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Influence of Therapeutic Proton Beam on Glioblastoma Multiforme Proliferation Index — A Preliminary Study

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The goal of the presented study was to compare the proliferation of different U118 MG and U251 MG glioblastoma cell lines irradiated with proton beam or X-rays in dose range 0.5–10.0 Gy. Cytokinesis-block micronucleus (CBMN) assay was carried out to study changes in proliferation presented as nuclear division index (NDI). Preliminary results suggest that protons and X-rays influence GBM (glioblastoma multiforme) cellular proliferation differently. Therapeutically, a decrease in NDI values with the increase in both types of radiation dose was found only for U251 MG cell line, and thus can be classified as more radiosensitive than U118 MG cell line. Also for U251 MG GBM cell line, a therapeutic proton beam was more effective in inhibition of proliferation than X-rays. Genetic differences between GBMs are supposed to be involved in the increased radiosensitivity, which is planned to be studied further by gene expression analysis.

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PACS/topics: Protons, X-rays, glioblastoma multiforme

1. Introduction

Glioblastoma multiforme (GBM) is one of the most common and aggressive types of primary brain tumors with an expected survival of usually less than 2 years [1]. GBM cancer is difficult to treat because it contains heterogenous proliferating and radioresistant cells. Furthermore, these cells may penetrate neighboring tissues [2]. Current treatments include surgery, systemic temozolomide (TMZ) chemotherapy, and radiotherapy [3]. For radiotherapy, there are a number of new prospects such as proton therapy which offers better dose delivery and distribution compared to photons, which can be exploited to reduce the probability of collateral normal tissue damage and post-treatment complication [4]. Radiation damage to DNA is believed to be a critical mechanism giving rise to cell death (apoptosis or necrosis) after exposure and predicting the response to treatment [5]. Recent studies reported that disturbance of the DNA damage checkpoint and enhanced DNA repair capacity in gliomas may lead to radioresistance [6]. The different cell cycle time, genomic instability, the efficacy of DNA repair processes or p53 (tumor suppressor) methylation status may also influence the effects of therapy [7]. Proliferation rate and intrinsic cellular radiosensitivity of tumor tissue are potential predictors of radiation response and may assist the choice of the best treatment for the individual patient [8]. Therefore, predictive assays of tumor growth for individual treatment of patients with cancer recurrence are required. Understanding of the cellular response and discovering the underlying mechanism is critical for optimization of the proton radiotherapy treatment scheme and may help to identify patients who will benefit from radiotherapy [9].

The goal of our study was to compare the impact of therapeutic proton beam irradiation (70 MeV) with the photon therapy (X-rays, 250 kV) in dose range from 0.5 Gy to 10 Gy on the proliferation of two glioblastoma multiforme U118 MG and U251 MG human cell lines (both classified as WHO grade IV). Studies were designed to compare morphology and confluency of GBM cells after different doses of proton beam irradiation vs. X-rays and analyze the dose-response effect between the nuclear division index (NDI) values and studied types of radiation.

2. Experimental details

2.1 Cell cultures

The human glioblastoma cell lines (U118 MG, U251 MG) were used as an *in vitro* model. The U118 MG was obtained from the ATCC® (American Type

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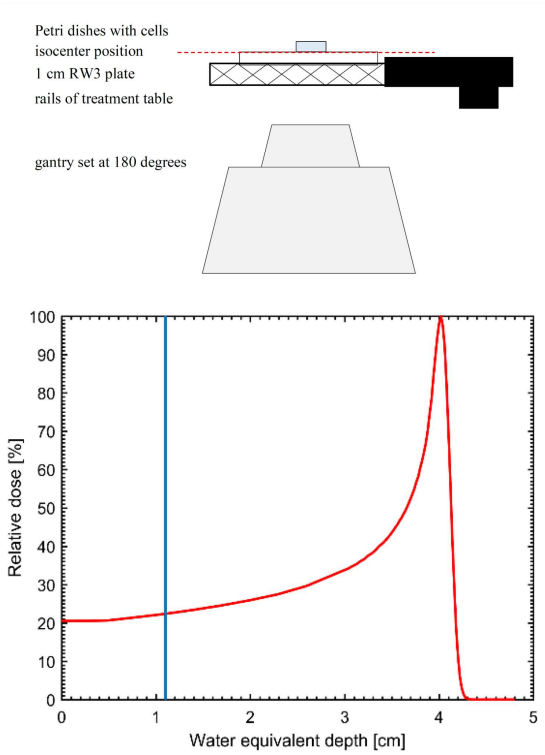

Fig. 1. The irradiation setup (top). The exemplary depth dose distribution of 70 MeV proton beam in water. The blue line marks the position of the cell at 1.1 cm water equivalent depth (bottom).

Culture Collection, Virginia, No. HTB-15^{TM}) and was continuously cultured in Dulbecco's modified Eagle's medium (DMEM, ATCC® No. 30–2002). The U251 MG (No. 09063001) human glioblastoma astrocytoma cell line was purchased from PHE (Public Health England, Salisbury, UK) culture collections and cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose, GlutaMAX^{TM}, Gibco®, No. 31966021). Culture medium for both cell lines was supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), cells were cultured at 37 °C, in a humid atmosphere of 5% CO_2. For the micronucleus assay, the cultures were trypsinized and plated (50 000 cells per 35 mm plastic dishes (Sarstedt) in 2 ml of growth medium). Culture plates were coated with 0.01% Poly-L-Lysine (Merck). The cell number was scored in a Bürker chamber. After 24 h the cells were irradiated in culture dishes with different doses of either proton beam (70 MeV, Proteus C-235, IBA) or X-rays (250 kV, model MCN 323, Philips X-ray machine) at the Institute of Nuclear Physics, Polish Academy of Sciences (IFJ PAN), Krakow, Poland. Irradiation was carried out at room temperature with doses 0.5 Gy, 2.0 Gy, 4.0 Gy, 6.0 Gy, 8.0 Gy, and 10 Gy for both types of irradiation. For irradiation of cells a pristine beam of energy 70 MeV and a field size of 15×15 cm^2 was chosen. The scheme of irradiation setup and the exemplary depth dose distribution of 70 MeV proton beam in water is presented on Fig. 1. Irradiations were performed at 1.1 cm water equivalent depth, which consists of a 1 cm RW3 slab phantom plate (IBA-dosimetry, Schwarzenbruck) and 0.1 cm of Petri dishes bottom while the isocenter was placed at 1 cm depth. The gantry was set at 180° (the beam was coming through the bottom of the Petri dishes). Prior to the experiment, dosimetry measurements were performed with Markus type ionization chamber calibrated in terms of absorbed dose to water D_w. The control (non-irradiated cells) was kept at room temperature for the same period of time as irradiated cell cultures.

2.2 Cytokinesis-block micronucleus assay and data processing

Cytokinesis-block micronucleus (CBMN) assay was carried out to study index and changes of proliferation presented as nuclear division index (NDI). CBMN assay was performed according to the method described by Fenech M. [10]. Immediately after irradiation, the cells were treated with 3 μM cytochalasin B (Merck) dissolved in dimethylsulfoxide. After 72 h of incubation, the cells were fixed in methanol: acetic acid (9:1 v/v) for 20 min, washed with distilled water and stained with Giemsa solution (Merck) what was described previously by Miszczyk et al. [11]. The specimens were evaluated under the Motic AE31 Elite Inverted Phase Contrast Microscope. The analysis was performed at magnification 400×. The nuclear division index of cells was calculated after scoring 500 consecutive cells twice (as an independent repetition of coded dishes for analyzing) and presented as a mean. Nuclear division index NDI and standard error of nuclear division index SE NDI were calculated according to the International Atomic Energy Agency recommendations [12]. Nuclear Division Index formula was as follows

$$\mathrm{NDI} = \frac{1}{N}\left(M_1 + 2M_2 + 3M_3 + 4M_4\right)$$

where M_1, M_2, M_3, M_4 represent the number of cells with one, two, three, or four nuclei, respectively, and N represents the total number of cells analyzed.

2.3 Statistical analysis

The data analysis was performed using the Microsoft Office Excel 2018. The NDI and dose-response curves were fitted in the OriginPro 2018b 64 bit (OriginLab Co. Northampton. MA. USA). For all data sets the 3-sigma limit test was used to determine whether the distribution of values was statistically relevant ($p < 0.05$).

3. Results and discussion

Our objective here was to determine the differences in the response of two different glioblastoma cell lines and the individual difference in dose-range of proton therapy vs. photon therapy of each cell line. The literature

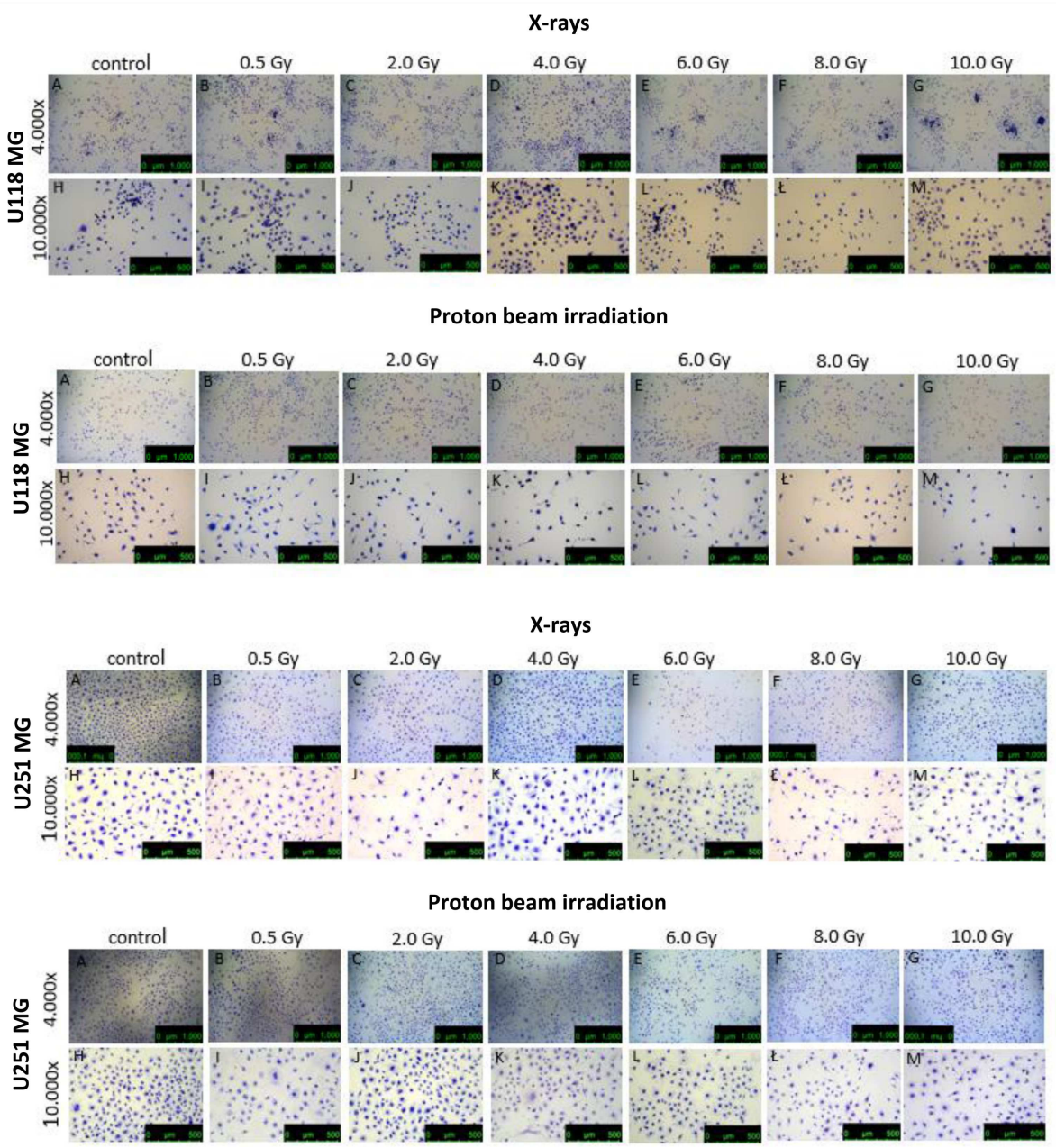

Fig. 2. Morphology and confluency of U118 MG and U251 MG cell line irradiated with either X-rays or proton beam. 4.000× or 10.000× magnification was created on Leica microsystems microscope (Leica Dmi8, incubator i8, LEICA LAS AF 4.0×).

data showed that the response of GBM to X-rays compared to proton therapy may be not satisfactory especially for tumors located near to critical organs at risk [13]. The growth potential of various GBMs results in re-growth of tumor after a specific time for a given patient, according to a rule that the higher proliferation rate is, the shorter is the turnover time [14]. Genetic background of each glioblastoma cell line also influences the response of cells to radiation exposure [15]. Recent studies showed that apart from the genetic background, the altered elasticity, shape, cytological structure, and adhesiveness of tumor cells may have an impact in tumor invasiveness and therefore, a response to therapies [16]. In our studies, morphologically the studied cell types look different. For illustration series of photos at two different magnification (4.000× and 10.000×) for both cell lines irradiated by protons or X-rays in the whole dose range are presented in Fig. 2. The protrusions in U251 MG cells were shorter and more branched than in U118 MG cells (Fig. 2). Nevertheless, for all doses, types of used radiation and both cell lines, the proliferation status was measured.

TABLE I

The fitting coefficients obtained for U118 MG and U251 MG cell lines post X-rays and proton irradiation using linear-quadratic model $y = A + B\,x + C\,x^2$.

	X-rays U118 MG	Protons U118 MG	X-rays U251 MG	Protons U251MG
A	1.1436 ± 0.0427	1.2613 ± 0.0493	1.8003 ± 0.1320	1.6694 ± 0.0604
B	0.0055 ± 0.0237	-0.0026 ± 0.0266	0.0114 ± 0.0587	-0.0987 ± 0.0295
C	$-8.7197 \times 10^{-4} \pm 0.0024$	0.0016 ± 0.0027	-0.0049 ± 0.0052	0.0051 ± 0.0027
Reduced Chi-Sqr	0.07	0.01	0.52	0.17
R-Square (COD)	0.07	0.039	0.69	0.90
Adj. R-Square	−0.39	0.09	0.53	0.85

NDI values as a function of X-rays and protons for U118 MG and U251 MG were presented in Fig. 3a and 3b, respectively. In our study for controls (non-irradiated cells), we observed statistically significant differences in mean values of proliferation index between U118 MG (1.19 ± 0.21) and U251 MG (1.78 ± 0.25). As accepted in radiobiological studies, to evaluate the dose-response effect, for both types of radiation, the linear-quadratic fitting was implemented by the least square method and the goodness of the fit was calculated using Chi-squared test [11, 12]. The fitting coefficients obtained for NDI distribution for U118 MG and U251 MG cell lines are presented in Table I. The R-Square values calculated for U251 MG achieved 0.69 for X-rays and 0.90 for protons, showing the dose-dependent relationship (Fig. 3b). A different case is observed when it comes to R-Square values for U118 MG cell line, where non-dependence dose relationship was observed (0.07 for X-rays, 0.39 post proton radiation). We also did not find statistically significant differences between NDI values for the same doses of protons and X-rays for U118 MG in the whole dose-range. The distribution of NDI values achieved constant-like-shaped from 0.0 Gy to 10 Gy (Fig. 3a) indicating that both radiation types appear to have a similar effect on the U118 MG cell line without dosage effect. Although, the U251 MG results show different radiation influence on the NDI values, only points 4.0 Gy and 8.0 Gy were statistically relevant ($p < 0.05$, Fig. 3b). For this type of GBM cells proton therapy inhibit strongly NDI, mostly in the dose range of 2–8.0 Gy. As a contrast in the whole study, the highest NDI values among studied cells and types of radiation were observed for U251 MG cells treated by X-rays. Surprisingly, the highest NDI was achieved for U251 MG after 4 Gy of X-rays. In order to examine if the obtained results for this point are correct, a blind test of repeated analysis with no opportunity to consult the results was performed. The measuring value was in agreement with previously achieved one for this dose.

Obtained results indicated that photon therapy may stimulate cells to faster growing to achieve higher values than control, that is in agreement with observations of other researchers. Ye et al. showed that Bmi-1 (B-lymphoma Mo-MLV insertion region 1) from the U87 MG cell line having the same grade as U118 MG has been proposed as an oncogene that suppresses the aging

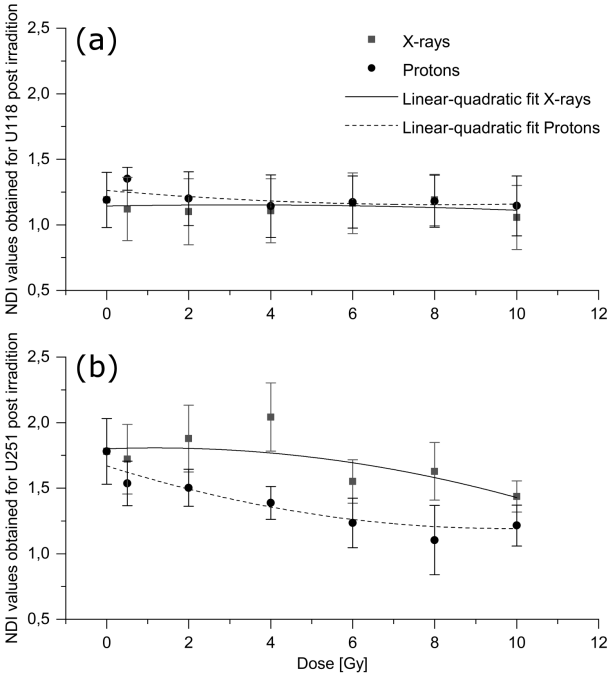

Fig. 3. Comparison between the nuclear division index of U118 MG (a) or U251 MG (b) cell lines irradiated with protons or X-rays determined by CBMN assay. The data represent the mean values of the nuclear division index (NDI) and SE(NDI) calculated for 500 cells, analyzed twice (as independent repetition).

of cancer cells and thus induces radioresistance. A dose of at least 6 Gy of X-rays resulted in an increase of Bmi-1 in U87 MG cell line [17]. Therefore, Bmi-1 might be important for cell radioresistance and might slightly change the NDI values of U118 MG cell line in comparison to U251 MG. It should be noted that U118 MG and U251 MG has been classified as the same grade IV, being the most malignant but were isolated from different donors [18, 19]. Actually, radiation is considered as a part of the care standard for the treatment of GBM, but individualized risk predictions and treatment decisions are made mainly of classified grade [20]. Our studies on glioblastoma multiforme proliferation index may have an impact on personalized treatment planning. The information from screening changes in the tumor cells genome

could guide clinical decisions making suggestions that not only classified grade but also the origin of tumor cells and genetic background may influence treatment outcome.

Numerous *in vitro* radiobiological studies for various cells using protons and photons have been carried out resulting in controversial data [21]. Authors showed that results are strongly influenced by the type of cells, culture condition, dose, dose rate, fractionation, and other physical features (i.e., LET, RBE) [22]. S.A. Amundson et al. published that proton irradiation compared to X or gamma irradiation increases gene expression level in both their number and strength of response [23]. In the case of rat C6 glioblastoma, similar to human glioblastomas grade IV, the significant decrease in proliferation was found at a dose of 5 Gy and 10 Gy of X-rays after 48 h of culture [24]. In human glioma MO54, 2 Gy of X-rays stimulated the expression of GAP-43 (growth associated protein 43). GAP-43 is known to be a crucial component of an effective regenerative response in the nervous system [25]. It is also postulated that X-rays can cause an increase in the production of CTSL (lysosomal cysteine protease cathepsin L), as well as increased migration and invasiveness of human glioma cells U251 MG [26]. In the human LN18 glioblastoma cell line, a dose below 2 Gy of X-rays had no effect on proliferation or apoptosis [27]. In our study, the effectiveness of proliferation inhibition by protons was more effective in comparison to X-rays, but only for U251 MG. Proton beam therapy mainly induces ROS-dependent mechanism of DNA damage and consequently brings to apoptosis of GSCs (glioma stem cells) in GBM patients. Thus, proton therapy may give better results than conventional photon therapy in growing inhibition of GBM [28].

While these data are preliminary, our laboratory is further exploring the influence of proton beam and X-rays as a reference on GBM cells response. Moreover, whole genes expression profile analysis of affected GBM cell lines is planned. Knowledge of genetic differences among GBM cell lines and studying their radio-sensitivity may enable to find genetic factors and other regulatory factors such as methylation status responsible for the radioresistance. Consequently, it can help to predict the radiosensitivity of tumors and thus develop appropriate treatment scheme to proton vs. photon therapy.

4. Conclusions

The main aim of the paper was to examine the influence of proton therapy and X-rays on the proliferation status of GBM human cell lines. The presented preliminary study shows that both types of radiation inhibit the proliferation, but to a different extent depending on the cell line used. A future experimental investigation involving measuring of micronuclei by CBMN assay, gene expression studies, and combined radiotherapy with TMZ chemotherapy will enable to better understand the molecular mechanisms of GBM response to applied therapies.

Acknowledgments

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