Radiotherapy and Oncology 139 (2019) 64–65

Contents lists available at ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com

Letter to the Editor

Response to letter regarding "An integrated physico-chemical approach for explaining the differential impact of FLASH versus conventional dose rate irradiation on cancer and normal tissue responses"

We are gratified that our mechanism-oriented proposal on how FLASH ultra-high dose rate ionizing radiation (FLASH-RT; >40 Gy/s) may protect against normal tissue damage while preserving tumor responses is sparking discussion in the research community [1]. The current use of fractionated ionizing radiation to treat cancer with relatively low-dose rates is designed to provide a differential toxicity between normal and cancer tissue based on approaches that maximize the presence of oxygen and reoxygenation in the tumor tissue during treatment. These approaches have been largely based on the assumption that normal tissue is always well-oxygenated radio-biologically and therefore relatively unaffected by O_2 tension. In stark contrast to the results with relatively low dose rates, excess normal tissue oxygenation beyond physiological concentrations (using carbogen breathing in animals) has recently been reported to significantly reduce the normal tissue protection seen in brain with the ultra-high dose rates used in FLASH-RT [2]. Our proposed mechanistic hypothesis for how FLASH-RT can minimize oxidative distress to normal tissue compared to tumor tissue is based on a thorough reconsideration of the fundamental principles of free radical radio-chemistry occurring both at the time of exposure and shortly thereafter that will modify the entire biological response following exposure to ultra-high dose ionizing radiation. We appreciate the comments made by our esteemed colleagues and have carefully considered each one to help guide experimentation focused on validating mechanistic studies for accepting or refuting our proposed hypothesis explaining the remarkable normal tissue sparing effects of FLASH-RT. The many estimations we made in this hypothesis were generated in the spirit of a "Fermi estimate". Several points for discussion that were initiated by our colleagues are addressed below:

1. **Time scale:** Indeed as Professor Wardman has summarized, time is undeniably a key and complex variable for the delivery of ionizing radiation and the subsequent biochemical and biological consequences [3]. Moreover, the time structure of the FLASH beam is an important characteristic, and will help distinguish between important parameters such as the mean dose rate and the instantaneous (intra-pulse) dose rate as critical determinants for the FLASH effect (8). Thus, given our current knowledge, the statement that the "pulsatile nature of the LINAC is likely irrelevant" is simply inaccurate. Importantly, our goal was to specifically emphasize that upon FLASH-RT a great deal, if not all, of the available tissue dioxygen could be consumed in a single, very short pulse. Well-known radiation chemistry would indicate that a substantial fraction of the dioxygen present at the "instant" of FLASH-RT will react rapidly with the downstream carbon-centered radicals ($R^{\cdot}$) formed, yielding oxidizing peroxyl radicals ($ROO^{\cdot}$). These peroxyl radicals will abstract hydrogen atoms from neighboring substances yielding an organic hydroperoxide (ROOH) and a new organic free radical ($R^{\cdot}$). These radicals can in turn initiate new chain reactions. This amplification could result in the complete depletion of oxygen. Any H_2O_2 formed can also feed into these chain reactions *via* Fenton Chemistry [4]. Thus, the time scale for the delivery of the ionizing radiation and the ensuing radiation chemistry with tissue O_2 are indeed important considerations.
2. **DSB and cell killing**: We agree that double strand breaks contribute to the cytotoxicity observed after FLASH-RT and we are well aware of the microdosimetric nuances of ionizing radiation and resultant "locally multiply damaged sites" [5]. Furthermore, data to date (unpublished) have not revealed a significant difference in the response to DNA damage between FLASH and conventional dose rate exposures, increasing the likelihood that late normal tissue toxicities (brain, lung) are driven by fundamentally different reactivities and signaling pathways. In this regard, the oxidative challenge posed by the different redox environment of tumor cells (relative to normal cells) may be overwhelming and contribute to cell death *via* additional pathways, *i.e.* normal cells are much better able to cope with the oxidative challenge presented by FLASH-RT, yielding the differential biological effects needed for successful enhancement of the therapeutic window. This will need to be investigated using manipulations of hydroperoxide metabolism as well as quantitative examination of the oxidative damage to critical biomolecules including lipids, proteins, and nucleic acids.

Cell culture systems are not the ideal approach for some of these studies as the ratio of extracellular water volume and cell volume is very large (a broad range on the order of 1000:1); whereas in tissues the ratio of interstitial fluid volume to cell volume is <1. Thus, the quantity of oxygen (moles) available per cell will be far greater in typical cell culture platforms than in tissue. To provide useful data cell culture experiments must be well designed with careful consideration of the quantitative aspects of the principal species of interest in relation to ratios more applicable to *in vivo* tissue/water ratios.

The point was also made that our first-order assumption that there would be negligible re-combination of $HO^{\cdot}$ to produce H_2O_2 may be inappropriate. However, any increase in the production of H_2O_2 could be a positive outcome as tumor cells in general have about 50% of the reserve capacity to remove H_2O_2 compared to normal cells, as determined with absolute quantitative

https://doi.org/10.1016/j.radonc.2019.07.009

approaches [6]. Furthermore, new data indicate that the production of H_2O_2 with FLASH-RT may actually be less than with conventional delivery approaches [2].

3. **Tissue pO_2 requirements for FLASH protection:** The goal of FLASH-RT is to deliver the total dose in a sub-second time-scale, *i.e.* a few milliseconds or less. We posited that in most settings it is possible to consume all the oxygen in the tissue during the FLASH-RT. The time frame of FLASH is much too short for re-oxygenation to occur. Thus, the detrimental effects typically associated with oxygen enhancement (indirect damage) are actually minimized; direct effects are primary. The differential effects between normal tissue and tumor tissue lie in their differing capacities to deal with oxidative distress [6–12]. Oxygen metabolism by mitochondria is indeed central to these metabolic differences between cancer *vs.* normal cells [13]. Thus, for testing our hypothesis it will be essential to determine oxygen levels and how this variable affects the responses tumor and normal tissue to FLASH-RT in the presence and absence of manipulations of hydroperoxide metabolism as well as redox active metal ions. As has been asserted, the metabolic rate of oxygen consumption in brain is quite high, $\approx$30 μM s^{-1}. To deliver a FLASH dose of 10 Gy (40 Gy s^{-1}), 250 ms would be required. Thus, basal metabolism is on the order of the potential radiochemical consumption of oxygen and would be a contributor to depletion of tissue oxygen. The goal of FLASH-RT is ultimately to deliver the dose at a rate of >100 Gy s^{-1} [14]. Although oxygen levels in the lipid regions of membranes are expected to greater than the surrounding water space, the partition coefficient, K_p, is on the order of 3, not 10 [15]. This oxygen will enhance local lipid oxidation reactions, including free radical-mediated lipid peroxidation chain reactions, but the total oxygen "stored" in membranes makes a relatively small contribution to total oxygen in tissue.
4. **Peroxides and peroxyl radicals:** We fully appreciate the informative work of Dr. Ward et al. in comparing the effects of H_2O_2 and ionizing radiation on the type and extent of DNA damage [16]. They observed that hydrogen peroxide was relatively ineffective at causing DSB. However, their work was carried out at 4 °C, a temperature that does not promote Fenton chemistry and subsequent DSB production. It must also be kept in mind that H_2O_2 does not react with DNA in the absence of redox active metal ions [17]. Iron or Cu or some other redox active metal is required to catalyze Fenton chemistry to generate hydroxyl radical from H_2O_2 or an alkoxy radical from ROOH. We emphasize that a key element in our proposed mechanism is iron because of its ubiquitous distribution in biological matrices and central role in metabolism. We propose that FLASH-RT will greatly increase the amount of iron in the labile iron pool of cells, much like we see with other oxidative challenges [18,19]. This will likely be greater in tumor tissue than in normal tissue due to the higher steady-state level of superoxide in cancer cells; this superoxide will dislodge iron from many protein sites such as ferritin, electron transport chains, and Fe-S clusters thereby increasing the labile iron pool [19]. This iron will be a key factor in inducing additional oxidative distress in tumor tissue compared to normal tissue, including damage to DNA, lipids, and proteins.

Conclusions

With the aforementioned careful considerations of our colleague's comments on our theoretical model [1], we have presented a careful and testable reconsideration of the free radical chemistry that would occur during and following FLASH-RT; this could provide a mechanism-based understanding for how the remarkable increase in the therapeutic window could be achieved. While vetting new ideas and criticisms is part of any healthy scientific process, it is often the case that critiques of new ideas are proffered in the absence of alternative and/or more plausible alternatives. At this point and based on the data sets available, we have proposed a theoretical framework for testing these ideas *in vivo*, albeit not an easy task as stated by our learned colleague. Furthermore, since all tools exist to test the causality of this proposed new hypothesis for FLASH-RT, we feel confident that these assumptions will be rigorously and thoroughly tested in the coming decade.

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Available online 16 August 2019