Response of Human HTB140 Melanoma Cells to Conventional Radiation and Hadrons

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Received December 7, 2010
Accepted March 16, 2011
On-line July 19, 2011

Summary

Conventional radiotherapy with X- and γ-rays is one of the common and effective treatments of cancer. High energy hadrons, i.e., charged particles like protons and ^{12}C ions, due to their specific physics and radiobiological advantages are increasingly used. In this study, effectiveness of different radiation types is evaluated on the radio-resistant human HTB140 melanoma cells. The cells were irradiated with γ-rays, the 62 MeV protons at the Bragg peak and in the middle of the spread-out Bragg peak (SOBP), as well as with the 62 MeV/u ^{12}C ions. The doses ranged from 2 to 24 Gy. Cell survival and proliferation were assessed 7 days after irradiation, whereas apoptosis was evaluated after 48 h. The acquired results confirmed the high radio-resistance of cells, showing better effectiveness of protons than γ-rays. The best efficiency was obtained with ^{12}C ions due to higher linear energy transfer. All analyzed radiation qualities reduced cell proliferation. The highest proliferation was detected for ^{12}C ions because of their large killing capacity followed by small induction of reparable lesions. This enabled unharmed cells to preserve proliferative activity. Irradiations with protons and ^{12}C ions revealed similar moderate pro-apoptotic ability that is in agreement with the level of cellular radio-resistance.

Key words

Protons • Carbon ions • HTB140 Melanoma • Survival • Proliferation

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Introduction

The secret of modern radiotherapy is to place the beam on the target with the desired dose, while sparing surrounding normal tissue. In the context of improving radiotherapy transition from conventional, i.e., photon radiation such as X- and γ-rays, to hadronic beams like protons and heavier ions is under way (Blattmann 1994, Amaldi and Kraft 2005).

Particles like protons and heavier ions have advantages for therapy compared to conventional radiation, such as well defined range, small lateral scattering and high energy deposition just before the end of the range, defined as the Bragg peak. The Bragg peak results from the increased ionization density or linear energy transfer (LET), leading to the rise of biological effectiveness toward the end of the particle range. Protons and heavier ions are considered as high LET radiation, although heavier ions have rather higher LET values. Therefore, the effect on DNA of heavier ions with respect to protons can be compared as the effect of "cannon ball" to "bullet" (Gerweck and Kozin 1999).

The commonly used parameter for assessment of radiation efficiency is the relative biological effectiveness (RBE), which reflects biological effectiveness of the specific type of ionizing radiation in comparison to the reference radiation, i.e., X- or γ-rays. The RBE increases with LET to reach its maximum value in the range from ~ 30 to 200 keV/μm (Barendsen 2000, Belli *et al.* 2000). It depends on the relative dose, ion species, initial energy of the beam, tissue and the analyzed biological end-point

(Kempe *et al.* 2007).

Since the Bragg peak is very narrow, in order to encompass the treatment volume it has to be spread-out by modulating the particle energy. For the improvement of radiotherapy, investigations of the effects obtained within the spread-out Bragg peak (SOBP) are of particular importance (Egger *et al.* 2003). Carbon (^{12}C) ions have some advantages, as compared to γ-rays, but also relative to protons. The main advantage of carbon beams with respect to protons is a favorable depth profile of the RBE. The RBE values for ^{12}C ions, due to higher LET attain larger values than for protons (Amaldi and Kraft 2005). Clinical results have shown that ^{12}C ion therapy can provide a sufficient dose to the tumor volume with a rather low destruction of the surrounding normal tissues (Tsujii *et al.* 2007).

The spectrum of induced lesions such as base damage, sugar damage, single strand breaks (SSB), double strand breaks (DSB), DNA-DNA and DNA-protein cross links, as well as their spatial distribution depends on radiation quality (Schwartz *et al.* 1991, Belli *et al.* 2002). Low LET radiation usually induces reparable single damages in DNA chain, while high LET radiation provokes multiple irreparable DNA breaks (Mulford *et al.* 2005).

Experimental results presented in this study are a part of an ongoing long-term research program accomplished within international collaboration of the Vinča Institute of Nuclear Sciences, Belgrade, Serbia and Istituto Nazionale di Fisica Nucleare, Laboratori Nazionali del Sud, Catania, Italy. These results represent chosen segments of an extensive investigation of radiobiological parameters and viability levels of a resistant human HTB140 melanoma cell line after exposure to conventional and different high ionizing radiation. To analyse and predict success of potential therapeutic irradiations for the radio-resistant limit case, assessment of the effects of two types of proton beams (at the Bragg peak maximum and along SOBP) and of a ^{12}C ion beam was performed.

Methods

Cell culture

Human melanoma HTB140 cell line was obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in the RPMI 1640 medium supplemented with 10 % FCS (Sigma Chemical Co., Steinheim, Germany) and penicillin/streptomycin (Sigma), in a humidified atmosphere of 5 % CO_2 at 37 °C (Heraeus, Hanau, Germany).

Irradiation conditions

Irradiations with protons and ^{12}C ions were carried out at the Istituto Nazionale di Fisica Nucleare, Laboratori Nazionali del Sud (INFN - LNS), Italy. Reference dosimetry was performed by the plane-parallel PTW 34045 Markus ionization chamber (Advanced Markus Chamber, 0.02 cm^2, Type 34045, PTW Freiberg, Germany), calibrated according to the International Atomic Energy Agency (IAEA) code of practice (IAEA-TRS-398 2000, Cirrone *et al.* 2004). All cell irradiations were carried out in air at room temperature.

For analysis of the effects of proton irradiations on the HTB140 melanoma cells, two experimental set-ups were applied. In the first case irradiations were performed with the monoenergetic 62 MeV proton beam. Irradiation position at the Bragg peak was simulated by inserting a 25 mm thick Perspex plate (Polymethyl methacrylate - PMMA) between the final collimator and the cell monolayer. Single doses delivered to the cells ranged from 8 to 24 Gy. The relative dose was 90.4±4 %, with the corresponding LET value of 9.08±0.38 keV/μm. In the second experimental set-up, the HTB140 melanoma cells were irradiated with the therapeutic 62 MeV proton beam. The irradiation position in the middle of the SOBP was obtained by interposing 16.3 mm thick Perspex plate between the final collimator and the cell monolayer. Single doses delivered to the cells were ranged from 2 to 16 Gy. The relative dose was 99.42±0.58 %, with the corresponding LET value of 4.71±0.15 keV/μm. The LET values were obtained by numerical simulations carried out with the GEANT4 code (GEANT4 1998). In both experiments with protons the average dose rate was 15 Gy/min.

For the analysis of effects of ^{12}C ions cells were irradiated with 62 MeV/u ^{12}C ions produced by the superconducting cyclotron in INFN – LNS, Catania. The position within the Bragg peak was obtained by interposing Perspex plate of 8.8 mm, giving the relative dose of 92.11±4.76 %. Single doses delivered to the cells ranged from 2 to 16 Gy at the dose rate of 11.03±0.27 Gy/min. The corresponding LET value was 415.27±42.84 keV/μm.

Irradiations with γ-rays, at the same dose levels, were performed using ^{60}Co source at the Vinča Institute of Nuclear Sciences in Belgrade, Serbia. Applied doses

ranged from 8 to 24 Gy, at the average dose rate of 1 Gy/min.

Clonogenic Assay

For the colony assay cells were harvested by trypsinization and seeded into 25 cm^2 flasks at a suitable number. After the incubation at 37 °C for 7 days colonies were fixed with methanol, stained with 10 % Giemsa solution and counted. Groups of more than 50 cells were scored as colonies. Survival was calculated by comparing the number of colonies in irradiated samples with untreated controls.

Proliferation assay

The 5-bromo-2-deoxyuridine (BrdU) assay (Roche Diagnostics GmbH, Mannheim, Germany) was used to measure cell proliferation. Assay was performed according to the manufacturer's instructions. The HTB140 melanoma cells were incubated with BrdU labeling solution for 2 h, fixed and anti-BrdU-POD antibody was added. After removing antibody conjugate, substrate solution was added and incubated until the color development was sufficient for the photometric detection (5-30 min). The reaction was stopped by adding 1 M H_2SO_4 solution. The absorbance was measured using a microplate reader (Wallac, VICTOR2 1420 Multilabel counter, PerkinElmer, Turku, Finland) at a test wavelength of 450 nm and a reference wavelength of 690 nm.

Flow cytometric analysis

Apoptotic cells were quantified by Annexin-V-FLUOS Staining kit (Roche Diagnostics GmbH, Mannheim, Germany). The assay was performed according to manufacturer's instructions. Cells were trypsinized, washed in ice-cold PBS and resuspended in 100 µl Annexin-V-FLUOS labeling solution (containing Annexin-V-FLUOS labeling reagent and propidium iodide - PI). After incubation for 15 to 30 min in the dark, 500 µl of incubation buffer was added per sample. In each sample, 10 000 cells were analyzed on the FACSCalibur (Becton Dickinson, USA). The number of apoptotic cells was calculated using the Cell Quest software.

Statistical analysis

Triplicate measurements were made during each experiment, while each experiment has been repeated three times, except for the cell proliferation assay that was performed in the quadruplicate experiments. The significance of differences between control and treated cells was assessed by the independent Student's t-test, with the level of significance set at $p<0.05$. Results were presented as the mean ± S.D. (standard deviation).

Results

Cellular responses to different types of radiation were analyzed on the radio-resistant human HTB140 melanoma cell line. Clonal survival as the most important parameter that describes the level of cellular radio-sensitivity was analyzed 7 days after irradiation, a time that is necessary for the development of the radio-biological response of this cell line. Experimental data were fitted to the linear-quadratic equation: $S = \exp(-\alpha D - \beta D^2)$, where S is the surviving fraction for the dose D, while α and β are the fitting parameters. Data obtained by the clonogenic assay and the fitted curves are shown in Figure 1.

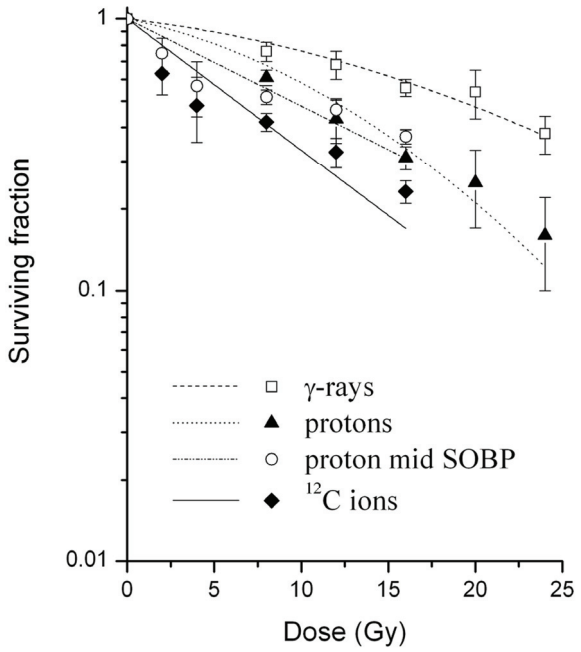

Fig. 1. Dose dependent surviving fractions of HTB140 cells irradiated with γ-rays, protons at the Bragg peak, protons in the middle of the SOBP and ^{12}C ions.

Considering very high radio-resistance and to overcome a moderate response the HTB140 cells were irradiated with γ-rays and protons at the Bragg peak using also the doses that are higher than those used in therapy, i.e. 8, 12, 16, 20 and 24 Gy. Although the applied doses were so high, the response remained moderate. For the irradiations with the protons in the middle of the

therapeutic SOBP and with ^{12}C ions the doses employed where those belonging to the therapeutic range, i.e. 2, 4, 8, 12 and 16 Gy. The levels of cell inactivation produced by both types of proton irradiation, as well as by carbon ions are stronger than that of γ-rays in the whole dose range. Also, ^{12}C ions have shown better killing ability than protons in the dose range from 2 to 16 Gy.

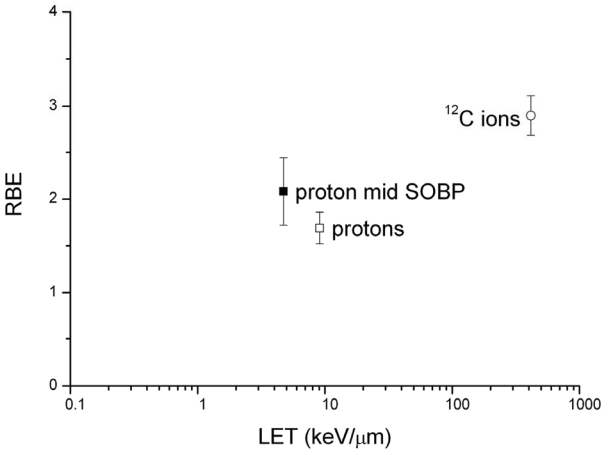

Fig. 2. RBE as a function of LET for protons at the Bragg peak, protons in the middle of the SOBP and ^{12}C ions.

The efficiency of protons and ^{12}C ions to inactivate cells with respect to conventional radiation (γ-rays) was analyzed through relative biological effectiveness at 2 Gy, RBE (2 Gy, γ), representing the dose ratio for the isoeffect. Analyzed types of radiation have shown different RBE for applied LET values and are given in Figure 2. The RBE values for protons, proton mid SOBP and ^{12}C ions were 1.69±0.17, 2.09±0.36 and 2.9±0.21, respectively.

Another important biological end-point for the analysis of the level of cellular radio-sensitivity is the cell proliferation. To compare the three types of radiation the chosen doses for this assay were 8, 12 and 16 Gy. Figure 3 shows that the proliferation capacity of the HTB140 melanoma cells after γ-irradiation was from 34 to 40 %, when compared to the control value. Similar results were obtained after the irradiation with protons close to the Bragg peak. When irradiation position was in the middle of the proton SOBP, the proliferation of melanoma cells was higher than in the two previous cases, ranging from 43 to 46 %. The highest level of cell proliferation was attained after irradiations with ^{12}C ions and was from 75 to 87 %, as compared to non-irradiated control.

The percentage of apoptosis was evaluated 48 h after irradiation. For the analysis of apoptosis, the applied doses were 8, 12 and 16 Gy. In previous studies, it was shown that γ-rays did not induce apoptosis in the HTB140 melanoma cells (Petrović *et al.* 2006). After proton irradiation close to the Bragg peak the level of apoptosis was ~ 18 %, in the whole dose range. Irradiations with ^{12}C ions did not additionally affect apoptosis. The level of apoptotic cells was around ~ 15 % for all three doses applied.

Discussion

Investigation of the effects of different radiation types, particularly regarding their LET values and track structure, were undertaken on the HTB140 human melanoma cells, representing the limit case of radio-resistance (Petrović *et al.* 2006, Ristić-Fira *et al.* 2007). The aim of this study is to reveal how far it is possible to go in inactivation, also considered as cell killing, of this cell line using radiation species with progressive efficiency. Moreover, the obtained results would serve for the characterization of the therapeutic beam at INFN – LNS, as well as for the provision of reliable experimental data for the validation and further development of the treatment planning system. Cellular responses to two different types of protons and carbon ions are evaluated through biological endpoints that were cell survival, proliferation and induction of apoptosis.

Due to the increased dose levels, the fitted curves for γ-rays and protons at the Bragg peak shown in Figure 1 display the well known shoulder shape (Bettega *et al.* 2000, Petrovic *et al.* 2006), although the curvatures are modest with a weak inclination. However, the stronger curvature and inclination of the proton curve illustrates their better killing ability. The fitted curves for the protons in the middle of the therapeutic SOBP and for ^{12}C ions are straight lines, because the lower doses applied produced data that allowed fitting only to the linear component of the linear-quadratic equation. Still, these two straight lines have larger slopes than the other two curves. The one for ^{12}C ions revealed the highest level of cell inactivation. Literature data suggested that ^{12}C ions with LET values of 20, 40 and 80 keV/μM showed better effectiveness on glioblastoma cells and fibroblasts (Tsuboi *et al.* 2007).

The RBE that are presented in Figure 2 pointed out that the highest value is obtained for the highest LET that is produced by ^{12}C ions. As for the two proton types of irradiation, higher RBE value with lower LET is attained with protons at the mid of the SOBP as

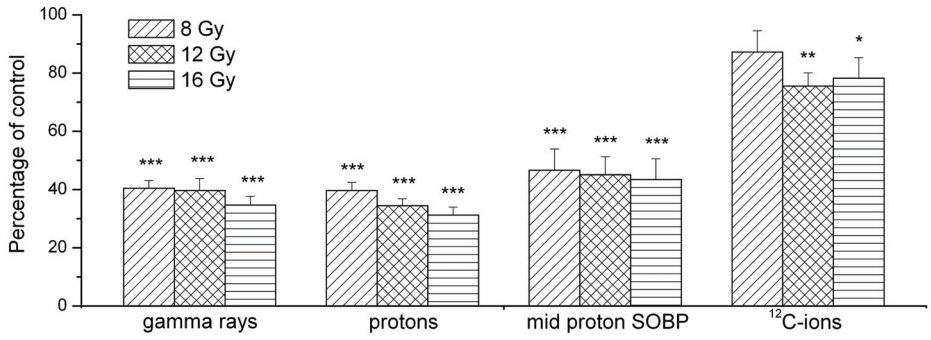

Fig. 3. Proliferation of HTB140 cells irradiated with γ-rays, protons at the Bragg peak, protons in the middle of the SOBP and ^{12}C ions. Statistical significance *vs.* control: * 0.01<p<0.05; **-0.001 <p<0.01; ***-p<0.001.

compared to the protons at the Bragg peak. Although this may seem contradictory, the explanation is as follows. In the case of ^{12}C ions and protons at the Bragg peak the beams are practically monoenergetic, meaning that the higher LET the greater cell inactivation is obtained, i.e. RBE. The beam of protons at the mid of the SOBP, due to its spreading is not monoenergetic and is characterized by a wide spectrum of proton energies. Therefore, its LET represents the mean value obtained from the series of high and low LET with obviously very different killing potential that is not linear. The contributions of the high LET components to the mean LET value and to the RBE are not the same. High LET components in the middle of the SOBP contribute considerably more to the inactivation of cells. This gives higher RBE for lower mean LET than in the case of the monoenergetic proton beam with the single although higher LET value.

Cell proliferation was evaluated after exposure of cells to irradiations with the therapeutic doses, i.e. 8, 12 and 16 Gy. Obtained results are shown in Figure 3. Irradiations with γ-rays and protons at the Bragg peak significantly reduced the HTB140 cell proliferation with a large and similar number of cells being arrested in order to repair radiation damage or being killed due to irreparable lesions. According to Figure 1 a greater portion of cells are killed after irradiation with protons at the Bragg peak. Similar results for antiproliferative effects of protons were reported for lung epithelial cells (Baluchamy *et al.* 2010). After irradiation in the mid of the SOBP again significantly low cell proliferation is obtained. Still, it is somewhat higher with respect to γ-rays and protons at the Bragg peak. This is caused by the contributions of higher LET components that increase cell killing, as given in Figure 1, and reduce cell arrest. Carbon ions, due to their even higher LET and greater killing, reveal high cell proliferation because more irreparable, but much less reparable lesions are induced.

Previous studies revealed that γ-rays did not induce apoptotic cell death in the HTB140 cells (Petrović *et al.* 2006). Similar results were reported in two different glioblastoma cell lines that were resistant to the induction of apoptosis with γ-rays (Tsuboi *et al.* 2007). The level of apoptosis after proton irradiation is in the range already shown for other resistant cell lines (Kumala *et al.* 2003). For protons and ^{12}C ions, 48 h after irradiations, the percentage of apoptotic cells ranges from 13 to 18 %. A non-linear dependence of apoptosis on dose (8 to 16 Gy) is in agreement with the non-linear killing capacity in the same dose range (Fig. 1) that was already noticed (Petrović *et al.* 2010). Similar pro-apoptotic level for protons and ^{12}C ions were reported for human glioma, PC3 prostate adenocarcinoma, Ca301D thyreoid carcinoma and MCF7 mammary adenocarcinoma cells (Di Pietro *et al.* 2006, Jinno-Oue *et al.* 2010).

Obtained results point out that protons and ^{12}C ions have better antitumor activity on resistant melanoma cells than conventional radiation. The best effectiveness in elimination of very resistant melanoma cells was achieved by ^{12}C ions due to their higher LET.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by the Ministry of Science and Technological Development of Serbia (grants 173046 and 171019) and Istituto Nazionale di Fisica Nucleare, Laboratori Nazionali del Sud, Italy. The authors would like to thank Lela Korićanac on her participation in data collection during irradiations.

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