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Application of an ultra-high dose rate (FLASH) proton beam for the 3D cancer cell model – a proof of concept

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ABSTRACT

Ultra-high dose rate (FLASH) proton radiotherapy is a promising treatment method for cancer patients. In our research, we want to compare the FLASH method with a conventional radiation method to show what effect they have on the biochemical structure of the tumour (3D model – spheroids) and the secretion of extracellular vesicles (EVs) and their cargo. The use of a modern method of creating spheroids will enable us to create conditions that are better able to mimic the tumour microenvironment.

KEYWORDS

FLASH proton radiotherapy, spheroids, melanoma cancer, extracellular vesicles

INTRODUCTION

The key task of radiotherapy, which is required in the treatment of cancer in 60–70% of patients, is to eliminate the tumour without causing undesirable effects in non-malignant tissue [1, 2]. Ultra-high dose rate (FLASH) radiotherapy is considered one of the most promising breakthroughs in cancer treatment. This is the administration of ultra-high dose rate radiation (>40 Gy/s) several orders of magnitude higher than what is currently used in standard of care (SOC) radiotherapy (0.5–5 Gy/min) [3, 4]. Different modalities are used to generate FLASH dose rates, depending on the type of radiation. For electrons, including electron beam-based FLASH linear accelerators or synchrotron, light sources are used, and for proton FLASH usually proton accelerators are tested [5]. It is also worth mentioning that there is ongoing development of new methods for flash proton beam range monitoring [6–8], and for monitoring the degree of tissue hypoxia by positronium produced in the body during proton beam irradiation [9–11].

The first clinical trials focus on the use of proton FLASH radiation therapy for bone symptomatic cancers with painful bone metastases in the extremities [12, 13]. In the FLASH Radiotherapy for Skin Cancer (LANCE) clinical trial, is planned to use the electron beam-based in a 22 Gy single dose and a fractionated dose (5 × 6 Gy) for small (T1) or large (< 2 cm T2 ≤ 4 cm) Cutaneous Squamous Cell lesions [14]. Only a few clinical trials have been published with high-dose proton beam therapy for non-ocular malignant melanoma, showing promising results for sinonasal mucosal melanoma treatments [15].

STATE-OF-THE ART

Despite huge progress in moving FLASH radiotherapy to clinical trials, the molecular radiobiology underlying the FLASH effect is scarcely understood and in vitro experiments are necessary. An emerging need is to develop a preclinical model for radiotherapy dose monitoring in FLASH proton therapy, where tumourspheres are a proven research tool [8, 16].

Previous studies conducted on 2D melanoma cell lines have shown that conventional proton beam irradiation at doses of 1, 3, or 5 Gy, causes a decrease in beta-1-integrin in the Mel270 cell line, an increase in the level of vimentin (EMT marker) in the BLM cell line, and an inhibition of migratory capacity [17]. Another study conducted by Jasińska-Konior et al. showed that low-LET proton beam causes changes in the organisation of the actin cytoskeleton and reduces cell elasticity, which remains at a reduced level long after irradiation [18]. A cell sublethal dose (3 Gy) of proton--beam radiation causes a 2.3-fold decrease in the vimentin level, which is a marker of metastatic properties and the epithelial to mesenchymal transition of melanoma [19].

Extracellular vesicles (EVs) are double-membrane vesicles, of size 100–1000 nm, which are secreted by cells into the extracellular space [20]. A cargo of EVs consists of proteins, lipids and nucleic acids. EVs may serve as a therapeutic target or therapeutic medium in radiotherapy. In proton therapy for glioblastoma, miR-574-3p carried by EVs may be considered a biomarker for monitoring the efficacy of radiotherapy, because of an explicit decrease in the level of such miRNAs in plasma exosomes after irradiation [21]. EVs derived from mesenchymal stem cells (MSCs), combined with radiotherapy, are determinant in the enhancement of radiation effects observed in the control of the metastatic spread of melanoma cells [22]. Exosomes increase the levels of mRNAs and survival-promoting pathway proteins, carcinogenic miRNAs (miR-889), but also decrease the levels of tumour suppressive miRNAs (miR-365 and miR-516) and mRNAs, hence EVs are supposed to promote the survival rate of cells exposed to 3 Gy or 12 Gy doses [23].

Hypoxia (low oxygen) is a common feature of solid tumours, which is significantly associated with radiation outcomes and poor patient survival [24–27]. FLASH irradiation might induce the protection of mammalian cells through transient hypoxia (including cancer cells) [28–32], whereas hyperoxia can eliminate FLASH effects in mice [33].

METHODOLOGY

The use of common methods of studying tumourspheres, i.e. histology (reflection of the cellular structure of the tumoursphere) and confocal microscopy (the role of hypoxia after tumoursphere irradiation, e.g. HIF-1 staining), wound healing and angiogenesis assays (evaluation by vascular formation on matrigel), in combination with modern methods, i.e. spectral flow cytometry (for EVs analysis – annexin V, CD9, CD63, CD81 and CD87 – uPAR), confocal Raman microscopy and FTIR (analysis of protein and lipid spectra in spheroids), will allow us to delve into the changes occurring within the irradiated tumourspheres (Fig. 1.). In our study, we plan to develop a methodology to produce melanoma and glioblastoma tumourspheres, which are three-dimensional (3D) cell aggregates (about 1–2 mm diameter) that can mimic tissues and microtumours (Fig. 2.). The use of a 3D model undoubtedly reproduces the conditions of the tumour to a greater extent compared to 2D cultures, but it should be remembered that in the human body the size of the tumour is much larger. A study of 104 glioblastoma scans showed the median area of cross--sectional method on 2D was 1151 mm^2 [34].

We decided to use melanoma cells because of the possibility of referring to the 2D studies conducted by Jasińska-Konior and the currently conducted clinical trial on FLASH radiotherapy. As the second research model, we chose glioblastoma because of its aggressiveness and a two-year survival rate of 10.4 percent (total dose 60 Gy, fractionated focal irradiation in daily fractions of 2 Gy, given 5 days per week for 6 weeks). Studies in mice have shown that FLASH-RT administered in hypo-fractionated regimens (daily fractionated doses of 4 × 3.5 Gy or 2 × 7 Gy; or 3 × 10 Gy spaced by 48 hours) is able to spare the normal brain from radiation-induced toxicity without compromising cancer treatment [35].

We would like to focus on the use of the proton beam with FLASH intensity (60 Gy/s) and deliver doses of 6 and 20 Gy and a dose of 3 Gy, using SOC proton radiotherapy (0.15 Gy/s) to compare the effect of two different dose rates on the biological systems of tumourspheres and on EV secretion and origin.

STUDY OBJECTIVES

We would like to answer the following questions:

1. Do EVs released from tumourspheres differ depending on dose rate?

2. Do secreted EVs affect vascular cells?

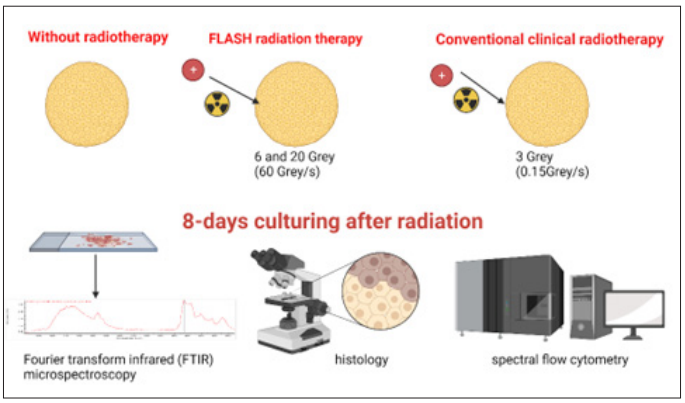

Fig. 1. Research scheme.

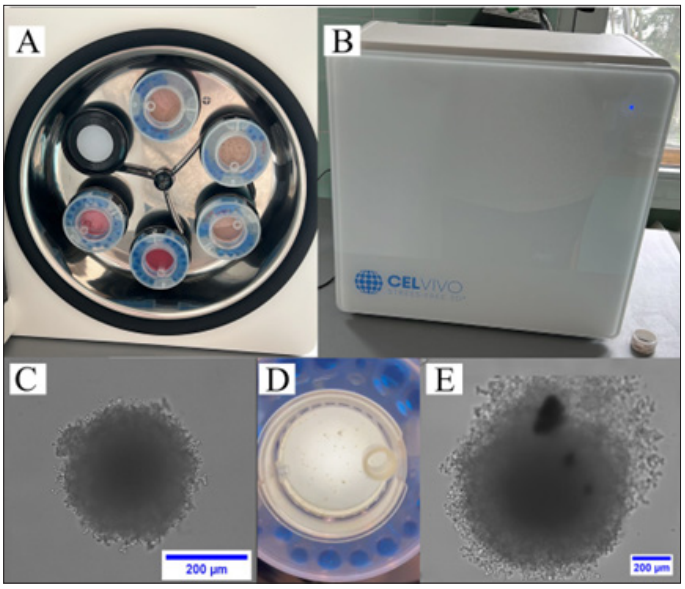

Fig. 2. (A) Celvivo ClinoStar Incubator; (B) Celvivo ClinoStar Incubator – external appearance of the device; (C) Example image of spheroid produced in 96-well plate; (D) The rotor with spheroids; (E) Example image of spheroid produced in ClinoStar Incubator.

3. What is the relationship between tumour hypoxia and the response to radiotherapy depending on the method used?

4. How do different radiotherapy methods change the biochemical composition of spheroids (lipid – protein content)?

EXPECTED RESULTS

Raman spectroscopy and IR microscopy are label-free optical and non-destructive spectroscopic techniques with the potential for in vivo tracking of overall biomolecular changes in tissue and cells. We expect to show a decrease in the spectra for amide 1 and 2 in the irradiated spheroids compared to the control spheroids. A greater decrease should be observed in spheroids

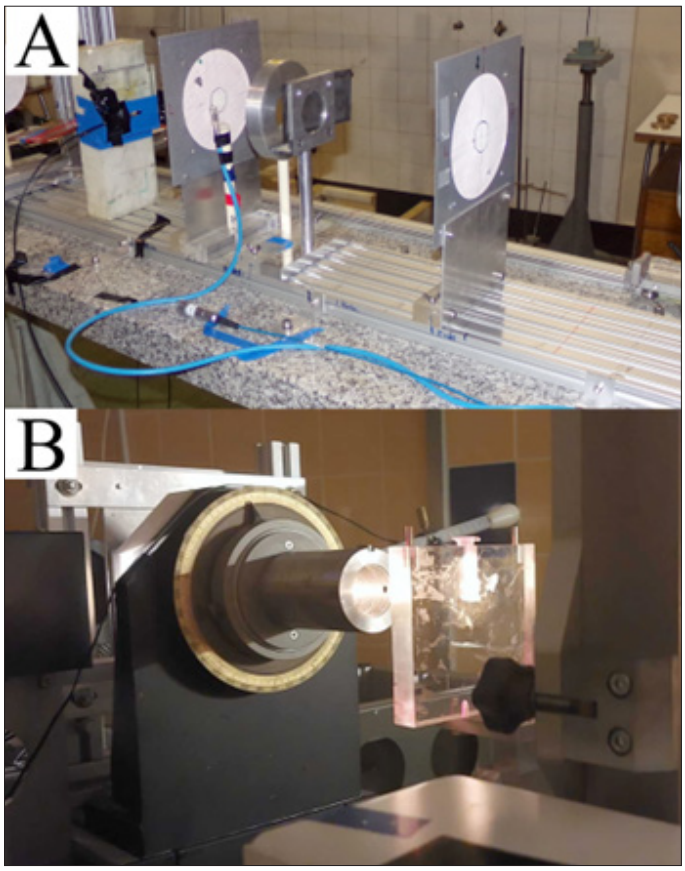

Fig. 3. (A) Sample in Eppendorf vessel located in PMMA phantom at proton irradiation station; (B) configuration of proton FLASH irradiation line.

irradiated using the conventional method, where the irradiation time is much longer than the FLASH method. Within a short time (approx. 24 hours) after irradiation, lipid oxidation and lipolytic activity are increased in cells, which is associated with a decrease in lipid content. After about 48 hours after radiation, their level increases as a result of cellular ER stress response or apoptotic processes [36]. We expect to obtain an increased signal from lipids spheroids irradiated with ultra-high dose rate proton FLASH (approx. 60 Gy/s).

Our pilot studies so far have shown that spheroids secrete EVs. We expect to demonstrate that EVs secreted by irradiated spheroids differ in their cargo and therefore can modify processes related to carcinogenesis (i.e. angiogenesis, wound healing assay).

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