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Original research paper

THE STUDY OF BIOLOGICAL EFFECTIVENESS OF U-70 ACCELERATOR CARBON IONS USING MELANOMA B-16 CLONOGENIC ASSAY*

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Abstract. *The study was carried out using the system of accelerators (I-100, U-1.5, U-70). Ultra-precise equipment to position biological objects was applied. The dependency of melanoma B-16 cells survival on the dose of ^{12}C ion irradiation was obtained. The carbon beam was studied within three main ranges: Bragg peak, areas before and after the peak. Dose dependence in the peak and in the area before the peak had a distinct linear pattern. In the distal part of the Bragg peak, linear-quadratic dependence was observed. Carbon ions RBE were 4.5, 1.7 and 2.4 for the peak, the areas before and after the peak, respectively.*

Key words: *Carbon ions, high-LET radiation, radiotherapy, U-70, RBE, clonogenic assay, melanoma B 16 cells*

1. INTRODUCTION

Carbon ion therapy is one of the most promising methods in oncology. Its advantage over the conventional radiotherapy is proved for tumors of different etiology. According to some studies [1, 2], carbon ions are more appropriate for head and neck tumors, lung cancer, prostate and hepatocellular carcinomas. It is shown in simulation study [3] that carbon ions are more effective comparing to protons in case of deep-seated tumors with a diameter of up to 4 cm.

The action of carbon ions on biological tissue as well as the exposure of any other ions due to specific energy transfer patterns should be described by at least three main areas. Range A is the plateau area before the Bragg peak (linear energy transfer – LET in case of used in the study system of accelerators – 10÷16 keV/μm). The second area – range B is the Bragg peak (LET was 180±26 keV/μm). Range C follows the Bragg peak and can have almost the same LET (20±1 keV/μm) as in Range A.

Available data on biological effectiveness of carbon ions are mainly related to ranges A and B [1, 4-7]. Range C, the area that follows the peak, has not been studied enough. It is also supposed that radiobiological effects in this area are insignificant. However, range C conforms the area of the healthy tissues located behind the target (tumor) and doses related to the area have to be taken into consideration while planning radiotherapy.

The subject of the present study was a precise estimation of the biological effectiveness of the carbon ion beam according to tumor cell survival.

2. MATERIALS AND METHODS

2.1. B-16 cells

The studies were carried out using mice melanoma B-16 cells. The cell culture was irradiated in the monolayer condition in the late log phase. In the course of irradiation, cell cultures were in 25 or 75 cm^2 flasks (Corning, USA) containing RPMI-1640 medium (Paneko, Russia) and 2% fetal calf serum (Biosera, France).

Melanoma is one of the most aggressive tumors, with high growth rates. According to some data [8] melanoma is considered as cell line with low radio-sensitivity and an appropriate choice for conventional fractionation schemes. The used cell clone was B16F10. In accordance with our estimates the cell culture doubling time was 11±3 hours, cell density in monolayer was 1423±72 cells per mm^2, cell diameter (suspension condition) – 14±2 μm. This mice melanoma cell clone according to some data [9] could

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be considered as more metastatic (and thus more radio-resistant) than standard B16 cell culture.

2.2. Clonogenic assay

Survival of the irradiated cells was estimated by cell clonogenic activity. After the irradiation, cells were removed from the plastic surface of the flasks using a trypsin solution with 0.25% EDTA (Paneko, Russia). The cells were resuspended in RPMI-1640 medium and counted. Afterwards, cells were seeded (from 1000 to 140 000 cells depending on the dose) in 100 mm Petri dishes (Corning, USA) containing RPMI-1640 medium and 10% fetal calf serum. Petri dishes were then kept in an incubator (Sanyo, Japan) with 5% CO_2 at +37°C for 8-10 days until the formation of visible colonies.

At the end of the incubation and colony growth, the medium was removed and cell colonies were stained with 2% solution of methylene blue in 50% ethanol [10]. Counting of the colonies was conducted using a manual counter (Interscience Scan 100, France). An irradiated cell was considered alive if its colony consisted of at least 50 cells. The survival fraction for a certain dose was determined by dividing the number of living cells by the total number of seeded cells. The survival endpoint was calculated as a ratio of irradiated and non-irradiated cells' survival fractions.

The relative biological effectiveness (RBE) of the radiation was estimated as a ratio of iso-effective doses of studied and standard exposures.

2.3. Irradiation sources and procedures

The source of the unmodified (not spread-out) carbon ion beam was the system of accelerators: I-100, U-1.5, U-70 of the Institute for High Energy Physics (Protvino, Russia). The average ion energy was ~ 455.8 MeV/nucleon. The dose rates were 0.1, 0.2 and 0.05 Gy/min in range A, B and C, respectively. Irradiation doses were 2.2-5.3 Gy in range A (plateau before the Bragg peak), 0.6-3.8 Gy in range B (Bragg peak) and 1.4-4.0 Gy in range C (area after the peak).

The standard exposure to gamma-rays was provided by ^{60}Co with an average energy of ~ 1.25 MeV. The dose rate was ~ 1.0 Gy/min, and the dose range was 2.0-10.0 Gy.

In the course of irradiation with ions, the flask with the cells was located in the air chamber, vertically and perpendicular to the beam. This chamber was, in turn, a moveable part of a bigger container filled with water (a necessary condition to form the ion specific energy transfer pattern). This two-chamber system was completed with the equipment for ultra-precise movement of the objects (Figure 1).

Comprehensive schemes of the irradiation equipment, dosimetry and monitoring devices, and a technique for irradiation have been published earlier [11].

Cell irradiation with gamma-rays was carried out without the described object moving system. The flask with the cells was located horizontally and perpendicular to the source of gamma-rays.

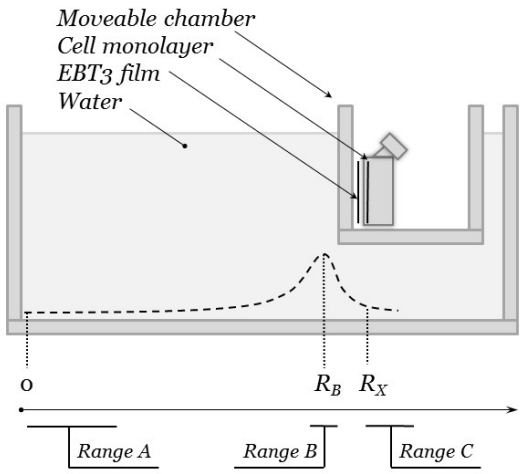

Figure 1. Two-chamber system for biological object irradiation with carbon ions: R_X – distance between the cell monolayer and the beginning of water layer, R_B – Bragg peak location

2.4. Statistics

The cell survival data was processed using the statistical software R 3.2.3 [12] and graphics application Veusz 1.23.2. The linear-quadratic model was applied to receive dose-survival dependencies (1). The agreement between the experimental data and the model was estimated using the χ^2 criterion.

$$S = 100 \cdot \exp\left(-\alpha \cdot D - \beta \cdot D^2\right) \qquad (1)$$

where:
α – coefficient related to single-track events,
β – coefficient related to two-track events,
D – irradiation dose.

The error of dose measurements was 5% for gamma-rays and 15% for carbon ions. The error of cell survival was estimated according to equation 2 and included the errors of hemocytometer (a – up to 10%), micropipettes (b – up to 3%) and Poisson error of grown colony number (c – up to 5%). In case of survival endpoint, the error doubles in accordance with the rule of relative errors summation. The errors of the dose-survival curves obtained by the linear-quadratic model were considered to be equal to 5%; RBE errors in compliance with relative error summation were 10%.

$$Sx = \sqrt{a^2 + b^2 + c^2} = 11.6\% \qquad (2)$$

3. RESULTS

The dependence of melanoma B-16 survival on irradiation dose according to clonogenic assay is shown in Figure 2. The curves are related to gamma-rays and three ranges (A, B and C) of carbon ions. Dose-survival dependencies in case of ranges A and B were linear. β-coefficient for range A (plateau) was minimal, for range B (the peak) equaled 0. By contrast, range C (are after the peak) had a distinct β-coefficient. The dose

dependence for range C was linear-quadratic. It is more specific for low-LET radiation than for carbon ions.

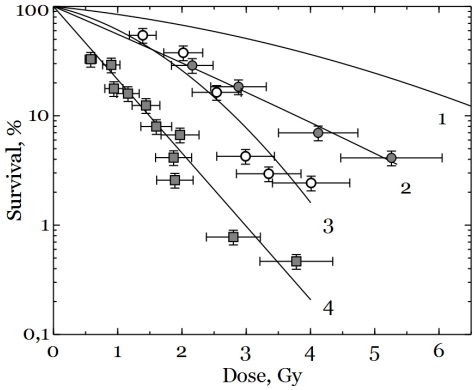

Figure 2. The dependence of melanoma B-16 cell survival on irradiation dose for: gamma-rays (1), carbon ions in range A (2, ●), range C (3, ○) and range B (4, ■)

Linear-quadratic equation coefficients are shown in Table 1. This table is supplemented with findings for fast neutrons published earlier [13-14]. The statistical significance (*) of the coefficients is also shown where possible.

Table 1. Linear-quadratic model coefficient values related to melanoma B-16 cell survival after the irradiation with gamma-rays, carbon ions and fast neutrons

Radiation	$\alpha \pm S_x$	$\beta \pm S_x$
Gamma-rays	0.177±0.027*	0.025±0.003*
Carbon-ions: range A	0.579±0.084*	0.007±0.020
Carbon-ions: range B	1.543±0.283*	0
Carbon-ions: range C	0.316±0.250	0.179±0.095
14 MeV neutrons	0.856±0.061*	0.001±0.020

According to data from Table 1 and Figure 1, RBE values could be determined. The calculated values are shown in Table 2. The estimation was performed for different survival rates: 10, 37% and for survival corresponding 2 Gy gamma-rays irradiation (conventional daily fraction in radiotherapy). The table is also supplemented with α-coefficients ratios (studied radiation / gamma-rays). The last parameter reflects the maximum value of RBE.

Table 2. RBE values related to melanoma B-16 clonogenic assay for carbon ions and fast neutrons

Radiation	10%	37%	2 Gy	α/α
Carbon-ions: range A	1.7±0.2	2.2±0.2	2.6±0.3	3.3±0.3
Carbon-ions: range B	4.5±0.5	5.7±0.6	6.8±0.7	8.7±0.9
Carbon-ions: range C	2.4±0.2	2.3±0.2	2.1±0.2	1.8±0.2
14 MeV neutrons	2.5±0.3	3.2±0.3	3.8±0.4	4.8±0.5

According to Table 2, RBE for range B (Bragg peak) in case of 10% survival level outweighs 4 units. RBE values for ranges A and C were comparable to each other and to fast neutron effectiveness. Depending on the survival level, RBE values of range A and B were growing while RBE was increasing. It reached its maximum value in case of RBE estimated by α-coefficients ratios. By contrast, RBE of the range C (area after the peak) due to the significant β-coefficient was falling when survival level was increasing.

4. Discussion

RBE values related to range A (1.7 at 10÷16 keV/μm) confirm available data. Depending on cell culture and LET (13-20 keV/μm), it varies from 1.5 up to 2.0 [4, 5, 7].

In the present study, range B (Bragg peak) was provided by an unmodified (not spread-out) peak with LET on the level of 180 keV/μm. The RBE obtained in the experiments was 4.5±0.5. This value exceeds data available in literature for the modified (spread-out) peak. The RBE in case of the modified peak is from 2.5 to 3.0 at average LET of 125 keV/μm [5, 7] and from 2.3 to 3.6 at 40-44 keV/μm [4].

Unusual linear-quadratic shape of the dose-survival curve in case of range C was an important part of the study results. Due to certain high-LET nature, all three ranges of the carbon ion radiation should provide linear (in log-scale) shape of survival curves. This effect may be explained by secondary particles forming in the biological tissue [3]. The ratio of doses related to primary radiation (carbon ions) and secondary particles differs sharply in ranges A and B and in range C.

The RBE value obtained in range C (2.4 units) exceeds RBE values available in literature. In case of LET of about 20 keV/μm, RBE should be on the level of 1.5 [4]. But this data is related to the plateau area (range A – in the present study) not to the area after the peak. The equality of the LET before and after the peak is a distinctive trait of the charged particles. Therefore, carbon ions provide a unique opportunity to study the influence of the secondary particles forming in the tissue. According to the results of the study, LET could not be considered as the only characteristic of radiation that influences its RBE.

5. Conclusion

The results obtained in the study require further investigations. It is related to the necessity of more accurate RBE values and RBE-LET dependency. This data will ensure more precise schedules of carbon ion therapy. Another important direction is related to the obtained biological effects of the area after the peak. The results of the present study substantiate its possible significant impact on doses corresponding to normal tissues surrounding the tumor. These issues are tightly associated with radiotherapy efficacy, patient life quality after the treatment and adverse long-term effects.

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preclinical trials of the source for radiotherapy with carbon ions.

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