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Effects of 60 MeV Protons and 250 kV X-Rays on Cell Viability

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Particle radiotherapy such as the one using proton beams, provides a successful treatment approach in many cancer types. However, the cellular and molecular mechanisms by which proton irradiation induces cell death, particularly in a human peripheral blood lymphocyte model has not been examined in detail. Comparative studies of the biological effects, such as cell death, of particle therapy versus conventional X-rays treatment are of utmost importance. Here, we compared the viability of human peripheral blood lymphocyte following *in vitro* irradiation with protons (therapeutic 60 MeV proton beam) and photon beam (250 kV, X-rays), by applying separate doses within the range of 0.3–4.0 Gy. Cell viability was assessed 1 and 4 h after irradiation with protons and X-rays by the FITC-Annexin V labelling procedure (Apoptotic & Necrotic & Healthy Cells Quantification Kit, Biotium). Results showed that irradiation with both radiation types reduced the number of viable cells in a dose-dependent manner, as assessed as a function of the duration of post-irradiation time. Protons proved more fatal to the cells treated than X-ray photons. This demonstrates a difference in cell viability after irradiation with protons and photons in a human peripheral blood lymphocyte model.

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1. Introduction

The use of proton beam in radiation therapy has increased considerably in the past few years and this trend is likely to continue in the coming years. Due to the inverted depth-dose profile and enhanced biological effectiveness in cell killing, protons generally seem to offer advantages over X-rays in the treatment of a variety of tumours [1]. Because of the higher efficacy in cell killing, concerns about possible damage to normal tissue also needs to be addressed. Maximum ionization is achieved at a precisely controlled position within the tissue, just before the end of the beam range, which is known as the Bragg peak [2]. In order to target a whole of the tumour volume, extending over a certain depth range, a spread-out Bragg peak (SOBP) mode is made use of, whereby the energy of incident particles is varied according to the penetration depth required [3]. Therefore, dose optimization is needed at the Bragg peak to maximize tumour cells killing and reduce collateral normal tissue damage [3, 4]. We recently characterized the response of human peripheral blood lymphocyte (HPBL) in terms of cellular proliferation and cytogenetic damage after proton and X-ray irradiations to better understand the differences in the mechanisms of biological effects of these two radiation types, based on the differences in the intracellular distribution of energy and biological effectiveness [5].

Little is known on the differences in cell viability in the HPBL model following irradiation with protons and X-rays. Most research performed to date with protons included studies using plasmid DNA, cell lines, or animals to understand the biological mechanisms triggered by different types of radiation [1]. Although, Apoptotic & Necrotic & Healthy Cells Quantification Kit allows for studying viability and two types of cell death modes, due to preliminary character of this work, we presented here only cell viability of HPBL as a function of radiation dose, at 1 and 4 h after *in vitro* irradiation with protons and photons.

2. Experimental details

2.1. Blood collection

Whole peripheral blood was collected after obtaining informed consent from healthy, non-smoking donors (3 male and 2 female), aged between 36 and 56 years, who had no known history of exposure to ionizing radiation, other than routine diagnostic medical exposures.

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Peripheral blood was collected by phlebotomy into vacutainers containing lithium heparin in the laboratory of the Institute of Nuclear Physics of Polish Academy of Sciences in Kraków, Poland (IFJ PAN) by the company "Diagnostics" that routinely performs phlebotomy for diagnostics. Lymphocytes were separated by density gradient separation using Histopaque-1077®(Sigma Aldrich, St. Louis, United States) according to the procedure described by Panek et al. [6]. The human bioethical committee of the Regional Medical Board in Krakow approved the informed consent form donors used in this study (No. 124/KBL/OIL/2013).

2.2. Proton and X-ray irradiation and dosimetry

The proton beam facility beam delivery, monitoring system, X-ray irradiation and dosimetry have been previously described in detail [5, 7, 8]. Following density gradient separation, lymphocytes were resuspended in a RPMI 1640 culture medium (PAA Laboratories GmbH, Pasching, Austria) at a concentration of 5×10^4 cells/ml and were exposed to proton doses: 0.3, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 Gy at an average measured dose rate of 0.15 Gy/s. The samples were irradiated in SOBP with 29 mm range and modulation of 29 mm. During irradiations the plastic chambers (the Eppendorf vials) with lymphocytes were placed inside a specially designed poly(methyl methacrylate) (PMMA) phantom in the mid of SOBP and in the centre of the flat beam field with diameter of 40 mm, according with comparable conditions (irradiation of whole blood samples in plastic chambers) during proton irradiations done by Joksic et al. [9]. For X-ray irradiation, lymphocytes were exposed to same doses as during proton irradiation, but at a rate of 1 Gy/min using a Philips X-ray machine (model MCN 323) at 250 kV, 10 mA operating at the IFJ PAN. Both proton and X-irradiations were carried out at room temperature. A non-irradiated part of the sample served as control (0.0 Gy).

2.3. Measurement of cell viability and data analysis

Cell viability was determined at 1 and 4 h after irradiation with protons and X-rays using Apoptotic & Necrotic & Healthy Cells Quantification Kit (Biotium, Inc., USA), according to the manufacturer's procedure. The cell viability quantitation kit provides a convenient method for quantifying viable (blue only) cells within a cell population by fluorescence microscopy. At least 100 cells per dose were analysed by two independent scorers under a fluorescent microscope, which was coupled to an image-analysis system, according to the criteria described by Zhang et al. [10]. Experiments were repeated twice each. The results obtained by two independent scorers were statistically not different, hence are presented as an average. The Pearson correlation coefficient for cell viability evaluation after 1 h post-irradiation for X-rays was 0.85 and 0.91 for protons ($p < 0.05$) and after 4 h: 0.86 and 0.88 ($p < 0.05$), respectively.

All slides were coded and blinded to the scorer. Decoding was done only after completing the microscopic examination of all slides used for the study. The data were analysed using Microsoft Office Excel 2007. The dose–response curves were fitted with the OriginPro 9.0 32 bit (OriginLab Co., Northampton, MA, USA). The error calculation for the cell survival is presented as the mean ±SD (standard deviation) for 5 donors. Student's t-test was performed to assess the significance of differences among analysed experimental groups. To investigate if the results derived by two scorers are comparable, the Pearson correlation coefficient was calculated. The significance level was set at $p < 0.05$.

3. Results and discussion

Our objective here was to determine the differences between proton and X-ray irradiation within the first few hours following irradiation. Most studies using cells (thymocytes, lymphocytes or lymphomas) showed that cell death i.e. apoptosis, often started within minutes, 1–3 h following irradiation, *in vivo* or *in vitro*, and reached a maximum at 6 h or less. None of those papers presented data beyond 8 h [11].

Viability of lymphocytes as a function of X-ray dose after 1 and 4 h of irradiation is shown in Fig. 1A. Mean value of viability for all studied donors was 92.7 ± 1.2 in non-irradiated cells. X-ray irradiation resulted in a decrease in viability with increasing radiation dose, dropping the value to $81.2 \pm 2.9\%$ (1 h) and $76.9 \pm 5.4\%$ (4 h) after exposure to the highest dose of 4.0 Gy. In general, following this initial drop there was no significant change in lymphocyte viability between 1 and 4 h of X-ray irradiation, except at two doses of 0.3 and 2.0 Gy where the difference in viability was statistically significant ($p < 0.05$).

The dose–response curves comparing cell viability [%] after 1 and 4 h of proton irradiation for all the donors are shown in Fig. 1B. After irradiation with protons statistically significant differences in lymphocyte viability between 1 and 4 h were observed ($p < 0.05$), except at doses of 0.3, 2.5, and 4.0 Gy.

Protons were more efficient in inducing cell killing compared to X-rays, which is evident by the significant decrease in the number of viable cells at a 4.0 Gy dose to $45.8 \pm 9.2\%$ after 1 h and to $46.1 \pm 2.7\%$ after 4 h compared to $81.2 \pm 2.9\%$ after 1 h and 76.7 ± 5.4 after 4 h for X-rays (Fig. 1C and D). Decrease in lymphocyte viability is relatively rapid after proton irradiation compared to X-rays. Our results are in agreement with the observations of others using cell lines [1, 12–14]. Cellular response to proton irradiation could be different from response to photon radiation [1, 12]. Particle therapy is an emerging treatment option for many cancer. Therefore, studies on the effect of proton irradiation on cell viability is important to understand on normal tissue injury, possible within hours after irradiation, as difference in damage response pathways following irradiation will not only have bearing on adverse acute effects, but also on manifestation of late

injury [1]. It is well known that radiation-induced cell death is mediated via DNA damage. Previously, using V79 cells it was shown that there is a clear difference in the fraction of remaining double strand breaks 2 h after proton and photon irradiation [14]. This difference is due to a difference in the complexity of DNA damage induced by these two radiation types. A higher level of clustered DNA damage lesions and locally multiply damaged sites (LMDS) has been previously reported after proton irradiation [15]. Observed differences might also be due a difference in cell death pathways [16]. Therefore, studies on differences in gene expression related to these cell death pathways are necessary and laboratory is pursing studies in this direction.

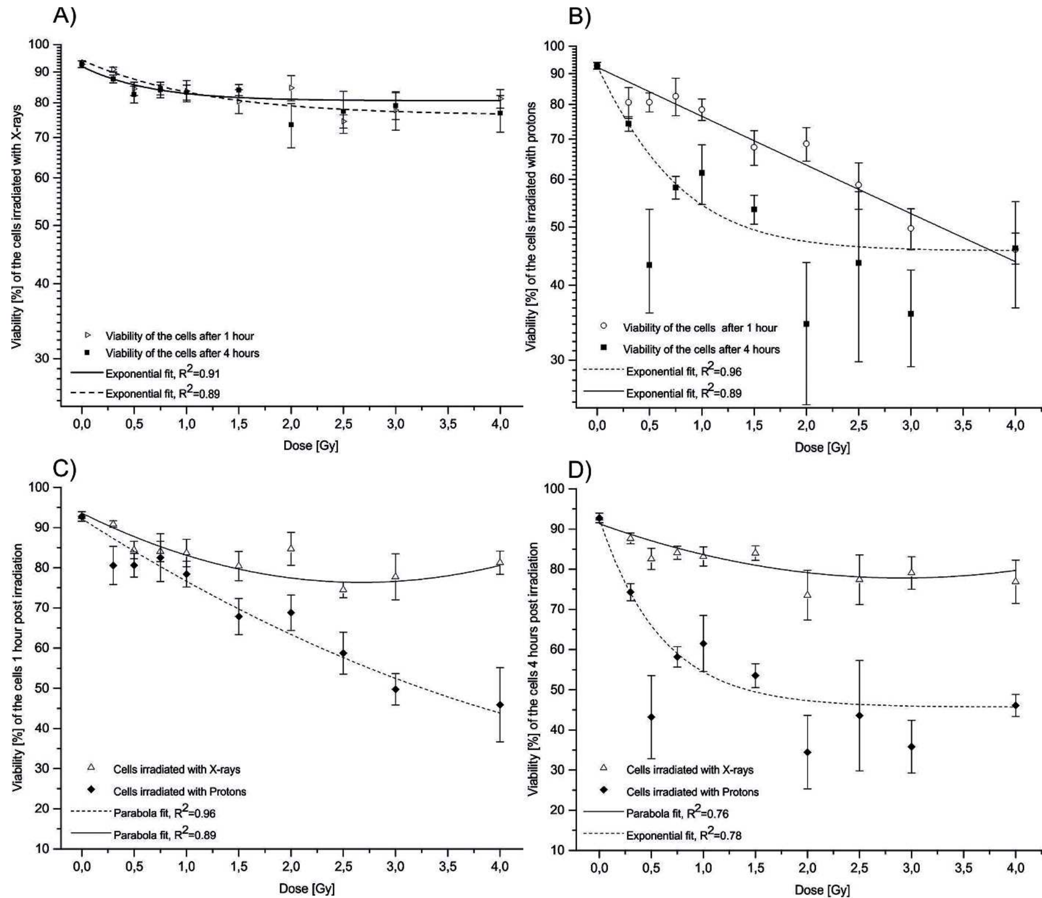

Fig. 1. Extent of viability [%] of the lymphocytes irradiated with X-rays (A) and protons (B) after 1 and 4 h, differences between X-rays and protons after 1 h (C) and 4 h (D) after irradiation as determined through the FITC-Annexin V staining procedure.

In our dose response studies involving both radiation types, we observed also inter-individual differences. Generally, for each donor viability decreased with dose, but the extent of this difference was different among individual donors. Such variations of different response of HPBL by *in vivo* irradiation have been seen in our previous study [5]. Individual cellular sensitivity may depend on many factors i.e. cellular repair capacity, genetic differences, etc. [17]. The biological responses of irradiated cells are also dependent on the dose rate [11]. A reduction in the dose rate favours accumulation of cells in the G_1 and G_2 phases of the cell cycle because of prevalence of repairable DNA damage. This cell cycle delay provides time for DNA to repair and allows recovery of cells in contrast to apoptosis induction at higher dose rates [5]. The measurement of cell viability plays a fundamental role in predicting of tissue/cell response to different types of radiation [18, 19]. While these data are preliminary, our laboratory is further exploring the differences in the modes of cell death in the *in vitro* HPBL model following irradiation with protons and photons. Such an understanding might help advance our knowledge on the effects of proton irradiation on mechanisms of cell death, which might be useful in improving proton therapy protocols.

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