Clinical and Translational Radiation Oncology 9 (2018) 23–29

Contents lists available at ScienceDirect

Clinical and Translational Radiation Oncology

journal homepage: www.elsevier.com/locate/ctro

Original Research Article

Do protons and X-rays induce cell-killing in human peripheral blood lymphocytes by different mechanisms?

J. Miszczyk [a,*], K. Rawojć [b], A. Panek [a], A. Borkowska [a], P.G.S. Prasanna [c], M.M. Ahmed [c], J. Swakoń [a], A. Gałaś [d]

[a] Institute of Nuclear Physics Polish Academy of Sciences, PL-31342, Poland
[b] Department of Endocrinology, Nuclear Medicine Unit, The University Hospital, Kraków, Poland
[c] Radiation Research Program, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA
[d] Department of Epidemiology, Chair of Epidemiology and Preventive Medicine, Jagiellonian University Medical College, Kraków, Poland

ARTICLE INFO

Article history:
Received 25 July 2017
Revised 31 December 2017
Accepted 15 January 2018

Keywords:
Proton biology
Human peripheral blood lymphocytes
Cell-killing modes
Apoptosis
Necrosis
Proton therapy
Photon therapy
Radiotherapy

ABSTRACT

Purpose: Significant progress has been made in the technological and physical aspects of dose delivery and distribution in proton therapy. However, mode of cell killing induced by protons is less understood in comparison with X-rays. The purpose of this study is to see if there is any difference in the mode of cell-killing, induced by protons and X-rays in an *ex vivo* human peripheral blood lymphocyte (HPBL) model.

Materials and methods: HPBL were irradiated with 60 MeV proton beam or 250-kVp X-rays in the dose range of 0.3–4.0 Gy. Frequency of apoptotic and necrotic cells was determined by the Fluorescein (FITC)-Annexin V labelling procedure, 1 and 4 h after irradiation. Chip-based DNA Ladder Assay was used to confirm radiation-induced apoptosis and necrosis. Chip-based DNA Ladder Assay was used to confirm radiation-induced apoptosis.

Results: *Ex vivo* irradiation of HPBL with proton beams of 60 MeV or 250 kVp X-rays resulted in apoptotic as well as necrotic modes of cell-killing, which were evident at both 1 and 4 h after irradiation in the whole dose and time range. Generally, our results indicated that protons cause relatively higher yields of cell death that appears to be necrosis compared to X-rays. The analysis also demonstrates that radiation type and dose play a critical role in mode of cell-killing.

Conclusion: Obtained results suggest that X-rays and protons induce cell-killing by different modes. Such differences in cell-killing modes may have implications on the potential of a given therapeutic modality to cause immune modulation *via* programmed cell death (X-rays) or necrotic cell death (proton therapy). These studies point towards exploring for gene expression biomarkers related necrosis or apoptosis to predict immune response after proton therapy.

Introduction

Protons with energies from 60 to 250 MeV are being used in the treatment of certain types of cancer (e.g., paediatric, head and neck, brain, gastrointestinal, lung, genitourinary, eye tumours) [1]. Compared to conventional radiotherapy, they offer better dose delivery and distribution, and thus lower probability of collateral normal tissue damage and lower risk of post-treatment complications [1,2]. Several phase I and II clinical trials are ongoing to explore the advantages of proton therapy over X-rays [2]. The physical properties of proton beams used in therapy have been widely characterized [3]. Despite of the well understood physical aspects of proton therapy, proton biology and its clinical relevance are still less understood [4]. The results of ongoing studies suggest that the biological response following proton irradiation is modulated differently than after X-ray exposure [5]. A deeper understanding of dissimilarity in cell killing induced by proton beams in comparison to photons is necessary. Previously, we characterized the response of Human Peripheral Blood Lymphocytes (HPBL) to therapeutic proton radiation of 60 MeV, by studying the nuclear division index and DNA damage and compared the results with X-rays [6]. A spatial difference in the energy deposition with proton irradiation in comparison to X-rays resulted in a localized manifestation of cytogenetic damage at cellular level [6]. These studies led us to believe that there might be differences in

* Corresponding author at: Department of Experimental Physics of Complex Systems, Institute of Nuclear Physics Polish Academy of Sciences, Radzikowskiego 152 Street, Kraków, PL-31342 Poland.
E-mail address: justyna.miszczyk@ifj.edu.pl (J. Miszczyk).

https://doi.org/10.1016/j.ctro.2018.01.004
2405-6308/

cell-killing modes between protons and photons, due to a difference in the spatial distribution of energy, which might be clinically relevant in evoking or suppressing immune response.

Radiation induces cell-killing through different modes: apoptosis, necrosis, necroptosis, autophagy, senescence, and mitotic catastrophe [7]. Apoptosis and necrosis are two major cell death modes, controlled by different physiological processes and molecular pathways [8]. Generally, irradiation induces apoptosis in most normal cells, but it also occurs in some tumour types [9], and peaks at 3–5 h after irradiation depending on cell type and radiaton dose. Susceptibility to apoptosis is a major determininant of radiosensitivity for most cells [9,10]; higher radiosensitivity of lymphocytes is due to their propensity to apoptosis [11].HPBL are predominantly in a resting phase (G0) of the cell cycle, they are a synchronous and homogeneous cell population, which is in continuous trafficking throughout the body and represent normal tissue. Lymphocytes are involved in many key mechanistic roles following exposure to radiation therapy of tumours, which include, systemic responses at distant sites, enhancement of anti-tumour innate and adaptive immune response, enhanced tumour recognition and killing *via* up-regulation of antigen presenting machinery and induction of positive immunomodulatory pathways due to trafficking of lymphocytes into the tumour microenvironment [12]. It has recently become apparent that particle therapy may distinctly affect cell death pathways, leading to an increased immunogencity [13]. Since proton treatment will minimize exposure of normal tissue [1], thereby exposure of normal lymphocytes in relation to photon irradiation, immunogenecity is likely to be less compared to photons in circulating lymphocytes [13]. Among patients treated with C-ions for esophageal, uterine and cervical cancers in peripheral blood lymphocytes level of cytogenetic damage was lower compared to X-rays [14].

Since HPBL traffic throughout the body, which include irradiation field, could potentially be used to interrogate radiation injury to normal tissue during irradiation of tumours. There is a need to understand the differences in cell-killing mechanisms induced by currently used radiation therapies; not only cell-killing in the tumour tissue but also in normal tissue, since total sparing of normal tissue within the treatment volume is not feasible. In this article, we present the results of our studies that looked at the differences in modes of cell-killing in an HPBL model, which represents the normal tissue, after *ex vivo* irradiation with photons and protons. Also, we discuss the possible mechanistic reasons for these differences, limitations, and potential implications for radiation therapy in light of emerging literature in this rapidly evolving field.

Materials and methods

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, in the same conditions as described earlier [6]. Lymphocytes were isolated by density gradient separation using Histopaque®-1077 (Sigma–Aldrich, St. Louis, United States). Cell viability was tested by the trypan blue exclusion test. The number of dye-excluding cells was 100% for all donors. The human bioethical committee of the Regional Medical Board in Krakow approved the informed consent form used in this study (No. 124/KBL/OIL/2013).

Proton and X-ray irradiations and dosimetry

Proton and X-ray irradiation procedures have been previously described in detail [6]. Briefly, HPBL irradiations with X-rays and protons were performed at the Institute of Nuclear Physics, Polish Academy of Sciences (IFJ PAN), Krakow, Poland. After acceleration, proton beam was delivered to the treatment room by a small field horizontal beam line. The parameters of a fully modulated proton beam with Spread Out Bragg Peak (SOBP) were as follows: 30-mm range, 30-mm modulation (measured in water phantom) and field diameter was collimated to the 40-mm lateral diameter. Parameters of the radiation field ensured homogenous distribution of the dose throughout the irradiated samples placed in eppendorf vials in a cell container. At the center of the cell container position, i.e. at the depths 15-mm of the SOBP, the dose-averaged Linear Energy Transfer (LET) was 2.9 keV/μm. Within the sample position in the SOBP the dose-averaged LET ranged from 2.5 keV/μm to 3.8 keV/μm [15]. The proton beam dosimetry was done as described previously [6], according to the TRS-398 protocol recommended by International Atomic Energy Agency [TRS-398] using a reference dosimeter consisting of a PTW TM31010 semiflex ionization chamber and a PTW UNIDOS Webline Electrometer (PTW, Freiburg, Germany). The dosimeter set was calibrated at the IFJ PAN at Theratron 780 Co-60 treatment unit. Lymphocytes were irradiated in 2 ml eppendorf vials (Eppendorf, Hamburg, Germany) with doses: 0.3, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 Gy for protons and X-rays. The cell number was scored in a Bürker chamber and then resuspended in 1.5 ml RPMI 1640 culture medium (PAA Laboratories GmbH, Pasching, Austria). The final concentration of cell suspension was 5×10^4 cells/ml. A specially designed PMMA-Poly (methyl methacrylate) phantom was placed at the irradiation setup isocentre (in the middle of SOBP) and in the centre of the flat beam. The average dose rate was 0.075 Gy/s. For X-ray irradiation, samples from the same donors were irradiated with the same doses as used for proton irradiation with a dose rate of 0.15 Gy/s by a Philips X-ray machine at the same conditions as described previously [6].

Both proton and X-ray irradiations were carried out at room temperature. Post-irradiation incubation of lymphocytes was done at 37 °C in RPMI 1640 culture medium supplemented with 10% heat-inactivated foetal bovine serum (Gibco, Carlsbad, United States). A non-irradiated part of the sample served as control (0.0 Gy).

Apoptosis and necrosis quantification

To quantify apoptosis and necrosis in our *ex vivo* HPBL model; Apoptotic, Necrotic and Healthy Cells Quantification Kit (Biotium, Inc., Hayward, USA) was used. The kit allows simultaneous quantification of apoptotic, necrotic and healthy cells. Identification and discrimination of apoptotic and necrotic cells *in vitro* can be challenging, especially late stage apoptosis from necrosis [16]. Biotium kit cannot distinguish late apoptosis from necrosis. We preferred to use fluorescence microscopy with the Biotium kit over flow cytometry for quantitative measurements of apoptosis and necrosis and then used apoptotic ladder kit for confirmation of apoptosis (see descriptions below). In this test, HPBL were washed in PBS and resuspended in 1X binding buffer, then 5 μl of FITC-annexin V, ethidium homodimer III and Hoechst 33,342 solution was added to each tube and incubated for 15 min at 21 °C in dark. HPBL were then washed 2 times with 1X binding buffer, fixed with 2% formamide, placed on a glass slide and covered with a glass coverslip.

Generally, 4–6 representative fields of at least 100 cells per dose, per time point were analyzed separately from 3 independent triplicates (slides), by two independent scorers using fluorescent microscopy coupled to an image analysis system (MetaSystems™, Altlussheim, Germany), according to the criteria described by Zhang et al. [17]. Experiments and irradiations were repeated twice each. All slides were coded, blinded to scorers. Sample decoding