Effect of Epithermal Neutrons on Viability of Glioblastoma Tumor Cells *in Vitro*

L. A. Mostovich, N. V. Gubanova*, O. S. Kutsenko, V. I. Aleinik, A. S. Kuznetsov**, A. N. Makarov**, I. N. Sorokin**, S. Yu. Taskaev**, G. I. Nepomnyashchikh***, and E. V. Grigor'eva**

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We studied *in vitro* effect of epithermal neutrons in various doses on viability of glioblastoma U87 tumor cells. Increasing the dose from 1.9 to 4.1 Sv promoted cell death. Cytofluorimetric analysis revealed no activation of apoptosis in the irradiated cells, which attested to necrotic death of the tumor cells exposed to epithermal neutron radiation.

Key Words: *glioblastoma; proliferation; apoptosis; boron neutron capture therapy; cytofluorimetric analysis*

Boron neutron capture therapy (BNCT) is a promising method of the therapy of diffuse tumors of various localities. The method is based on introduction of boron isotope ^{10}B into the tumor followed by irradiation of this tumor with external source of epithermal neutrons. This irradiation is characterized by lower energy in comparison with other types of radiation used in medicine and produces less damage to the adjacent tissues. However, capture of a neutron by ^{10}B atom results in its decay into ^{7}Li and α-particle ^{2}He. These high-energy particles destroy tumor cells. Therefore, BNCT should selectively kill tumor cells with minimal damage to adjacent normal tissue [11].

Clinical studies of the efficiency of BNCT in the treatment of malignant neoplasms and multiple distant metastases [4,8,9,10] went ahead the fundamental research in this field. BNCT is a promising tool for the treatment of glioblastomas and various diffuse tumors, but it is still at the stage of experimental testing [7,10]. In many respects, it is related to the lack of fundamental data on the effects of the priming epithermal neutron irradiation itself and molecular mechanisms of its action on biological objects both *in vivo* and *in vitro*. Dual irradiation employed within BNCT (by epithermal neurons and α-particles) was shown to affect viability of carcinoma cells in the oral cavity and their potency to form colonies predominantly due to cell cycle arrest in G1-phase and triggering apoptosis [5], the effect being dependent on mutations in gene-suppressor of *p53* tumor [3].

Our aim was to examine the effect of priming radiation with epithermal neutrons on viability of tumor cells of human glioblastoma *in vitro*. In experiments we used the accelerator-based source of epithermal neutrons developed in Institute of Nuclear Physics (SD RAS) [1,2].

MATERIALS AND METHODS

The study was carried out on human glioblastoma U87 tumor cells and normal mouse fibroblasts. The cells were cultured in a 6-well plastic plate in

Institute of Molecular Biology and Biophysics, Siberian Division of the Russian Academy of Medical Sciences; *Institute of Cytology and Genetics, Siberian Division of the Russian Academy of Sciences; **Institute of Nuclear Physics, Siberian Division of the Russian Academy of Sciences; ***Institute of Regional Pathology and Pathomorphology, Siberian Division of the Russian Academy of Medical Sciences, Novosibirsk, Russia. ***Address for correspondence:*** mostovichl@gmail.ru. L. A. Mostovich

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IMDM+glutamine+penicillin/streptomycin+10% FCS (Sigma). Cells of each type were subdivided into control (not irradiated) and experimental (irradiated) subgroups. The experimental plates were taken from the incubator to be exposed to various doses of epithermal neutrons (1.9, 2.9, 3.0, and 4.1 Sv) for 2-3 h. The control plates with the same cells were also taken away from the incubator for the time equal to the radiation exposure. Then the control and experimental cells were returned to the incubator. The cells were daily examined under an "Axiovert 40" (Carl Zeiss) light microscope equipped with a digital camera (Canon).

Viability of normal and tumor cells was assessed by specific staining with trypan blue. The analysis is based on the fact that dead cells lose membrane integrity and become permeable for the dye, while live cells remain unstained. Seventy-two hours after irradiation, the control and experimental cells of both types were routinely harvested from plates with trypsin/EDTA (Sigma) and washed two times in phosphate buffered saline. An aliquot of cell suspension was stained with 0.1% trypan blue and stained (dead) and live (unstained) cells were counted in a Goryaev chamber under a light microscope. The remaining cells were fixed in 70% ethanol and stored at -20°C.

Apoptotic death of damaged cells was analyzed by flow cytofluorimetry with propidium iodide (PI), which made it possible to assess quantitatively the content of apoptotic cells in a sample. For cytofluorometric analysis of the cell cycle and apoptosis activation, the cells were washed in phosphate buffered saline and stained with PI (25 μg/ml) for 20 min. Stained cells were analyzed in a BD FACSCanto II flow cytofluorimeter. The phases of cell cycle and the percentage of the apoptotic cells were determined using FACS Diva software.

RESULTS

Evaluation of the effect of epithermal neutrons on viability of tumor and normal cells exposed to various irradiation doses (Fig. 1) revealed morphological changes in U87 cells with increasing the irradiation dose: irradiated cells retained the monolayer type of cell growth for a long period (Fig. 1, *a*), while in

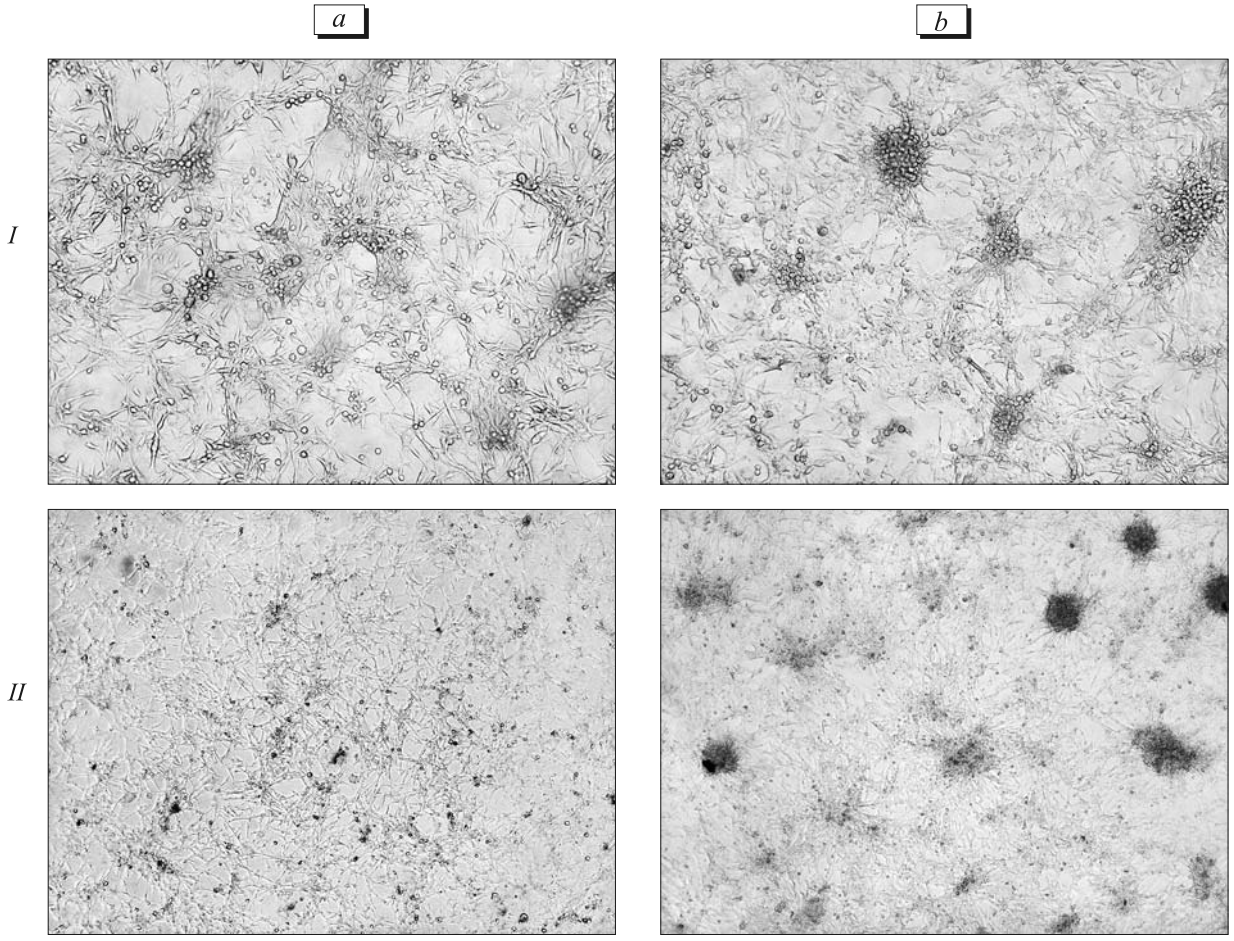

Fig. 1. Effect of epithermal neutron irradiation at the doses of 2.9 Sv (*I*) and 4.1 Sv (*II*) on glioblastoma U87 tumor cells, ×20. *a*) irradiated tumor cells; *b*) control (not irradiated) tumor cells.

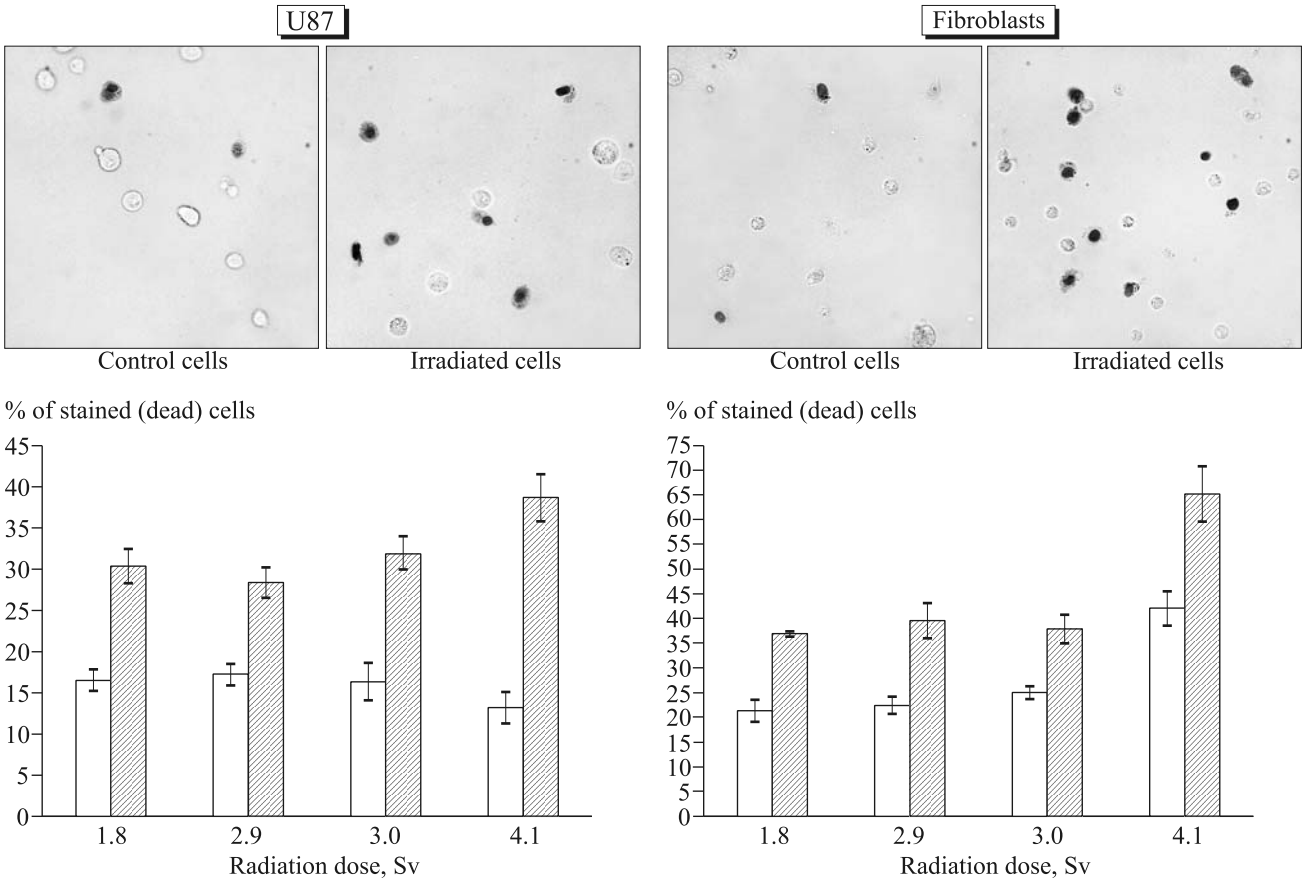

Fig. 2. Effect of epithermal neutron irradiation on cell death, ×30. Dead cells were counted 72 h postradiation by trypan blue staining. Light bars: control cells; shaded bars: irradiated cells.

control (not irradiated) U87 cells, disruption of cell monolayer and formation of spherical cell clusters characteristic of this cell line were seen (Fig. 1, *b*). The density of irradiated tumor cells in the plates was significantly lower in comparison with that of control tumor cells, which indirectly attests to accelerated death of tumor cells exposed to epithermal neutrons. To test this hypothesis, the tumor and normal cells were exposed to various doses of neutron radiation and 72 h after irradiation the content of live and dead cells was determined (Fig. 2).

Analysis of viability of tumor cells after irradiation revealed intensification of cell death with increasing the irradiation dose. Promotion of cell death by neutron radiation is demonstrated in Figure 2 for a dose of 3 Sv, which increased the content of dead tumor cells from the control value 28% to 36%. The corresponding values for normal (fibroblast) cells were 38 and 63%. These data showed that the effect of neutron irradiation at the used dosage is not therapeutically efficient, because the death of even 38% tumor cells is far from being acceptable. Thus, this study

TABLE 1. Effect of Epithermal Neutron Radiation on the Death of Glioblastoma U87 Cells (*M*±*m*)

Experimental conditions	Irradiation dose			Type of cell death, %
	1.9 Sv	2.9 Sv	4.1 Sv	
Control	16.5±1.3	17.3±1.2	13.2±1.9	Total cell death (trypan blue)
	5.1±0.8	9.9±0.9	10.2±1.7	Apoptosis (FACS, PI)
Irradiated cells	30.4±2.2	28.4±1.9	38.7±2.8	Total cell death (trypan blue)
	6.2±1.6	14.1±0.9	11.8±0.7	Apoptosis (FACS, PI)

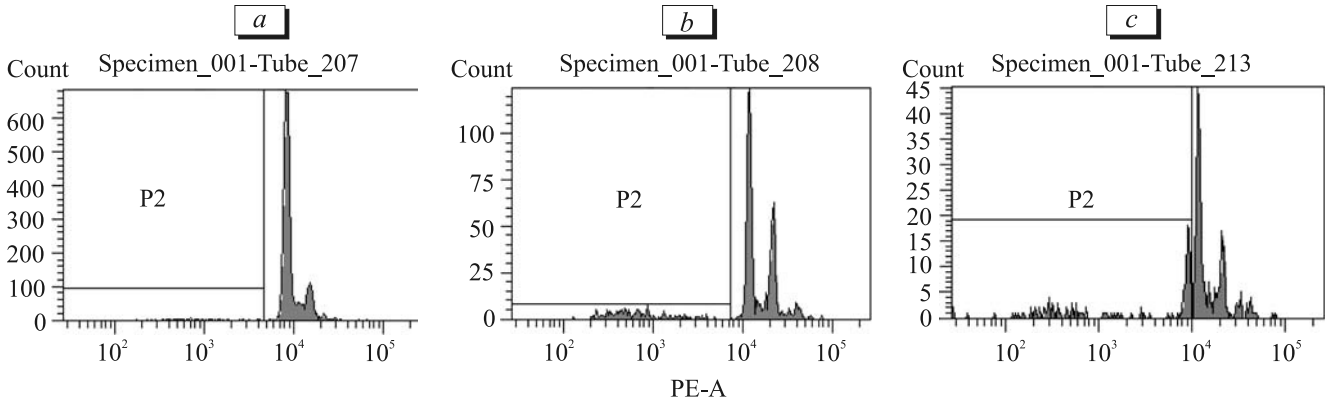

Fig. 3. Cytograms of U87 tumor cells irradiated with epithermal neutrons and stained with PI. The radiation doses: 1.9 (*a*), 2.9 (*b*), and 4.1 Sv (*c*).

corroborated the view on the necessity to sensitize the tumor with boron-containing agents to make it specifically vulnerable to external neutron radiation. Within BNCT technique, such sensitization is performed with sodium borocaptate BSH or boronophenylalanine BPA, which greatly increase the damaging effect of the tumor cells [6].

Although the method used in this study can assess the total fraction of dead cells, it cannot reveal the mechanism of cell death and its acceleration after irradiation. We hypothesized that the neutron radiation triggers apoptosis in the cells damaged by radiation. To test this hypothesis, we used flow cytofluorometry with PI. This agent binds stoichiometrically to nucleic acids to produce fluorescence proportional to DNA concentration in the examined sample (Fig. 3).

Cytometric analysis of cells stained with PI showed a wide hypodiploid peak (Sub-G1) characteristic of the apoptotic cells, which could be easily distinguished from the narrow peak characteristic of normal (diploid) DNA. The existence of sub-G1 peak attests to cell cycle arrest in the G1 and G2 phases and triggering of the apoptotic process.

Comparative analysis of the content of apoptotic cells in the control and experimental groups performed for each radiation dose revealed no significant differences between these groups. In other words, neutron irradiation did not activate apoptosis (Table 1).

The irradiation dose of 1.9 Sv doubled the content of dead cells in samples (from 16 to 30%). Further increasing the radiation dose from 1.9 to 4.1 Sv even more increased the content of dead cell (from 13 to 38%). The absence of apoptosis activation in irradiated cells and the dose-dependence of the biological effect of neutron radiation attest to the necrotic mechanism of the death among the tumor cells.

This study showed that irradiation of human glioblastoma U87 cells with epithermal neutrons promotes necrotic death of tumor cells. Increasing the irradiation dose to 4.1 Sv even more enhances cell death indicating a principal possibility to apply neutron radiation as an efficient tool to kill tumor cells. The therapeutic potential of neutron radiation should be further studied.

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