Response of Human HTB140 Melanoma Cells to Conventional Radiation and Hadrons

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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 12C 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 12C 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 12C ions due to higher linear energy transfer. All analyzed radiation qualities reduced cell proliferation. The highest proliferation was detected for 12C 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 12C 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

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.
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 (\(^{12}\text{C}\)) ions have some advantages, as compared to \(\gamma\)-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 \(^{12}\text{C}\) ions, due to higher LET attain larger values than for protons (Amaldi and Kraft 2005). Clinical results have shown that \(^{12}\text{C}\) 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 \(^{12}\text{C}\) 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 % \(\text{CO}_2\) at 37 °C (Heraeus, Hanau, Germany).

**Irradiation conditions**

Irradiations with protons and \(^{12}\text{C}\) 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-TS-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/\(\mu\text{m}\).

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/\(\mu\text{m}\). 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 \(^{12}\text{C}\) ions cells were irradiated with 62 MeV/u \(^{12}\text{C}\) 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/\(\mu\text{m}\).

Irradiations with \(\gamma\)-rays, at the same dose levels, were performed using \(^{60}\text{Co}\) 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 cm² 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 H₂SO₄ 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 radiosensitivity was analyzed 7 days after irradiation, a time that is necessary for the development of the radiobiological response of this cell line. Experimental data were fitted to the linear-quadratic equation: \( S = \exp \left( -\alpha D - \beta D^2 \right) \), where \( S \) is the surviving fraction for the dose \( D \), while \( \alpha \) and \( \beta \) are the fitting parameters. Data obtained by the clonogenic assay and the fitted curves are shown in Figure 1.

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, protons in the middle of the SOBP and ¹²C ions.

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 ¹²C ions.
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.

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 (Bettgea
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
compared to the protons at the Bragg peak. Although this may seem contradictory, the explanation is as follows. In the case of 12C 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 12C 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 12C ions were reported for human glioma, PC3 prostate adenocarcinoma, Ca301D thyroid carcinoma and MCF7 mammary adenocarcinoma cells (Di Pietro et al. 2006, Jinno-Oue et al. 2010).

Obtained results point out that protons and 12C 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 12C ions due to their higher LET.

Conflict of Interest
There is no conflict of interest.

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