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Macrophage and tumor cell responses to repetitive pulsed X-ray radiation

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Abstract. To study a response of tumor cells and macrophages to the repetitive pulsed low-dose X-ray radiation. Methods. Tumor growth and lung metastasis of mice with an injected Lewis lung carcinoma were analysed, using C57Bl6. Monocytes were isolated from a human blood, using CD14+ magnetic beads. IL6, IL1-beta, and TNF-alpha were determined by ELISA. For macrophage phenotyping, a confocal microscopy was applied. "Sinus-150" was used for the generation of pulsed X-ray radiation (the absorbed dose was below 0.1 Gy, the pulse repetition frequency was 10 pulse/sec). The irradiation of mice by 0.1 Gy pulsed X-rays significantly inhibited the growth of primary tumor and reduced the number of metastatic colonies in the lung. Furthermore, the changes in macrophage phenotype and cytokine secretion were observed after repetitive pulsed X-ray radiation. Conclusion. Macrophages and tumor cells had a different response to a low-dose pulsed X-ray radiation. An activation of the immune system through changes of a macrophage phenotype can result in a significant antitumor effect of the low-dose repetitive pulsed X-ray radiation.

1. Introduction

It has previously been demonstrated by several studies that a pulse-modulated non-ionizing radiation with a frequency range of 6–25 pulse/sec can induce different types of biological responses [1-3]. However, short pulses of the ionizing radiation failed to produce a significant increase of biological responses [4]. The exposure of hamster cell lines to a few pulses of X-ray radiation with 5 min interval between pulses and an absorbed dose of 0.1 Gy did not affect a cell survival [5]. A low efficiency of the used regimens can be attributed to the suboptimal pulse repetition frequency, the pulse duration, the total dose, and the number of fractions. Previously, we have demonstrated that the low-dose nanosecond pulsed X-ray radiation inhibited the tumor cell proliferation in vitro up to 95% [6].



However, tumor cell reactions *in vivo* and possible mechanisms of antitumor effects of the low-dose nanosecond pulsed X-ray are insufficiently studied.

It is known that an immune system plays an important role in antitumor effects of the low dose ionizing radiation. Several studies demonstrated a reduced lung metastasis in animals irradiated with the low dose radiation (<1 Gy) [7-9]. This effect can be associated with an activation of natural killer cells and macrophages, as well as an increased production of cytokines that play an important role in anti-metastatic effects of the ionizing radiation. The ability of tumor associated macrophages to respond to a plethora of environmental factors (including the ionizing radiation) makes them perspective targets for a cancer therapy.

The application of pulsed X-ray radiation as a perspective therapeutic tool is based on the fact that biological responses are strongly modified when the pulsed radiation regime is used (i.e. every pulse is divided into a series of short pulses of a nanosecond duration and a certain repetition frequency). Consequently, this can result in the alteration of macrophage function, and the stimulation of immune responses, which influence the key stages of tumor progression, thereby enhancing the effects of anticancer therapy.

2. Materials and methods

2.1. Irradiation

Irradiation of the animals was performed using a generator of a pulsed periodic X-ray ("Sinus-150", Institute of High-Current Electronic SB RAS, Tomsk, Russia). A high-voltage pulse had a half-height duration of 4 ns. The calculated photon energy spectrum had a maximum at 90 keV, and most of the quantum flux was within 60-200 keV range. The pulse amplitude stability of the diode voltage in the operating mode was constantly controlled by a capacitance sensor. The absorbed doses were verified, using thermoluminescent dosimeters LiF. Direct-reading dosimeters ("Arrow-Tech", USA) in the plastic casing were also used for a continuous dose control. The pulse repetition rate was 10 pulses per second.

2.2. Analysis of tumor growth and metastasis

Female C57Bl6 mice (6-8 weeks old) were subcutaneously injected with 2×10^6 LLC cells. The irradiation started when the tumor volume reached 430 ± 25 mm³. The animals were exposed daily, for 5 days, to the local irradiation at 1.8 mGy/min so that a daily absorbed dose was equal to 0.02 Gy, and the total absorbed dose was 0.1 Gy. Control mice were sham-exposed (a generator at the off-mode) under identical conditions. The solid tumor weight and the size was measured on the 19th day after the tumor cells injection. For measurement of spontaneous metastases, lungs were fixed with Bowen fixative and amounts of colonies in every mouse were calculated under a dissecting microscope [10]. All animal studies were carried out in accordance with the regulations and with permission of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986).

2.3. Monocyte isolation and cultivation of macrophages

The isolation and cultivation of human monocytes/macrophages was done, using a magnet separation. Briefly, the cells were purified from individual buffy coats of healthy donors. Peripheral blood mononuclear cells (PBMC) were separated, using a ficoll, and monocytes were collected using a percoll gradient followed by CD14⁺ magnetic cell sorting, using a monocyte isolation kit (MiltenyiBiotec, Germany). Macrophages were cultured at 1×10^6 cell/mL in X-vivo 10 serum free medium supplemented with human M-CSF (Peprotech, USA) at a final concentration of 10 ng/ml. Monocytes were irradiated with absorbed doses 0.001, 0.01, and 0.1 Gy.

2.4. Cell viability assay

The viability of cells was measured as has been previously described in [11]. Briefly, 24 h after irradiation, the cells were incubated in 20 μ L MTT solution (5 mg/mL), and formazan crystals were solved with 100 μ L DMSO. The absorbance was determined at 490 nm, using a microplate reader Multiscan (Thermo Scientific, USA). The cell survival rate was measured as an absorbance compared to that of untreated controls. The results represent the average of 3 independent experiments.

2.5. ELISA analysis

IL1-beta, TNF-alpha and IL6 content in supernatants from monocytes cultures was determined by ELISA (R&D Systems, USA).

2.6. Immunofluorescence

The macrophage phenotype was analyzed using the confocal microscopy. Macrophages were stained using CD68 antibody (1:100) (PG-M1, Dako, Germany) and a custom-made rabbit anti-mouse/human stabilin-1 antibody (RS1), as a marker of M2 phenotype (1:1000) (PSL, Germany). As a fluorescent secondary antibodies, the goat anti-mouse Alexa488 (1:400) (Dianova, Germany) and the goat anti-rabbit Cy3 (1:400) (Dianova, Germany) were used. The samples were analyzed by the confocal microscope LSM 780NLO (Carl Zeiss, Germany).

2.7. Statistical analysis

The Mann-Whitney U test for non-parametric trials was used for the statistical analysis of the results, and p values lower than 0.05 were regarded as significant.

3. Results and discussion

The pulsed X-ray local irradiation of mice led to a significant antitumor activity against LLC tumors. A delay of tumor growth measured both by taking into account a tumor size and a tumor weight reached 42 % (figure 1A). To analyze an anti-metastatic activity of pulsed X-ray radiation, a number of lung colonies was used. The fractioned local irradiation, using pulsed X-ray, significantly decreased the number of metastatic colonies (figure 1B). The average number of colonies in a control group was 55.9 ± 4.41 . The irradiation of mice with a total absorbed dose of 0.1 Gy inhibited the colony formation by 60 % (22.2 ± 1.2). The results of the present study demonstrate that the local irradiation of mice with the doses of pulsed X-ray below 1 Gy leads to a significant suppression of the tumor growth and tumor colonies in a lung. Some authors showed that the traditional (non-pulsed) X-ray irradiation inhibits the growth of the primary Lewis lung carcinoma by 50 %, if we use 2 fractions of 20 Gy for each or 5 fractions of 8 Gy for each (40 Gy total absorbed dose) [12]. In the present study, the tumor growth delay was observed when we applied the 200-times lower total dose of the repetitive pulsed X-ray.

Presently, there is a lack of data about the *in vivo* antitumor efficacy of X-ray doses lower than 0.2 Gy. However, under certain experimental conditions, the delayed tumor growth in mice exposed to the low doses radiation was observed. Kojima et al. observed a significant inhibition of a tumor progression in mice only when the irradiation was applied before the tumor inoculation [8]. Ito M., et al. found that the total-body irradiation of mice with 0.2 Gy before the tumor cell inoculation significantly prolonged the mean time of the tumor progression [13]. The irradiation of mice 2 h before the tumor cell inoculation (the absorbed dose 0.1 – 0.2 Gy) resulted in 30-50% decrease of pulmonary tumor colonies [14]. Authors noted that the mechanism of such antitumor efficacy might be related to the activation of an immune system, i.e. the stimulation of cytotoxic activities of peritoneal macrophages and natural killer cells, which resulted in a decrease of pulmonary tumor colonies [8, 15-17].

We demonstrated that the irradiation of human monocytes with the total absorbed doses 0.1 and 0.5 Gy of the pulsed X-ray increased the secretion of IL6 and IL1-beta but not TNF-alpha by macrophages 3 days after irradiation (figure 2).

The macrophage phenotype was analyzed using the 6-day culture (5 days after irradiation). As it is shown in figure 3, the non-irradiated macrophages had CD68+/RS1- phenotype (M1 phenotype). After irradiation, an increase of the stabilin-1 expression was observed, indicating a transition to M2-skewed CD68+/RS1+ phenotype. The tumor associated macrophages which make up to 50% of total cells in certain tumors, are the potential targets for an anticancer therapy (including the ionizing radiation). In this case, the application of pulsed X-ray radiation, as a perspective therapeutic tool, is based on the fact that the biological responses are strongly modified when the pulsed radiation regime is used. Wunderlich R., et al. suggest that the X-ray radiation of low and moderate doses may alter the cytokine expression, the chemotaxis, the migration, and the phenotype of macrophages [18]. El-Saghire H., et al. showed that in contrast to the high doses, the low doses of radiation can be more efficient for the monocyte activation. Monocytes exposed to the low doses of ionizing radiation induced the immune-stimulatory and the pro-survival responses, while those exposed to the high doses induced the immuno-suppressive responses [19]. In addition, an exposure of macrophages to the low doses of X-ray radiation can lead to an increase of the phagocytic and the proliferative activity [20, 21].

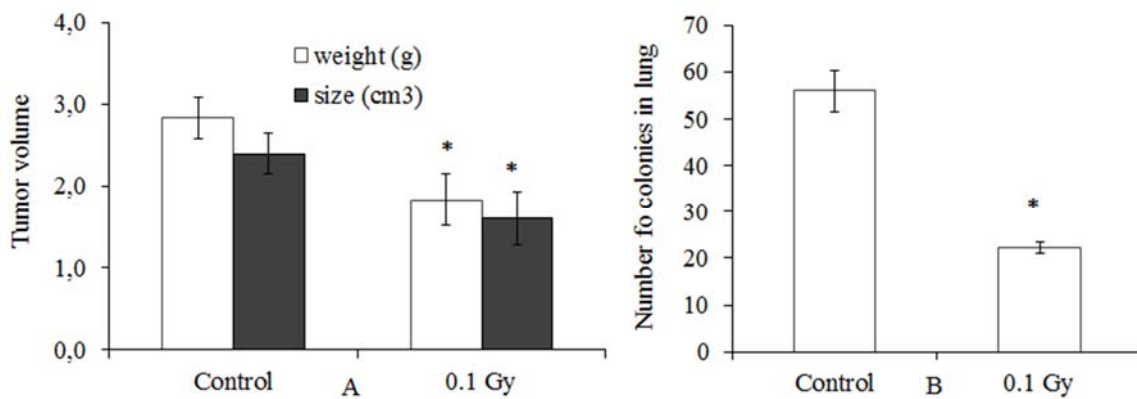


Figure 1. Volume of a solid tumor (A) and the number of metastatic colonies in the lung (B) of C57Bl6 mice after the exposure to pulsed X-ray at an absorbed dose 0.1 Gy. Error bars indicate the standard error of the mean (SEM) for n=3 independent experiments; * - indicates statistical significance ($p \leq 0.05$).

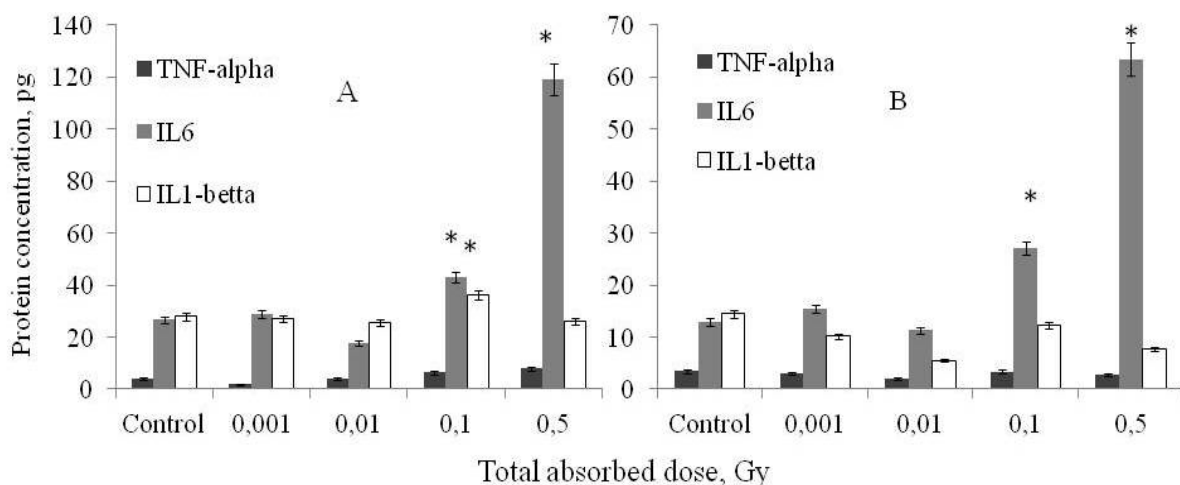


Figure 2. The cytokine concentration 3 days (A) and 6 days (B) after the monocytes exposure to the pulsed X-ray at the absorbed dose 0.001 – 0.5 Gy. Error bars indicate the standard error of the mean (SEM) for n=5 independent experiments; * - indicates statistical significance ($p \leq 0.05$).

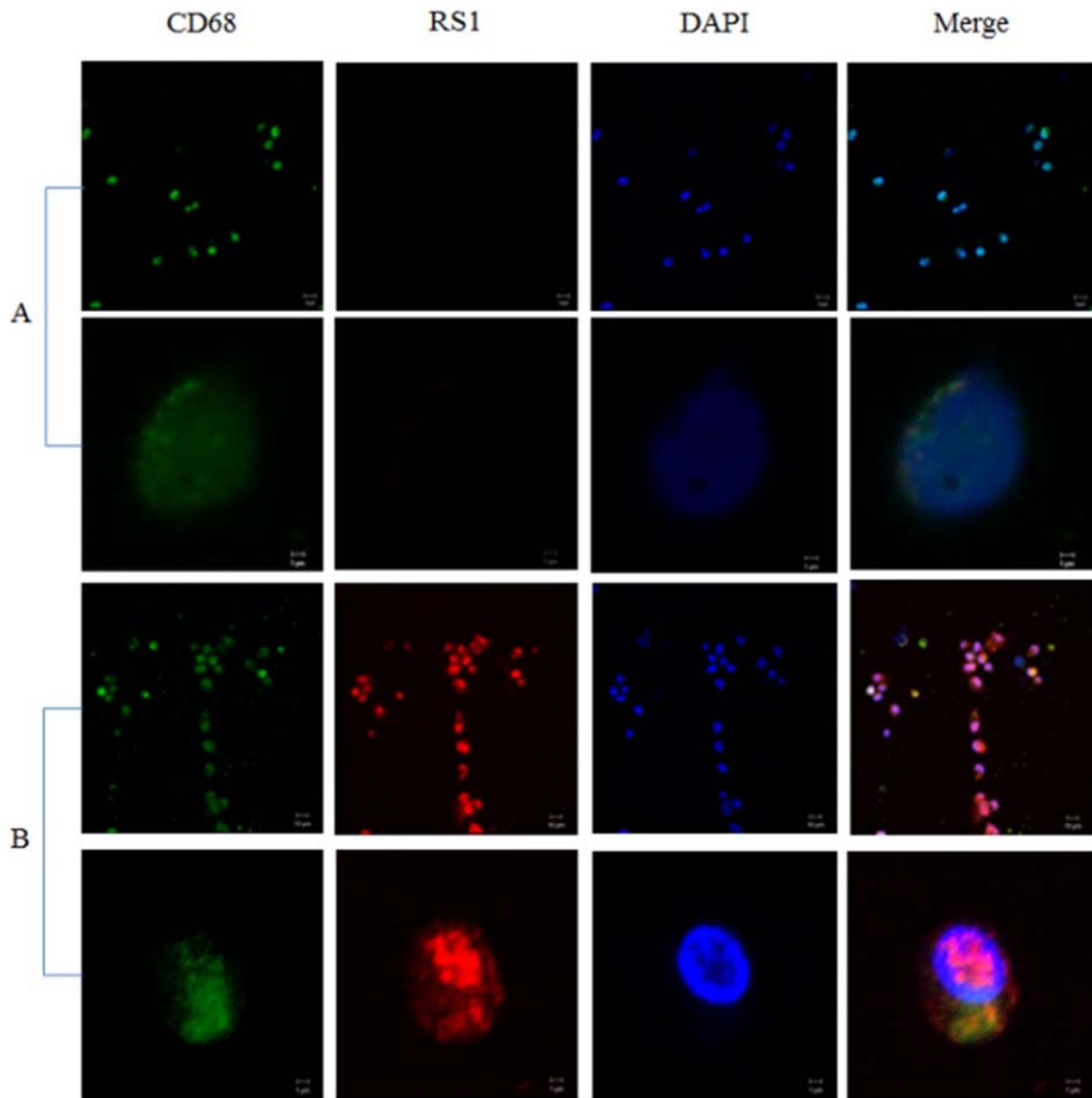


Figure 3. The volume of solid tumor (A) and the number of metastatic colonies in the lung (B) of C57Bl6 mice after exposure to the pulsed X-ray at the absorbed dose 0.1 Gy. Error bars indicate the standard error of the mean (SEM) for $n=3$ independent experiments; * - indicates statistical significance ($p \leq 0.05$). x630/6300

4. Conclusion

The described experimental data provide an explanation of the inhibitory effects of the low-dose repetitive pulsed X-ray radiation on the development and progress of tumors. The induction of the immune system through the macrophage activation can result in the tumor growth inhibition and the decrease of lung metastatic colonies.

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