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Cell cycle arrest, extracellular matrix changes and intrinsic apoptosis in human melanoma cells are induced by Boron Neutron Capture Therapy

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ABSTRACT

Boron Neutron Capture Therapy (BNCT) involves the selective accumulation of boron carriers in tumor tissue followed by irradiation with a thermal or epithermal neutron beam. This therapy is therefore a cellular irradiation suited to treat tumors that have infiltrated into healthy tissues. BNCT has been used clinically to treat patients with cutaneous melanomas which have a high mortality. Human normal melanocytes and melanoma cells were treated with BNCT at different boronophenylalanine concentrations for signaling pathways analysis. BNCT induced few morphological alterations in normal melanocytes, with a negligible increase in free radical production. Melanoma cells treated with BNCT showed significant extracellular matrix (ECM) changes and a significant cyclin D1 decrease, suggesting cell death by necrosis and apoptosis and cell cycle arrest, respectively. BNCT also induced a significant increase in cleaved caspase-3 and a decrease in the mitochondrial electrical potential with selectivity for melanoma cells. Normal melanocytes had no significant differences due to BNCT treatment, confirming the data from the literature regarding the selectivity of BNCT. The results from this study suggest that some signaling pathways are involved in human melanoma treatment by BNCT, such as cell cycle arrest, ECM changes and intrinsic apoptosis.

Significance

Metastatic melanoma remains a highly lethal disease, with an incidence that continues to increase faster than any other cancer and almost adjuvant treatments fail to control this malignancy. Boron Neutron Capture Therapy was used is this work with selective treatment for melanoma cells with minimum effects in normal cells. This therapy induces cell death by apoptosis and cell cycle arrest only in melanoma cells.

1. Introduction

Boron Neutron Capture Therapy (BNCT) is a binary treatment modality that involves the selective accumulation of boron carriers in a tumor, followed by irradiation with a thermal or epithermal neutron beam (Monti Hughes et al., 2011). The neutron capture reaction with a boron-10 (^{10}B) nucleus yields high linear energy transfer (LET) particles, alpha and ^{7}Li, with a range of 5–9 μm, thus BNCT is a putative cell-selective radiation therapy with the potential to control local recurrences of malignant tumors (Nakamura et al., 2011). These particles can only travel very short distances and, as such, release their damaging energy directly to the tissue that contains the boron compound. Cell death is triggered by the release of these charged particles, which create ionisation tracks along their trajectories, thereby resulting in cellular damage (Toppino et al., 2013).

BNCT has two advantages. Firstly, the dose of radiation given in the neutron beam can be quite low; secondly, the local decay and action allow the surrounding healthy tissue to be spared damage due to radiation (Barth et al., 2005).

BNCT has been used clinically to treat patients with cutaneous melanomas (Mishima, 1996). These patients were either not candidates for, or had declined, conventional therapy (Barth et al., 2004). Melanoma is the most aggressive skin cancer and frequently involves distant and locoregional spread, usually with no efficient

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treatment (Menéndez et al., 2009). Metastatic melanoma remains a highly lethal disease, with an incidence that continues to increase faster than any other cancer (González et al., 2004). Almost all adjuvant treatments fail to control this malignancy (Pawlik and Sondak, 2003).

BNCT has a strong local radiotherapy effect. The efficacy of the method in cancer therapy requires sufficient accumulation of boron into the tumor and an irradiation in tumor location (Joensuu et al., 2011). Only cells that have 10-boron are damaged by thermal neutrons. So, this therapy is a cellular radiation suited to treat local tumors or those infiltrate near healthy tissues (Esposito et al., 2008).

BNCT could be an attractive tool to improve response over the standard radiotherapy treatment delivering high dose to tumor while reducing normal tissue effect, due to the different boron uptake in normal and tumor cells (Menéndez et al., 2009).

There are no published results about the BNCT effect on normal melanocytes compared to melanoma cells, and these data are extremely important to know the effectiveness of BNCT versus the side effects incidence in healthy tissues. There is also no data about signaling pathways involved in the melanoma treatment. The aim of this study was to evaluate the selectivity and signaling pathways involved in melanocytes and melanoma treatment with BNCT.

2. Materials and methods

2.1. Cell lines

A human melanoma tumor cell line (SK-MEL-28) was cultivated in 75 cm^2 flasks with RPMI-1640 (Cultilab) medium supplemented with 10% inactivated fetal bovine serum (Cultilab), 2 mM L-glutamine (Sigma Chemical Company) and 0.1 g/mL streptomycin (FontouraWyeth AS). A human primary culture of melanocytes isolated from foreskin was cultivated with 254CF medium (Life Sciences®), supplemented with 10% HMGS growth factors (Life Sciences) and 0.1 mg/mL streptomycin (FontouraWyeth AS) as previously described (Fernandez et al., 2005).

Adherent cell suspensions were propagated by treatment of the culture flasks with 0.2% trypsin and then inactivated in 10% fetal bovine serum (FBS)-enriched medium. The nonadherent cells were centrifuged twice, resuspended in medium and then seeded in plates and allowed to grow for 24 h.

2.2. Boronophenylalanine (BPA)

^{10}B-enriched (>99%) BPA was purchased from KatChem and converted to a fructose 1:1 complex to increase its solubility (Coderre et al., 1994).

2.3. Cells treatment and BNCT irradiation for MTT and lipid peroxidation tests

Melanocytes were seeded in 96-well plates at concentration of 10^5 cells/mL and allowed to grow for 24 h. They were then treated with different concentrations of BPA, from 40 to 0.52 mg/mL, which corresponds to 2100–27.5 μg ^{10}B/mL for MTT assay and from 8.32 to 0.52 mg/mL, which corresponds to 440–27.5 μg ^{10}B/mL for lipid peroxidation test. After incubation with BPA for 90 min, the cells were irradiated at the BNCT research facility at the Nuclear and Energetic Research Institute (IPEN, Brazil) Coelho et al., 2002 for 120 min using the IEA-R1 nuclear reactor operating at a power of 3.5 MW. The thermal neutron flux, epithermal neutron flux and fast neutron flux at the irradiation position were $(2.31 \pm 0.03) \times 10^8$, $(4.60 \pm 0.10) \times 10^6$ and $(3.50 \pm 0.10) \times 10^7$ n/cm^2 s, respectively. The gamma dose rate in air at the irradiation site was 3.50 ± 0.80 Gyh^{-1}. Before irradiation, the BPA-enriched incubation medium was removed and the cells were washed in 0.9% saline solution. Another cell group was irradiated without BPA (beam only) and was designated as the "irradiated control". A non-irradiated and BPA-free group was also studied and was designated as the "control". Images of the control and treated cells were recorded by a camera (Sony Cyber-shot 7.2 mega pixels) coupled to an optic inverted microscope (Carl Zeiss), magnified by 40×.

2.4. Cells treatment and BNCT irradiation for soluble collagen quantification and flow cytometry tests

Melanocytes and SK-MEL-28 melanoma cells were seeded in 24-well plates at a concentration of 10^5 cells/mL and allowed to grow for 24 h. SK-MEL-28 melanoma cells were treated with 3.7 mg/mL BPA in all flow cytometry tests (this value is equivalent to 192.0 μg ^{10}B/mL), which corresponds to the inhibitory concentration of 50% (IC_{50}) for this compound in this cell line (Faião-Flores et al., 2011a). Melanocytes were treated with 34.4 mg/mL BPA in all flow cytometry tests (this value is equivalent to 1.8 mg ^{10}B/mL), which corresponds to the IC_{50} for this compound in this cell line. After 90 min of incubation with BPA, the cells were irradiated at the BNCT research facility at the Nuclear and Energetic Research Institute (IPEN, Brazil) Coelho et al., 2002 for 30 min, using the IEA-R1 nuclear reactor operating at a power of 3.5 MW. The analysis was performed 6 h after BNCT treatment. The thermal neutron flux, epithermal neutron flux and fast neutron flux at the irradiation position were $(2.31 \pm 0.03) \times 10^8$, $(4.60 \pm 0.10) \times 10^6$ and $(3.50 \pm 0.10) \times 10^7$ n/cm^2 s, respectively. The gamma dose rate in air at the irradiation site was 3.50 ± 0.80 Gy h^{-1}. Before irradiation, the BPA-enriched incubation medium was removed and the cells were washed in 0.9% saline solution. Another cell group was irradiated without BPA (beam only) and was designated as the "irradiated control". A non-irradiated and without BPA group was also studied and was designated as the "control".

2.5. Cellular viability assay – MTT

The cellular viability of the melanocytes was determined using a colorimetric methodology known as MTT (3-(4,5-dimethylthiazol-2-y1)2,5-diphenyl tetrazolium bromide) (Sigma) Mosmann, 1983. MTT is reduced in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. The color is then quantified by spectrophotometry. After irradiation, the culture medium was removed for lipid peroxidation (LPO) analysis and 10 μL MTT reagent (5 mg/mL) (Sigma–Aldrich Corp.) was added to each well. The plates were incubated at 37 °C for 3 h, protected from light. Blue formazan crystals thus formed were pelleted to the bottom of the well by centrifugation, separated from the supernatant and dissolved in 150 μl of dimethylsulfoxymide. The optical density at 540 nm was determined by absorbance spectrometry using a microplate reader. A linear relationship between cell number and absorbance was established, enabling an accurate, straightforward quantification of changes in proliferation. The mean values of several experiments were presented in a linear regression model, and the IC_{50} was calculated.

2.6. Lipid peroxidation (LPO)

The oxidative stress on unsaturated lipids in cell membranes was evaluated by determining the amount of malondialdehyde (MDA), which is the final product of fatty-acid peroxidation that reacts with thiobarbituric acid (TBA) to form a colored complex. Thiobarbituric acid reactive substances (TBARS) were quantified