Cellular Viability and Apoptosis of Human Breast Cancer MDAMB-231 Cell Line After Co^{60} Irradiation

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Abstract

Co^{60} photon radiotherapy is indicated to the breast cancer treatment. The goal of the present research is to investigate the tumor cell line behavior after gamma irradiation. The human breast carcinoma MDAMB-231 cell line was exposed to a Co^{60} source at an unique fraction of 10Gy, 25Gy and 50Gy doses at 44cGy/min rate. Cellular viability was evaluated by MTT assay and apoptosis was observed by Propidium Iodide assay at 1h, 24h, 48h and 72h after Co^{60} irradiation. Cellular viability was evaluated by observing formazan's crystals formation and by measuring it's optical density at 595nm. Apoptotic cells stained by Propidium Iodide were observed by fluorescence microscopy. We observed that high dose of irradiation do not promote significant level of apoptosis in MDAMB-231 cell line when compared to apoptosis induced by 10% methanol. We showed that clinical dose used in treatment of human breast carcinoma did not reduce cellular viability in the same cell line. Indeed, viable cells were observed at level of 50Gy suggesting a radioresistent behavior.

Keywords: Apoptosis, breast cancer, gamma irradiation, MDAMB-231

CORRESPONDENCE TO

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Introduction

Breast cancer is a type of malignancy tumor frequently diagnosed in women and the one with higher mortality rate. This disease is most common in 45-65 year old women *(1,2)*. Breast cancer is a public health issue in South America, with about 70.000 cases and 30.000 deaths according to the World Health Organization database. Notably, Argentina has one of the highest incidences of breast cancer in the world, while in Brazil breast cancer is the second most frequent type of cancer *(3,4,5,6)*. In 1996 this carcinoma was the primary cause of cancer mortality among brazilian women *(7)*. The surgical method associated with radiotherapy and chemotherapy is the primary clinical intervention in patients without distant metastasis *(8)*. Radiotherapy is recommended as the standard treatment after breast conserving surgery using two tangential fields in 25-29 fractions of 2Gy up to a total dose of 50-58Gy, spread over a total treatment period of 33-39 days. Locoregional control is observed with 45Gy to 50Gy photons dose *(9,10,11)*.

Radiation effects on cells have been studied *in vitro* using several methodologies *(12)*. Exposure of cells to ionizing radiation leads to a variety of biological effects which include cell cycle arrest, transformation, cell killing. Stimulation of octamer factor DNA binding activity with 5Gy and 15Gy in MDAMB-231 human carcinoma cells and PC-3 prostate carcinoma cells was found *(13,14)*. For instance , it was shown in osteoblast culture that radiation induces alteration of factors related

to bone remodeling *(15)*. Irradiation of melanoma cells and squamous cell carcinoma with Co^{60} gamma rays at dose of 7Gy blocks cell cycle after 40-60 hours. Moreover, radiation resistance mechanisms were investigated in two bladder carcinoma cell lines after 2Gy Cesium source exposure *(16,17)*.

The relationship between tumor radiosensitivity and apoptosis has been evaluated in several tumors cell lines *(18)*. It was shown that caspase 8 inhibitor partially prevented apoptosis in Ataxia Telangiectasia cells and normal lymphocytes exposed to low doses of radiation. These results suggest that caspase 8 activation is involved in mediating radiation-induced apoptosis *(19)*. Cyclin B1 protein levels rapidly increase during low dose (2Gy) gamma radiation, inducing apoptosis in human Burkitt`s lymphoma line, promyelocytic leukemia cell and thymocytes. *(20)*.

In the current work, we investigated the effects of the clinical gamma radiation dose, by radiotherapy with a Co^{60} source, non fractionated, on MDAMB-231 human breast cancer cell line. We verified the cellular viability by MTT metabolisation assay, as well as apoptosis through Propidium Iodide method, after gamma irradiation and methanol apoptosis induction. The apoptotic nuclei were observed by fluorescence microscopy and apoptotic cells quantified.

Materials and Methods

Materials. MDAMB-231 cells, a human breast carcinoma cell line, was donated by Pharmacology Department of Instituto de Ciências Biológicas of Universidade Federal de Minas Gerais – ICB/UFMG. T-25 tissues culture flasks from Costar® and 96 wells microtiter plates from Corning® were used. PBS, RPMI-1640 medium, gentamicin, penicillin, streptomycin and MTT were obtained from Sigma Chemical Company. Fetal Bovine Serum was from Laborclin® and Propidium Iodide from Calbiochem Inc. Cell culture were maintained in RPMI-1640 medium supplemented with 10% FBS and antibiotics gentamicin (50µg/µL), penicillin (500U/mL) and streptomycin (500mg/mL) in T-25 tissues culture flasks in a humidified atmosphere containing 5% CO_2 at 37°C. The medium was changed every 2-3 days.

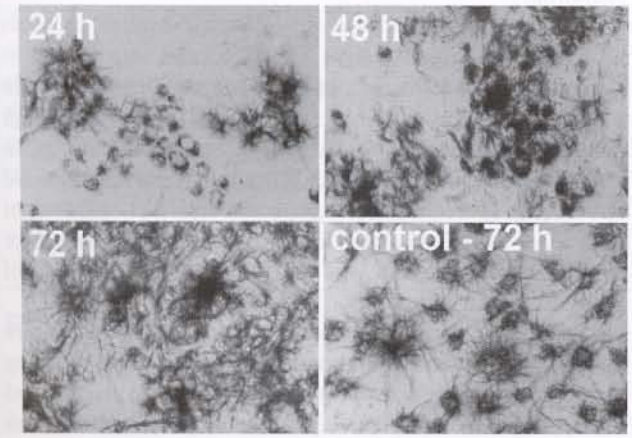
Co^{60} Irradiation. The irradiations were performed using a Co^{60} GammaCell-220 (Nordion International Inc., Canada), from Centro de Desenvolvimento da Tecnologia Nuclear–CDTN/CNEN, at 44cGy/min dose rate and radiotherapy equipment Theratron 80 (Atomic Energy Canada Limited), Instituto de Radioterapia São Francisco, at 190 cGy/min. dose rate. The cells were exposed to 10Gy, 25Gy and 50Gy at room temperature. After the exposure the cells were returned to the CO_2 incubator.

MTT assay. 10^4, 10^5 and 10^6 MDAMB-231 cells were loaded in 96 wells plate and irradiated by gamma-rays. After the irradiation the cells were maintained in 5% CO_2 at 37°C. At the appropriate times, MTT (5mg/mL) was added to the cells and incubated for 2h. This assay is based on the ability of cells to reduce 3-(4,5dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide to formazan, a purple crystal, that can be easily visualized in the viable cells, by optical microscopy. This assay allows quantification of cellular viability by addition of 10% SDS-HCl to the cells and further incubation at 37°C 5% CO_2 for 18h. The solubilized formazan crystals can be now quantified by measuring the optical density at 595nm in an ELISA reader.

Propidium Iodide assay. MDAMB-231 cells were incubated for 1, 24, 48 and 72h after gamma irradiation with 10Gy, 25Gy and 50Gy doses. At the indicated times, cells were washed with 5mL of phosphate buffer saline (PBS) and incubated for 5min at room temperature with 50µg/mL Propidium Iodide, a fluorescent dye that stains nuclei of cells. The cells were washed again and apoptotic nuclei were viewed by fluorescence microscope (Olympus I x 70). Photographic images were taken using Kodak films (Ektachrome, 100 ASA). Apoptotic cells quantification were done by counting nuclei in 8 fields, for each specific time and dose. Each field contained approximately 100 cells stained and the results were expressed as percentage of apoptotic cells per field. To compare apoptosis induction by irradiation and other inducement agent, MDAMB-231 cells were treated with RPMI medium supplemented with 10% methanol added on the cells and incubated in 5% CO_2 to 12h. This method was used as positive control to Propidium Iodide assay.

Results

MTT assay was used to study cellular viability after radiation of cell with Co^{60} gamma beam. Figure1, panel A shows the formazan's crystals formed by viable cells after 50Gy dose at the indicated times. A specific set of cells metabolizing MTT is observed at 24h and a gradual increase on cells metabolizing MTT is observed at 48h and 72h respectively. Interestingly, 72h after irradiation, the number of viable cells seems greater than in control non-irradiated cells. The same pattern of MTT metabolysis was observed when cells were irradiated with 10Gy and 25Gy (data not shown). Figure 1, panel B shows cellular viability of MDAMB-231 through optical density measured at 595nm after gamma irradiation. A progressive increase of viability is observed in the control cells. Similar levels of cellular viability are observed at 1, 24 and 48h after irradiation with 10Gy and 25Gy. At 50Gy dose an increase in cellular viability is already observed at 48h. However, the rate of MTT metabolism is larger in comparison with control at 72h.. This profile is also observed when cells were plated at 1 x 10^4 or 1x 10^5 cell density (data not shown).



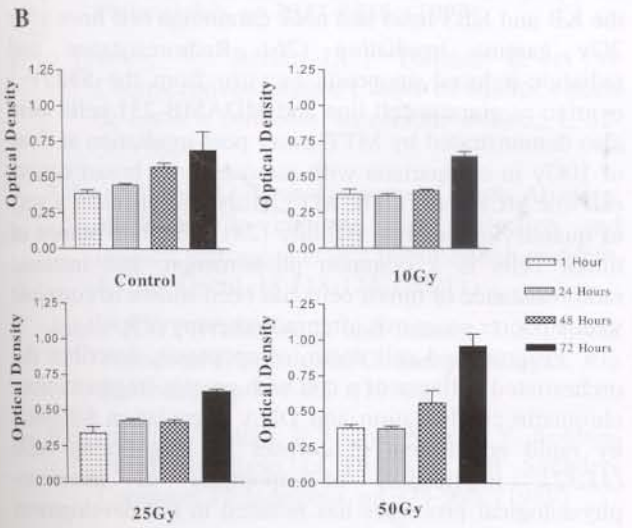


Figure 1. Gamma irradiation effects on cellular viability. 1x 10^6 MDAMB-231 cells were plated one day before gamma-irradiation with 10Gy, 25Gy and 50Gy. At the indicated times post irradiation, viable cells were evaluated by MTT assay. (A) Photomicrographs (X 400) shows formazan's crystals by MTT assay after 50Gy. Viable cells are observed in all times. At 72h the number of viable cells is greater than in non-irradiated cells control. (B) Quantification of cellular viability. Optical density measurement of soluble formazan crystals. Results represent average ± standard error (Mean ± SE) of quadruplicates from 3 experiments.

Morphology of MDAMB-231 cells after irradiation suggested the existence of processes equivalent to apoptosis. Figure 2 shows non-irradiated MDAMB-231 cells (left) and cells 24h after 50Gy irradiation (right) with presence of apoptotic bodies and vacuoles. Therefore, we decided to investigate apoptosis through nuclear staining by Propidium Iodide. Figure 3, panel A shows irradiated cells in phase contrast while panel B shows these cells stained by Propidium Iodide. Only apoptotic nuclei is observed. Panel C shows merged images with apoptotic and non-apoptotic cells. Apoptosis was observed in positive control induced by 10% methanol added to RPMI viewed in figure 3-D. The insert in figure 3-D shows nuclear detail with chromatin fragmentation, indicative of apoptosis process.

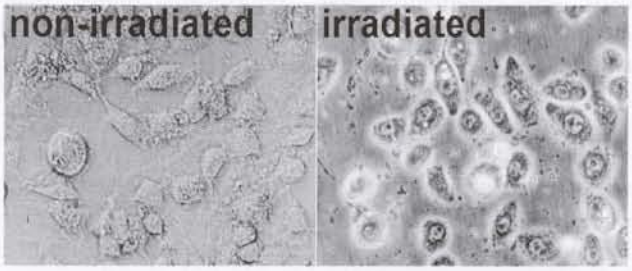


Figure 2. MDAMB-231 cellular morphology after irradiation. Photomicrographs (X 400) shows normal MDAMB-231 (left) and morphological alterations similar to apoptotic bodies (right) in phase contrast after 50Gy and 24h post irradiation.

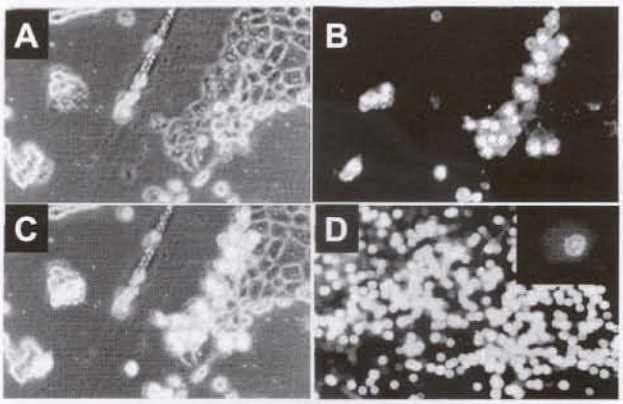


Figure 3. Propidium Iodide stained cells. Photomicrographs (X 400). (A) Shows cells after 50Gy irradiation in phase contrast. (B) shows the fluorescence field with apoptotic nuclei stained by Propidium Iodide. (C) shows the merged of these fields with apoptotic and non-apoptotic cells. (D) shows positive control of apoptosis induced by 10% methanol in fluorescence and an insert showing chromatin condensation .

We also quantified the percentage of apoptotic cells after some values of irradiation doses. Table 1 shows the percentage of apoptotic MDAMB-231 cells after gamma irradiation and after induction by 10% methanol viewed by fluorescence microscopy. The percentage of apoptotic cells after irradiation was significantly higher than control. However, these percentages were lower in comparison with apoptosis induced by 10% methanol, suggesting radioresistance. The percentage of apoptosis induced on the cells submitted to irradiation was significantly low when compared to apoptosis level in these cells treated with 10% methanol. These results are compatible with the MTT assay, which shows a higher level of viability at 72h suggesting radioresistance behavior.

Table 1: Apoptosis percentage quantified by the Propidium Iodide method.

Cell Treatment	1 h	24 h	48 h	72 h
None	0.4 ± 0.2	2.7 ± 1.7	11.1 ± 1.7	6.2 ± 2.4
10Gy	22.2 ± 4.3	26.4 ± 3.1	32.6 ± 4.2	20.5 ± 4.5
25Gy	39.1 ± 4.3	30.3 ± 2.4	38.7 ± 6.3	23.6 ± 6.3
50Gy	37.8 ± 3.1	33.7 ± 4.7	42.1 ± 8.8	29.0 ± 2.4
Methanol	-	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

Data show percentage of apoptosis of MDAMB-231 cells taken from Propidium Iodide assay after 10Gy, 25Gy and 50Gy, at the indicated times post irradiation. Eight fields with approximately 100 stained cells were selected and apoptotic and non-apoptotic cells were counted. The number of apoptotic cells were normalized by the total number of counted cells. Methanol results were measured after 12h. Results represent average ± standard error (Mean ± SE) from different experiments.

Discussion

The breast carcinoma is the most common malignancy in women with an incidence of 60 per 100.000 per year. The higher incidence is between subjects with 45 and 70 year old. Unfortunately, the clinical relevance of early diagnosis of metastatic breast carcinoma is currently limited by paucity of therapeutic options for this disease. Factors related to intrinsic radioresistance or hypoxia suggest that large radiotherapy fractions may be more effective in a subset of tumors *(21,22,23)*. Breast cancer research has been developed over the last decades. Recent discoveries promise to provide individualized treatment options, increased long-term survival for women with breast cancer, and the possibility of moving toward curative intent in the treatment of advanced breast cancer *(24)*. In this paper we investigated the effects of gamma irradiation in human breast carcinoma cell line. The MDAMB-231 cell line was selected due to the well known low response to radiation.

Our results shows radioresistance in high dose levels at 10Gy, 25Gy and 50Gy. The clinical control dose to human breast cancer did not decrease cellular viability in MDAMB-231 cell line. An unique 50Gy fraction at high dose should be enough to induce apoptosis similar to positive control 10% methanol treatment but this fact did not occur. Moreover, the apoptosis data did not present statistical variations with dose at indicated times. Tumor dose control in human was not found *in vitro* at level of 50Gy to induce apoptosis.

Several studies demonstrated radiation effects in cellular response. Clonogenic repopulation during fractionated radiotherapy using 3Gy fraction in 24h and 48h intervals on human squamous cell carcinoma was found similar under ambient conditions *(25)*. Construtive NF-κB level influences the intrinsic radiosensitivity of the KB and KB3 head and neck carcinoma cell lines after 2Gy gamma irradiation *(26)*. Radioresistance and radiation-induced apoptosis *in vitro* from the SKOV-3 ovarian carcinoma cell line and MDAMB-231 cells were also demonstrated by MTT assay post irradiation at dose of 10Gy in comparison with radiosentivity breast cancer cell line MCF-7 *(27)*. The MTT assay also provides a way of quantifying cellular viability *(28)*. Radioresistance of tumor cells is a common phenomenon, and intrinsic radioresistance of tumor cells has been shown to correlate with a poorer prognosis after radiotherapy *(29,30)*.

Programmed cell death, or apoptosis, describes the orchestrated collapse of a cell with protein fragmentation, chromatin condensation and DNA degradation followed by rapid engulfment of corpses by neighboring cells *(31,32)*. Implication of apoptosis in numerous physiological processes has resulted in the development of numerous methods to detect apoptosis. Propidium Iodide is an easy technique able to evidence apoptotic cells *(33,34,35,36)*.

The present work suggests the presence of a radioresistance behavior on the irradiated MDAMB-231 cell line at the level of clinical radiotherapy dose control. Further studies are necessary to understand the mechanisms of radioresistance in human breast carcinoma cell lines to contribute to breast cancer treatment.

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