from 0 to 10 Gy. Seven days post irradiation the clonogenic proliferation was evaluated by counting the colonies containing 50 cells or more. Colonies were fixed with 10 % buffered saline formalin and stained with Toluidine Blue (T.B.). For cell treatment, Histamine (Hi) 10 μM or 0.02 ml of O-LM per ml of culture medium were added to flasks immediately after plating and continued up to 24 hrs post-irradiation.

2.2. Irradiation.

The employed irradiation unit was an IBL 437C, H type, equipment calibrated by the Argentine National Commission of Atomic Energy with a TLD 700 dosimeter, which were tested by the Argentine Nuclear Regulatory Authority. The equipment has three ^{137}Cs encapsulated sources (189 TBq, Dose rate 7.7 Gy/min).

2.2.1. Survival parameters.

The Survival Curves were plot and adjusted to the Linear-quadratic mathematical model [4,5] with the GraphPad Prism 4 software; the alpha, beta, alpha/beta radiobiological parameters, as well as the Dose needed to have a survival fraction of 0.01 and the Survival Fraction at 2 Gy, were obtained. The results represent the mean of three independent experiments in which each dose was evaluated by triplicate.

2.2.2. Morphologic studies.

Cells were fixed, stained and later analyzed with optical microscopy to evaluate morphologic alterations caused by ionizing radiation. The two irradiated cell lines were also processed to detect apoptotic cells with Apoptag detection kit.

2.3. Analysis of cellular superoxide and peroxide (ROS).

Dihydroethidium (HE) is a specific SO_2 dye, while dichlorofluorescein-diacetate (Da) is used to monitor H_2O_2 levels in cells [13]. Cells were seeded in 10 cm plates and cultured in complete medium until 80% confluence and then irradiated with a dose of 2 Gy. Immediately after medium was removed and HE or Da (both dissolved in DMSO and diluted with PBS to final concentrations of 5 μM) was applied to the cells and incubated for 20-25 min at 37ºC. Then cells were washed twice with PBS, harvested and subjected to flow-cytometric analysis.

3. Results and Discussion.

3.1. Radiobiological parameters.

From the Survival Curves the radiobiological parameters were obtained (Table I). These results show that both cell lines does not shows significant differences in any of the radiobiological parameters, regardless their metastatic capability. Figure 1 illustrates the Survival Curves for both cell lines: WM35 and M1/15, respectively, whilst Figure 2 illustrates their morphologic characteristics.

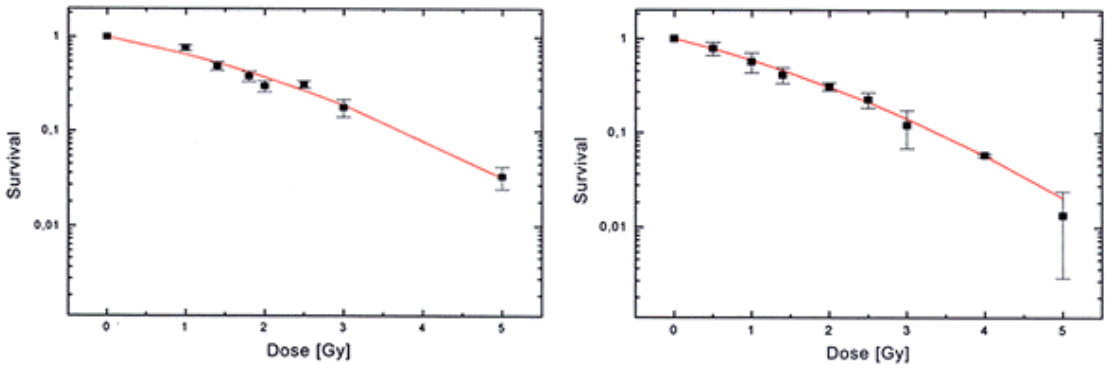

FIG 1. *Survival Curve from WM35 and M1/15 cell lines, respectively.*

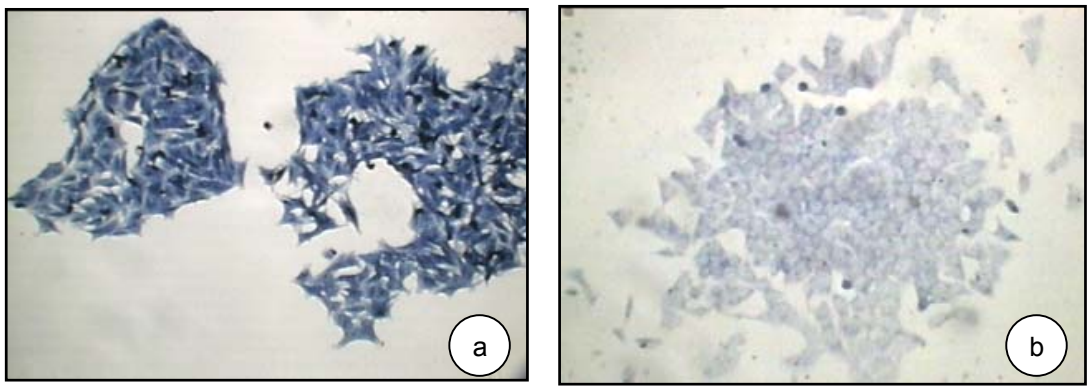

FIG 2. *ColoniesWM35 (2a) and M1/15 (2b). In both cases colonies show cohesive aggregation of cells (T.B. 100X).*

Table I. Radiobiological parameters of WM35 and M1/15 cell lines.

Cell line	α (Gy^{-1})	β (Gy^{-2})	α/β (Gy)	SF_{2Gy}	$Dose_{0,01}$ (Gy)
WM/35	0.37±0.07	0.060±0.020	6.07±1.50	0.52±0.09	6.0±0.5
M1/15	0.47±0.03	0.060±0.010	7.33±1.40	0.52±0.11	6.1±0.5

3.2. Morphological changes.

After irradiation with different Doses morphological changes were observed under optical microscopy. In both cell lines the radiation effects were evident even with low Doses. After irradiation with 2 Gy in the WM35 cell line colonies composed by polygonal cells with gigant nucleus and various nucleoli were predominant. An increase of chromatinic condensations compatible with nucleolus, and changes in cell size and progressive lost of cohesion capability among colony cells, were induced (Figure 3a and 3b). Similar changes were observed in the M1/15 cells (Figure 3c and 3d). Among 4 and 5 Gy the appearance of numerous multinucleate cells were observed, signaling alterations in the cytokinesis. No morphological change compatible with apoptosis induction could be observed, which was corroborated by the results from flow cytometry and DNA fragmentation. Special techniques to detect apoptosis (Apoptag) were negative in both cell lines. In fact both cell lines showed few morphological changes, compatible with its radioresistance.

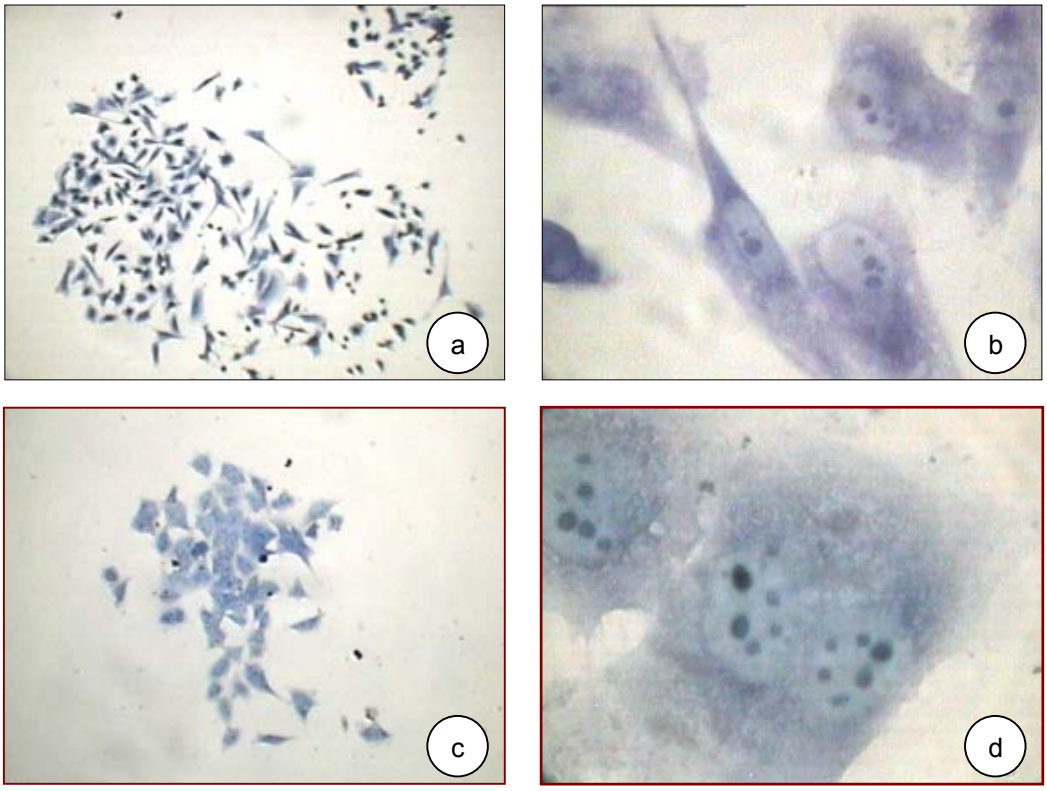

FIG 3. *Colonies from WM35 cells (3a), (3b) and M1/15 (3c), (3d) irradiated with 2 Gy (T.B. 100X and 400X). Both cell lines showed disperse cells and several nucleoli.*

3.3. Effect of irradiation on ROS generation in WM35 cells.

To compare the formation of intracellular H_2O_2 and SO_2 in control cells and after irradiation with 2 Gy, fluorescent intensities were measured (Table II). Irradiation significantly increased the SO_2 production while it decreased H_2O_2 levels as showed by the mean fluorescent intensities determined by flow cytometry. On the other hand, the treatment with Histamine 10 μM produced a significant increase in H_2O_2 level in control cells but it could not modify the modulation of ROS produced by ionizing radiation. The treatment with O-LM exhibited identical results (data not shown).

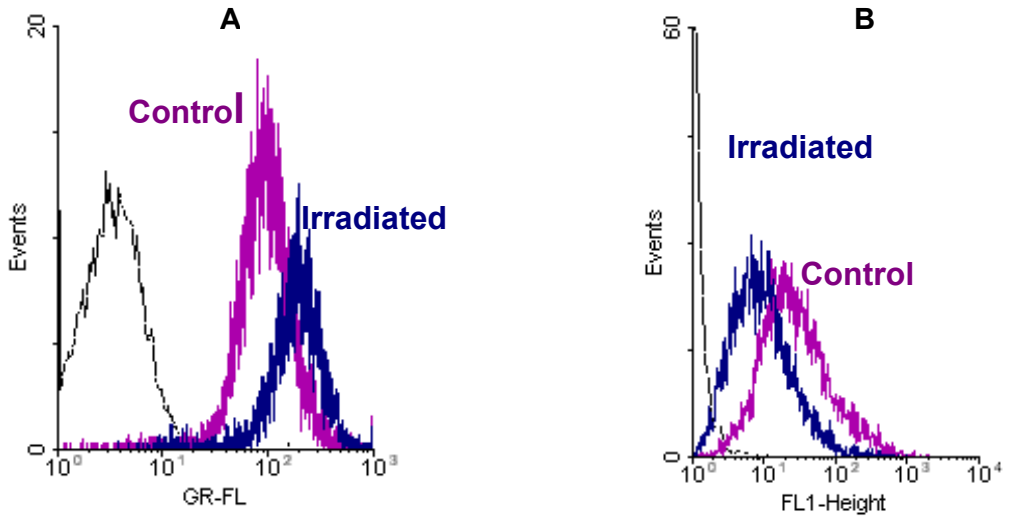

FIG 4. *Production of ROS in WM35 cells. Cells were stained with the peroxide-sensitive fluorescent dye Da or the superoxide specific HE for 20 min at 37ºC immediately after irradiation with 2 Gy or in control conditions. The fluorescence was analyzed by flow cytometry.* ***A.*** *Generation of Superoxide in control and in irradiated WM35 cells.* ***B****. Effect of irradiation on production of H_2O_2 in WM35 cells. In both graphics the first peak corresponds to the control of autofluorescence.*

Table II: ROS production in WM35 cells

	Non irradiated		**Irradiated**	
	Control	**Hi 10 μM**	**Control**	**Hi 10 μM**
Da mean fluorescence (H_2O_2)	60±24	164±14 [a]	16±0.6 [a]	7.2±2.2 [a, b]
HE mean flurescence (SO_2)	26±6	23±3	42±2 [a]	42±2 [a]

Table II: Cells were stained with 5μM Da or HE in PBS for 20 min at 37ºC. Resuspended cells were subjected to flow-cytrometric analysis. Irradiated cells received a dose of 2 Gy and were stained immediately after. [a] $p<0.01$ vs. control non irradiated, [b] $p<0.01$ vs. irradiated control.

4. Conclusions.

It is well known that radiotherapy constitutes an important alternative for the treatment of melanomas even when the great majority is radioresistent to radiations of low linear energy transference (LET), showing in consequence Survival Curves with ample shoulders [4, 14].

In this paper the results on the characterization of radiosensitivity parameters and morphological alterations obtained after irradiation with low LET radiations in two melanoma cell lines, WM35 and M1/15 are presented. The radiosensitivity parameter values are into the range reported for eucariot cells, including the human melanoma cell lines [15]. The SF_2 values obtained in our experiments clearly indicate that the two cell lines studied are radioresistant independently of their metastasic capacity. Furthermore, the radioresponsiveness of the cells was not modified for different treatments that produce significant inhibition on proliferation in both cell lines.

Considering the aggressive characteristic of this type of cancer the search for therapeutic alternatives is yet a challenge for the medicine. Already, many researches are addressed to the possible administration of drugs that, in addition to ionizing radiation, lead the melanoma cells to apoptosis [16]. The importance of certain genes possibly implicated in the radiosensitivity/radioresistence of these cells is also being widely reviewed [16, 17, 18].

Our results are in agreement with some reports which postulate that MnSOD sensitizes cells to oxidative stress, including ionizing radiation [19]. The decreased in H_2O_2 intracellular levels with a simultaneous increase in SO_2 will be part of a mechanism responsible for induction of cell radioresistance. This response triggered by irradiated cells could not be abrogated by different treatments like histamine or O-LM that in control cells are capable of modulating ROS production as well as inducing a significant inhibition of proliferation.

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