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Boron neutron capture therapy induces cell cycle arrest and DNA fragmentation in murine melanoma cells

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ABSTRACT

The melanoma is a highly lethal skin tumor, with a high incidence. Boron Neutron Capture Therapy (BNCT) is a radiotherapy which combines Boron with thermal neutrons, constituting a binary system.

B16F10 melanoma and L929 fibroblasts were treated with Boronophenylalanine and irradiated with thermal neutron flux. The electric potential of mitochondrial membrane, cyclin D1 and caspase-3 markers were analyzed.

BNCT induced a cell death increase and cyclin D1 amount decreased only in B16F10 melanoma. Besides, there was not caspase-3 phosphorylation.

1. Introduction

Malignant melanoma is the most aggressive form of cancer, which causes the majority of deaths from skin cancers. Although it represents less than 10% of skin cancers, it is responsible for more than 75% of skin cancer related deaths.

While the incidence of many solid tumors is gradually decreasing, the incidence of cutaneous melanoma is still on the rise, making this malignancy a significant clinical problem. Overall, melanoma accounts for 1%–3% of all malignant tumors and is increasing in incidence by 6%–7% each year (Bevona and Sober, 2002).

The prognosis of malignant melanoma is strongly related to the stage at which it is detected. Early stages of melanoma are, in majority of the cases, successfully treated with surgery, but advanced disease is generally refractory to conventional chemotherapy (Mendes et al., 2008).

Boron Neutron Capture Therapy (BNCT) is a radiotherapy procedure where the efficacy depends upon the deposition of ^{10}B in tumor cells. When these cells are irradiated with thermal neutrons a capture nuclear reaction is induced in ^{10}B converting it into ^{11}B, which decays by emission of highly ionizing alpha particles, quite toxic to cells. This therapy essentially combines a radiosensitizer, boron (^{10}B), with a nonionizing radiation, thermal neutrons, constituting a binary system. An advantage of a binary system would be that each component can be manipulated independently to maximize selectivity (Barth et al., 1992).

Once the melanoma takes up these ^{10}B compounds, thermal neutrons, which cause insignificant cell damage, are easily absorbed by nonradioactive ^{10}B, inducing the ^{10}B(n, alpha)^{7}Li reaction and releasing the high linear energy transfer (LET) particles to 14 μm melanoma cell diameter, destroying the tumor without damaging surrounding tissue (Mishima et al., 1989).

The radiation can kill the cell by apoptosis or mitotic catastrophe. This type of death results from DNA damage unrepaired (Dagrosa et al., 2008). The mechanisms that play a role in the tumor damage as produced by BNCT still are unknown. The aim of this paper is to find molecular mechanisms such as reduction of mitosis and tumor proliferation, as well as to suggest cell death pathways using BNCT treatment.

2. Materials and methods

2.1. Cell lines

Tumor cell lines of murine melanoma (B16F10) and normal fibroblasts (L929) were cultivated in flasks of 75 cm^2 in RPMI-1640 medium supplemented with 10% inactive fetal bovine serum (SFB), 2 mM of L-glutamine and antibiotics streptomycin 0.1 mg/mL.

Adherent cells suspensions were obtained by treatment of the culture flasks with trypsin 0.2% for 5 min and then inactivated in SFB. The nonadherent cells were centrifuged twice, next resuspended in supplemented RPMI-1640 medium, and cell concentration was adjusted to 5×10^5 cells/mL in plates with 96 wells for 24 h.

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2.2. Boronophenylalanine (BPA)

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

2.3. Cells treatment and irradiation

Tumor and normal cells were treated with 331 μg/mL of BPA in all experiments (this value is equivalent to 17 μg ^{10}B/mL), corresponding to 10% of the Inhibitory Concentration ($IC_{50\%}$) for this compound in these cell lines (data found by our research group), and after 90 min (the best time of boron-10 uptake for these cells) they were irradiated during 30 min, using the IEA-R1 nuclear reactor operating in the potency of 3.5 MW, at the Nuclear and Energetic Research Institute (IPEN, Brazil) facilities. The thermal neutron flux, epithermal neutron flux and fast neutron flux at the position was 2.3×10^8, 4.6×10^6 and 3.5×10^7 n/cm^{2}s, respectively. The gamma dose rate in air at the irradiation location was 4.2 ± 0.2 Gy h^{-1}. The control group was defined as cells without irradiation and without incubation with BPA compound. The culture medium with boron was removed before irradiation. Another cell group was irradiated without BPA in the same condition.

2.4. Assessment of mitochondrial membrane potential

Rhodamine 123 is a cationic lipophilic fluorescent chemical that accumulates specifically in mitochondria of living cells (Kennady et al., 2004). Then, this agent binds only in active metabolically mitochondria, resulting fluorescence emission.

After treatment by BNCT, the medium culture was removed and the adherent cells were trypsinized. B16F10 and L929 cells were pelleted by centrifugation at 1800 rpm for 10 min and resuspended in 5 μL of rhodamine 123 (5 mg/mL) for 30 min at room temperature, washed with phosphate-buffered-saline (PBS) and resuspended in Facs flow buffer. The samples were analyzed for fluorescence (FL-1 H detector) on a Becton Dickinson FACScan flow cytometer using the Cell Quest software.

2.5. Cell cycle phases analysis

After treatment by BNCT, the medium culture was removed and the adherent cells trypsinized. B16F10 and L929 cells were pelleted by centrifugation at 1800 rpm for 10 mins and washed in cold solution of PBS, fixed in 70% cold ethanol and stored at −20 °C. Before analysis, cells were washed in PBS, treated with 50 μg/mL RNase A and incubated with 50 μg/mL propidium iodide (PI) for at least 1 hour in the dark at 4 °C. The samples were analyzed for fluorescence (FL-2 H detector) on a Becton Dickinson FACScan flow cytometer using the Cell Quest software.

2.6. Cell cycle progression analysis by cyclin D1 quantification

The type D cyclins (with their partner CDKs) form a regulatory unit of the G1/S transition that is frequently impaired in neoplasia (Li et al., 2006).

After treatment by BNCT, the medium culture was removed and the adherent cells trypsinized. B16F10 and L929 cells were pelleted by centrifugation at 1800 rpm for 10 minutes and incubated with 1 μg of specific anti-cyclin D1 antibody (Santa Cruz, USA) and 10 μL of Triton X-100 (0.1%) for 1 h at 4 °C. After that, the cells were resuspended in Facs flow buffer. The samples were analyzed for fluorescence (FL-1H detector) on a Becton Dickinson FACScan flow cytometer using the Cell Quest software.

2.7. Caspase-3 activity

Caspases represent a family of cysteine proteases, which are common downstream effectors of apoptosis (Chen et al., 2001).

After treatment by BNCT, the medium culture was removed and the adherent cells trypsinized. B16F10 and L929 cells were pelleted by centrifugation at 1800 rpm for 10 min and incubated with 1 μg of specific anti-caspase-3 PE antibody (Santa Cruz, USA) and 10 μL of Triton X-100 (0.1%) for 1 h at 4 °C. After that, the cells were resuspended in Facs flow buffer. The samples were analyzed for fluorescence (FL-2 H detector) on a Becton Dickinson FACScan flow cytometer using the Cell Quest software.

2.8. Statistical analysis

The values are expressed as mean ± standard deviation. The data were analyzed using one-way analysis of variance (ANOVA) and significant mean differences were determined using multiple comparisons by the TUKEY-KRAMER test at the $p<0.05$ level.

3. Results and discussion

3.1. Mitochondrial potential decrease by BNCT

BNCT induced decrease of mitochondrial potential causing cell death in B16F10 murine melanoma. The more rhodamine is caught

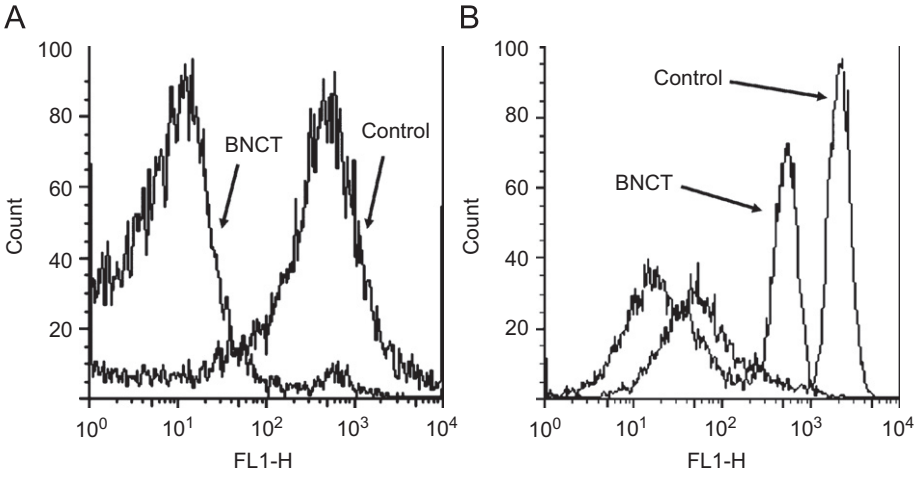

Fig. 1. Representative overlaps of fluorescence intensity from B16F10 (A) and L929 (B) cell populations treated by BNCT in comparison to control group.

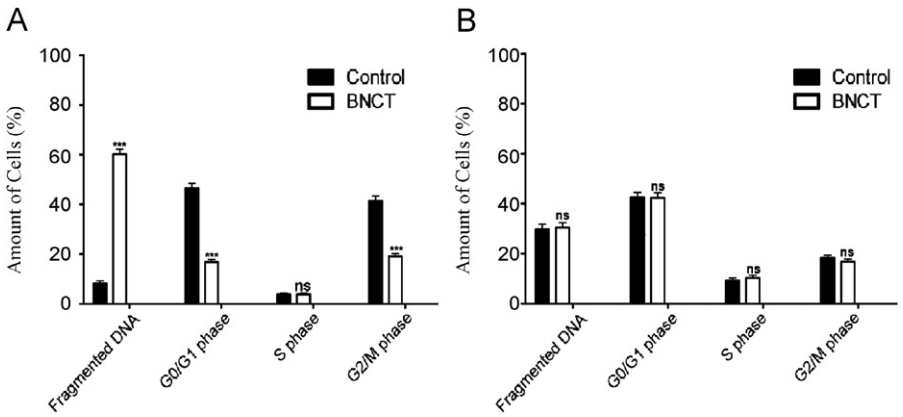

Fig. 2. Cell cycle phases distribution in B16F10 (A) and L929 cells (B), both treated by BNCT, plus the control group in each case.

Table 1
Comparison among percentage values of active/inactive mitochondria, viable/inviable cells (rhodamine 123) and positive/negative marker (cyclin D1 and caspase-3) of BNCT and control.

	B16F10				**L929**			
	Active/viable		Inactive/inviable		Active/viable		Inactive/inviable	
	Control	BNCT	Control	BNCT	Control	BNCT	Control	BNCT
Rhodamine 123	70.0	5.1	30.0	94.9	54.1	47.0	45.9	53.0
	Positive		**Negative**		**Positive**		**Negative**	
	Control	BNCT	Control	BNCT	Control	BNCT	Control	BNCT
Cyclin D1	14.3	3.1	85.7	96.9	17.3	19.4	82.7	80.6
Caspase-3	15.4	15.5	84.6	84.5	0.2	0.5	99.8	99.5

the higher is the fluorescence emission, and consequently the higher is the amount of living cells with active mitochondria. The electric potential in B16F10 murine melanoma was significantly reduced, while in L929 murine normal fibroblasts this effect was not observed. The L929 cell line remained without effects causing damages toward death (Fig. 1). The mitochondrial potential is reduced when the energetic metabolism is breached during cell death (Fuller and Arriaga, 2003).

3.2. Cell cycle arrest by decrease of cyclin D1 and cell death by DNA fragmentation

Dysregulation of the cell cycle in conjunction with other signaling pathway aberrations seems to play a role in therapeutic resistance (Smalley et al., 2008). BNCT treatment reduced the preparation for proliferation phase (G0/G1) and mitosis (G2/M). This was proven by the reduction of cyclin D1, one of the markers of the G1 proliferation phase. Moreover, BNCT increased the amount of fragmented DNA in melanoma cells, without affecting normal cells (Fig. 2).

3.3. Cell death induction with caspase-3 absence

DNA fragmentation in B16F10 melanoma was extremely significant, but death of these cells was not occurring by means of intrinsic apoptosis, because the increase of phosphorylated caspase-3 did not take place. In L929 cells this effect was not found too. These findings suggest that the cell death should be of another type, independently from direct phosphorylation of caspase-3 as e.g. necrosis or anoikis (Dagrosa et al., 2008).

It is shown in Table 1 all data for rhodamine 123, cyclin D1 and phosphorylated caspase-3 with BNCT treatment and control groups.

4. Conclusions

The findings of this study suggest that BNCT works by means of cell cycle arrest decreasing the amount of cyclin D1 in melanoma cells, without affecting normal cells. Additionally, we observed that cell quantities in the G0/G1 and G2/M phases decreased, with a significant increase in the DNA fragmented cells quantity.

Moreover, cell death does not occur by activation of caspase-3, indicating that this is accomplished by other types of cell death like necrosis, autophagy or anoikis.

All experiments were performed with a group exposed only to neutron irradiation without BPA. The results were not significant in any of the experiment and for this reason these data were not included.

Conflict of interest

None.

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