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Response of human lymphocytes to proton radiation of 60 MeV compared to 250 kV X-rays by the cytokinesis-block micronucleus assay

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A B S T R A C T

Particle radiotherapy such as protons provides a new promising treatment modality to cancer. However, studies on its efficacy and risks are relatively sparse. Using the cytokinesis-blocked micronucleus assay, we characterized response of human peripheral blood lymphocytes, obtained from health donors irradiated *in vitro* in the dose range: 0–4. 0 Gy, to therapeutic proton radiation of 60 MeV from AIC-144 isochronous cyclotron, by studying nuclear division index and DNA damage and compared them with X-rays.

Peripheral blood lymphocytes show decreased ability to proliferate with increasing radiation doses for both radiation types, however, in contrast to X-rays, irradiation with protons resulted in a higher proliferation index at lower doses of 0.75 and 1.0 Gy. Protons are more effective in producing MN at doses above 1.75 Gy compared to X-rays. Dose–response curves for micronucleus incidence can be best described by a cubic model for protons, while for X-rays the response was linear. The differences in the energy spectrum and intracellular distribution of energy between radiation types are also apparent at the intracellular distribution of cytogenetic damage as seen by the distribution of various numbers of micronuclei in binucleated cells.

Our studies, although preliminary, further contribute to the understanding of the mechanistic differences in the response of HPBL in terms of cellular proliferation and cytogenetic damage induced by protons and X-rays as well as intra-cellular distribution of energy and thus radiobiological effectiveness.

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Particle radiotherapy, such as the use of proton beams, provides a successful approach to treat cancer. However, experimental studies on the biological effects of proton irradiation are relatively sparse [1–3]. In particular, little is known on its relative effectiveness in inducing damage to a variety of normal tissues likely to be exposed during treatment [3–5]. During proton beam therapy, maximum absorbed dose is delivered to the tumor, while leaving normal tissue almost unaffected. This is possible due to the energy loss of incident ion particles occurring almost entirely at the end of their fly path in matter, known as the Bragg peak [1–3]. Even then, some degree of normal tissue injury is still inevitable. In most research performed to date, cancer cell lines were used for studying the biological mechanisms triggered by different types of radiation used during treatment [2,4]. Peripheral blood lymphocytes are distributed throughout the body, and are in constant circulation, hence chromosomal aberrations in these cells may reflect damage to normal tissue during radiotherapy. An understanding of the biological effects of proton beam irradiation on circulating lymphocytes can possibly help to optimize the proton therapy [3–5]. Further, radiation-induced chromosomal aberrations in peripheral blood lymphocytes may also serve as biomarkers of radiosensitivity that might differ among patients and may potentially allow treatment customization based on an individual's predicted response to therapy [6], and thus pave the way for "precision radiotherapy".

Several methods of different sensitivities are used to study the DNA damage induced by low- and/or high-LET (linear energy transfer) radiation [7]. In particular, the cytokinesis-blocked micronucleus (CBMN) assay was performed using human peripheral blood lymphocytes (HPBL), in which confounding effect of cell-division on the estimated frequency of micronuclei (MNi) is

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eliminated by preventing cytokinesis with cytochalasin-B. It is a simpler and faster method than the gold standard "dicentric chromosome" assay [8–11], as it estimates the frequency of micronuclei as a sensitive biomarker of radiation-induced cytogenetic damage. MNi are chromatin bodies found in the cytoplasm, originating from acentric fragments or whole chromosomes that are not included in the main nuclei following DNA replication and nuclear division [12–13], and represent chromosome breakage and/or loss [14]. They are measured in interphase cells, therefore a large number of cells can be analysed in a relatively short time, thus minimizing statistical uncertainty on the assessment of damage. This standardized method is now routinely used for population monitoring, determining genetic damage, screening of chemicals for genotoxic potential, predicting radiosensitivity of tumors, determining inter-individual variation in radiosensitivity, measuring chromosome breakage, chromosome loss, chromosome rearrangement (nucleoplasmic bridges), cell division inhibition, nondisjunction, necrosis, apoptosis, cytostasis etc. [10,15]. HPBL are widely used in biological dosimetry for assessing dose by measuring radiation-induced DNA damage [9]. HPBL are in the G_0 phase (a DNA presynthetic stage of the cell cycle) and consist of two main cell types, T and B cells. HPBL circulate throughout the body and can be stimulated by the phytohaemagglutinin (PHA) to undergo mitosis and offer many advantages over other cells in the study of radiation-induced chromosome damage [9,16].

In this paper, we applied CBMN assay to characterize the response of HPBL to irradiation by a therapeutic beam of 60 MeV protons delivered by the AIC-144 isochronous cyclotron. We studied cellular proliferation, MNi induction and distribution. The results were compared with those after the X-rays irradiation to explore the feasibility of using this endpoint in clinical proton radiobiology.

Materials and methods

Blood collection

Whole peripheral blood was collected after obtaining informed consent from healthy, non-smoking donors (2 male and 2 female), aged between 36 and 56 years, who had no known history of exposure to ionizing radiation, other than that necessary for routine medical diagnosis. Peripheral blood was collected into vacutainers containing lithium heparin by phlebotomy and then de-identified in the laboratory of The H. Niewodniczański Institute of Nuclear Physics Polish Academy of Sciences in Kraków, Poland (IFJ PAN); by the specialized company "Diagnostics". The human bioethical committee of the Regional Medical Board in Kraków approved the informed consent form used in this study (No. 124/KBL/OIL/2013).

Irradiation and dosimetry

Proton beam

The facility used for the proton irradiation was the AIC-144 isochronous cyclotron. It was originally designed and constructed at the IFJ PAN in late 1980s, and then adapted to medical applications between 2006 and 2009 [17–18]. The beam delivery system and the formation of proton beam were described in detail by Bakewicz and Swakoń [18–19]. The 60 MeV protons extracted from the cyclotron are delivered to the treatment room by a 25-m long beam-line system. The uniform lateral dose distribution is achieved by a single scattering system with a 25-μm tantalum foil located 10 m upstream of the isocentre. The collimated beam traverses through a six-sector ionization chamber and two beam monitors [17,18]. The Spread Out Bragg Peak (SOBP) homogenous dose distribution is controlled by a dedicated system located on an optical bench placed in the treatment room. It consists of a PMMA – Poly(methyl methacrylate) range-shifter wheel, range modulator with PMMA propeller and a set of four collimators placed in the beam line.

The proton beam monitoring system and the beam dosimetry

The dosimetry of the proton beam was accomplished with the PTW UNIDOS T10001 instrument and the semiflex ion chamber, PTW TM31010. The proton beam intensity was controlled by two transmission PTW ionization chambers, type TM7862, connected to electrometers. During irradiation the TM7862 ionization chambers carried out the function of dose monitors. The time- and spatial stability of the beam were controlled on-line by a 6 sector dedicated ion chamber, which consisted of 4 ion chambers in the form of quarters, a circle ion chamber and a ring one.

Dose measurements were performed in the middle of SOBP using a solid phantom (PMMA). The experiments were performed with a 40-mm diameter collimated beam. Overall uncertainty of dosimetry was about 3%, the precision of dose delivery was better than 0.5%. The average dose rate of the proton beam during irradiation was 0.075 Gy/s. Dosimetric equipment was calibrated at IFJ PAN, according to the TRS 398 protocol [20]. The therapeutic ^{60}Co unit-Theratron 780 E with the PTW Farmer ionization chamber type TM30010, a water phantom and the UNIDOS webline T10021 were used during calibration.

The proton and X-ray irradiation

For proton irradiation, heparinized whole blood samples in the amount 1.5 ml were exposed to doses in the range of 0.3–4.0 Gy (chosen doses were 0.3, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, and 4.0 Gy). For dose–response studies we used plastic chambers (Eppendorf vials) of 2 cm length, set in a specially designed PMMA phantom located at the isocentre, at a distance of 93 mm from the final collimator. The average dose rate used was 0.15 Gy/s.

For X-ray irradiation, heparinized whole blood samples in the same amounts of 1.5 ml from the same donors were used. The same dose range was applied to samples in sterile plastic tubes. The radiation was delivered at a dose rate of 1 Gy/min by a Philips X-ray machine (model MCN 323) at 250 kV, 10 mA operating at the IFJ PAN. The vials were held in a polyethylene box; the dimensions of the radiation field were 20×20 cm^2, and the source to the surface distance was 34.8 cm. Immediately after irradiation the tubes with blood were transferred to a box containing ice water and transported to the laboratory for cell culture. Prior to irradiation of cells, the X-ray dose was measured using the same chamber as for proton beam dosimetry. Both proton and X-irradiations were carried out at room temperature. A part of non-irradiated heparinized whole blood sample served as control (0.0 Gy).

Cytokinesis-block micronucleus assay

The CBMN assay was performed as previously described by Fenech [10]. Briefly, whole blood (0.5 ml) was added to 4.5 ml of RPMI 1640 culture medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% heat-inactivated foetal bovine serum (Gibco, Carlsbad, United States), L-glutamine (2 mM) and antibiotics (100 U/ml penicillin and 100 g/ml streptomycin; Polfa Tarchomin, Warszawa, Poland). Lymphocytes were stimulated by the addition of PHA (450 μl; PAA Laboratories GmbH, Pasching, Austria) and incubated for 72 h, at 37 °C at 5% CO_2 in a humidified incubator. After 44 h of initiation of the culture by the addition of PHA, 6 μg/ml cytochalasin-B in dimethylsulphoxide (Sigma-Aldrich, St. Louis, United States) was added to block cytokinesis. After 72 h of incubation, the cells were harvested by centrifuging and then re-suspending the cell pellet in a hypotonic solution (0.075 M KCl) and fixed with a fixative, methanol:acetic acid (3:1). After three washes with fixative, the cells were dropped onto

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