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Effects of Iron Ions and Iron Chelation on the Efficiency of Experimental Radiotherapy of Animals with Gliomas

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We evaluated the effect of iron ion chelation on the growth of gliomas and radiotherapy efficiency in tumor-bearing animals. The rats with transplanted glioma-35 received iron-containing water; desferroxamine was injected for metal chelation. Long-term treatment with iron-containing water promoted glioma growth in rats and improved the efficiency of radiotherapy due to combination of apoptosis and ferroptosis. Desferroxamine reduced the efficiency of this treatment and was inessential for the efficiency of radiotherapy alone.

Key Words: *rats; gliomas; radiotherapy; iron ions; desferroxamine*

Iron is the most prevalent and essential transitional metal in humans and animals. Iron content in mammals gradually increases with age, as its intake is higher than its loss and there are no special mechanisms of excretion of iron excess when its accumulation surpasses the physiological requirement [9]. Iron content is regulated mainly by the efficiency of its intestinal absorption changing in inverse proportion to its reserve in the body. As a transitional metal, iron is involved in redox cycles in cells. Iron is involved in oxygen transfer mediated by hemoglobin in mammals and is essential for activities of many enzymes, *e.g.* catalase modulating the level of peroxides. Iron and oxygen excess leads to oxidative stress.

Redox processes with participation of iron contribute to tumor induction [13]. Iron is involved in carcinogenesis [8], for example, it is essential for cell transition from G_1 to S phase of the cell cycle, which supports proliferation. Epidemiological studies demonstrate the potentiating role of iron augmenting the risk of tumor diseases [14]. On the other hand, injection of iron chelators, *e.g.* lactoferrin and desferroxamine (DFA) to animals inhibits tumor growth [6,11]. Iron depletion caused by bloodletting reduces the risk of tumor formation [15]. Discovery of iron transmitters, transcription regulation of activities of genes involved in iron metabolism, hepcidine, a peptide hormone produced by hepatocytes and regulating iron metabolism, and ferroptosis, a new form of cell death [1] provided new insight into mechanisms of iron activity, which is essential for analysis of carcinogenesis processes and approaches to improvement of cancer therapy.

We studied the effects of DFA, a specific chelator of iron ions, on the growth of a transplanted tumor and efficiency of experimental radiotherapy (RT) of rats with gliomas.

MATERIALS AND METHODS

Experiments were carried out on male Wistar rats (n=73) from Breeding Center of the Russian Research Center of Radiology and Surgical Technologies. Animal age at the start of the experiment was 10-12 months (body weight 0.25-0.30 kg). The rats were kept in vivarium on standard ration until natural death of tumor-bearing animals. The animals received tap water with Fe^{2+} concentration of 0.2-0.3 mg/liter or iron-containing water (ICW) with Fe^{2+} concentration of 60-63 mg/liter.

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Nitrosoethylurea-induced cerebral glioma (strain 35) was transplanted to rats subcutaneously into the hip [3]. The animals were taken into the experiment in 9-15 days after transplantation and formation of a tumor node of 1.3±0.2 cm^3. The tumor volume was calculated by the formula: $V=a\times b^2/2$ (cm^3), where a and b were the axes of the tumor.

Tumor-bearing animals were divided into groups: 1) rats drinking tap water *ad libitum* (n=18); 2) ICW *ad libitum* (n=7); 3) DFA (n=11); 4) RT (n=8); 5) RT+ DFA (n=6); 6) ICW for 3 days before RT in a dose of 15 Gy (n=9); 7) ICW for 3 days before RT and DFA 3 h after RT (n=9). Desferroxamine (Novartis Pharma AG) was injected intramuscularly in a dose of 80 mg/kg.

Experimental RT was carried out as follows. The rat hip area corresponding to 1.5 cm^3 of the transplanted tumor volume was exposed on a RUM-17 device to a single dose of 15 Gy at a dose power of 2.1 Gy/min (220 kV, 13 mA, filters: Al – 1 mm, Cu – 0.5 mm; distance from the skin focus 50 cm).

Hematological and biochemical values were measured 24 h after RT. Whole blood (0.1 ml) for analysis was collected from the caudal vein. Hematological studies were carried out by the standard methods: the blood counter pipette was used for evaluation of the total count of leukocytes and erythrocytes; hemoglobin concentration was measured on a photocolorimeter (CPC-2) at λ=540 nm and estimated by the calibration curve. Complete leukocyte count was evaluated on blood smears stained with Leischman's eosin under a microscope.

DNA supercoiling was evaluated by the DNA fluorescence coefficient (FC) of blood nucleoids formed after cell lysis in the presence of Na_2-EDTA, 2 M NaCl, and Triton X-100 for 3-5 min [2]. DNA structure in the nucleoid was close to native, but without complex-forming, protein, and lipid components, which facilitated binding of specific dyes to the substrate. Changes in the number of ethidium bromide molecules (final concentration 4 μg/ml) interacting with the nucleoid DNA, were caused by relaxation of the polynucleotide supercoiled structure in comparison with control, due to ruptures in its pentosephosphate chains after irradiation. The FC was calculated by estimating fluorescence intensity of ethidium bromide (Sigma) intercaling in DNA depending on its supercoiling in comparison with the fluorescence intensity of DAPI (Serva) externally binding to the ligand DNA spiral (ligand concentration 0.1 μg/ml). The measurements were carried out on a Model-850 spectrofluorimeter (Hitachi) at λ_{stim}=350 nm and λ_{reg}=450 nm for DAPI and λ_{stim}=510 nm and λ_{reg}=590 nm for ethidium bromide. Regression analysis showed a significant relationship between blood DNA FC and number of chromosome and chromatid ruptures in lymphocytes [5].

The results were statistically processed by nonparametric U test, Student's t test, and Spearman's correlations coefficient on the basis of intravital measurements of hematological and biochemical values, tumor size, and individual lifespan of each animal.

RESULTS

The tumor volume reached 52.6±9.2 cm^3 in group 1 rats over 28 days of the experiment (Fig. 1, *1*). Treatment with ICW instead of tap water (group 2) stimulated the growth of gliomas, which were larger on days 14 and 21, while iron chelation by DFA in group 3 animals somewhat inhibited the tumor growth in comparison with group 1. Tumor irradiation significantly inhibited its growth (Fig. 1, *4*). The increment of the tumor volume was the minimum in animals receiving ICW before irradiation (group 6), while injection of DFA (group 7) reduced the inhibitory effect of irradiation.

The lifespan of animals receiving different treatments in comparison with the control group (29.4±1.8 days taken for 100%) was presented in Figure 2. The lifespan of group 2 rats was shorter by 34% than in group 1. The lifespan of group 6 animals was 2-fold longer than of controls, while additional DFA injection (group 7) canceled this effect.

Rats with gliomas died from day 21 to day 73 after irradiation. Regression analysis showed a significant negative relationship between tumor volumes on day 21 after irradiation (V, cm^3) and lifespan of each animal (T, days), which could be presented by the equation:

$$T=68.04-0.41\times V;\ (R=0.817;\ p<0.05). \quad (1)$$

The protocol of DFA treatment used in the experiment caused no appreciable changes in hemoglobin

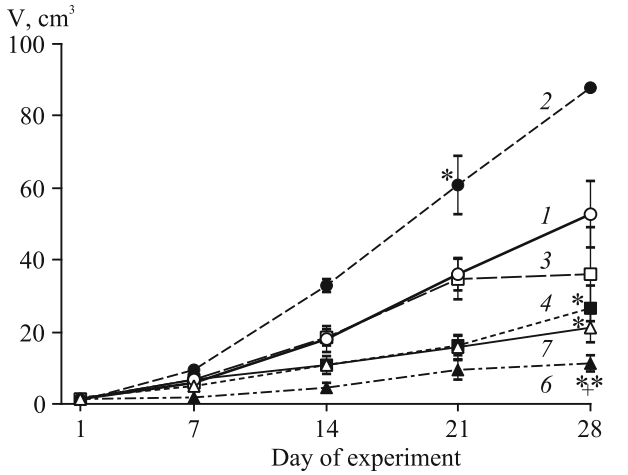

Fig. 1. Changes in tumor volume in rats after RT. Here and in Fig. 2: *1*) tumor control; *2*) tumor+ICW; *3*) tumor+DFA; *4*) tumor+RT; *6*) tumor+ICW+RT; *7*) tumor+ICW+RT+DFA. *p<0.05, **p<0.01 in comparison with *1*; +p<0.05 in comparison with *4*.

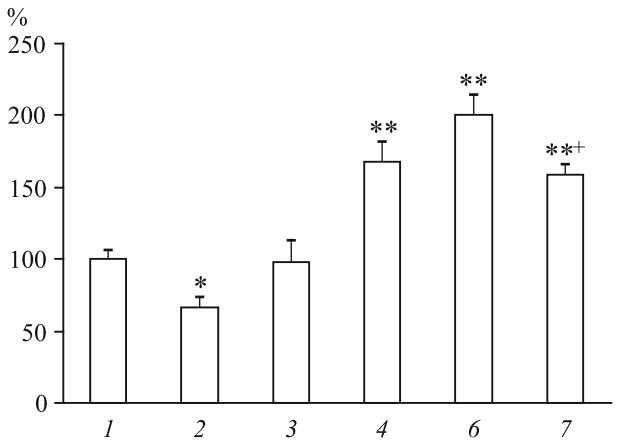

Fig. 2. Lifespan of rats with gliomas treated by ICW and DFA. *p<0.01 and **p<0.001 in comparison with *1*: +p<0.05 in comparison with *6*.

concentrations and erythrocyte counts in all groups of rats with tumors in comparison with control. All measured parameters in group 5 (data not presented in figures) did not differ from the parameters in group 4 animals.

Analysis of correlations of individual parameters in three groups of rats with tumors, receiving no RT (groups 1, 2, and 3), detected a significant negative relationship between the lifespan (in days) and blood monocyte count (Mon, 10^{-9} cells/liter):

T=53.2-6.8×Mon; (R=0.621>0.533; p<0.05), (**2**)

but not between the lifespan and any other changed hemocytological or biochemical values. By contrast, a significant relationship between the time course of individual lifespan and the blood DNA supercoiling (rel. units) was detected in the total group of animals with tumors which received RT and animals treated with ICW and DFA, in which the transplanted tumor growth was inhibited:

T=-1.97+18.77×FC; (R=0.592>0.575; p<0.01) (**3**)

and a somewhat less intense relationship between this parameter and the total leukocyte count (L, 10^{-9} cells/liter) in the blood:

T=55.4-1.3×L; (R=0.561>0.455; p<0.05). (**4**)

These results indicate the involvement of various mechanisms, mediating the effects of drinking water iron ions and their chelation on the growth of transplanted gliomas in animals, receiving and not RT.

According to formula (**2**), the lifespan of animals with tumors receiving no RT depends on the monocyte count. Monocytes form from bone marrow precursor cells and after their maturation are released into circulating blood. Delivered into tissues, they differentiate into macrophages and myeloid dendritic cells. Macrophages phagocytose invading microorganisms and generate an AOS flash, thus protecting the organism from infections. However, some DNA aberrations, such as 8-oxyguanin and thymine glycol, induced by ROS, lead to disorders in the DNA base coupling, genetic instability, mutations, and eventually to tumor formation [12]. Monocytes are presumably one of the components of the cell population opposite to the tumor host immune system of reacting to malignant cells. Hence, the efficiency of the immune system in tumor control increases with decrease in the blood monocyte count, this eventually leading to lifespan prolongation.

As for iron ions proper, it is known that they can be carcinogenic ecotoxicants [1,10]. This work has demonstrated that they stimulate the growth of transplanted gliomas. Transferrin receptors mediate the accumulation of iron and formation of ROS, the number of these receptors superexpressed in glioma cells [6]. Our data on iron ion chelation by DFA, leading to shrinkage of gliomas to 72±22% of control by day 28, was in line with previous data [6] on shrinkage of transplanted gliomas in mice in response to DFA to 63% *vs.* tumor control. Hence, reduction of blood iron level by iron chelation inhibits tumor growth stimulated by these ions.

As for combined effects of Fe^{2+} and radiation on rats with tumors, ICW before RT led to an increase of FC (reflecting the number of DNA ruptures) and stimulated monocytopenia as early as just 24 h after the exposure, due to hyperradiosensitivity of these cells [3,4]. We observed the maximum (*vs.* control) inhibition of glioma growth after this combined treatment due to apoptosis and ferroptosis. Ferroptosis as a cell death form differs morphologically and biochemically from apoptosis and depends on the level of intracellular iron, but not on other metals [7]. Addition of DFA to RT protocol reduced the efficiency of glioma growth inhibition, most likely because it switched off ferroptosis.

Hence, ICW before RT stimulates glioma cell death by a combination of apoptosis and ferroptosis, and hence, improves treatment efficiency. Addition of DFA to this combined therapy reduces its efficiency. RT protocol including ICW reduces the count of monocytes that hamper detection and elimination of cancer cells by the immune system and is recommended for clinical trials.

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