Radiation Induces Live-Cell Metabolic Fluxes: an *in vitro* Demonstration

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Radiation therapy is a very important component of cancer treatments. Considerable advancements in radiotherapy technology have allowed for more uniform dose distributions, as well as improved the ability to avoid organs at risk. These important innovations have led to reductions in normal tissue toxicities resultant from treatments. While improved dose-delivery methods have allowed for dose-boosting and spatial dose-painting, these improvements do not address the fundamental biological impact that radiation creates through the creation of free radicals and reactive oxygen species. This dissertation aims to quantify prompt changes in metabolic signature and to investigate HIF-1 signaling following radiation on the cellular level *in vitro*. Moreover, we evaluate changes in mitochondrial behavior in response to radiation *in vitro.* An understanding of how metabolic fluxes may create interplay between radiation therapy and radiosensitivity may improve cancer treatment efficacy in the future.

For this study, metabolic fluxes in two cell lines, a cancerous urothelial carcinoma cell line (T24) and a comparable normal cell line, Normal Oral Keratinocytes (NOK) were assessed by fluorescence lifetime imaging microscopy (FLIM) of the reduced form of Nicotinamide Adenosine Dinucleotide (NADH). Using FLIM we demonstrated that the cancerous T24 cells showed a strong shift in metabolic signature toward free NADH following irradiation of 10 Gy. In contrast, no such shift in metabolic signature was observed for NOK cells following irradiation. These changes occurred within 30 minutes, which is well ahead of the time-frame of any cell-death related changes. Free NADH is created when glucose is cleaved into pyruvate in the process of glycolysis. During oxidative phosphorylation (oxphos), NADH is bound to the electron transport chain (ETC) and oxidized. This shift toward free NADH in the cancer cells is evidence for increased aerobic glycolysis.

To link changes in metabolism with the local tumor microenvironment, we also assessed expression of transcription factor subunit HIF-1α via immunofluorescence staining for both cell lines. The HIF-1 complex serves as a transcription factor for several processes including glucose transport enzymes, glycolytic enzymes, and stimulates angiogenesis and erythropoiesis. It was found that following irradiation the T24 showed a three-fold increase in HIF-1α expression. However, in the case of the NOK cells, HIF-1α expression was actually found to slightly decrease by about 35%. Clonogenic survival assays found that T24 cells are approximately 100 times more radioresistant than NOK cells for high dose (>8 Gy) assays.

Because cellular mitochondria play a major role in cellular metabolism through oxidative phosphorylation (oxphos) and oxphos-generated reactive oxygen species (ROS), we quantified cellular ROS and mitochondrial membrane polarization (MMP) in each cell line following irradiation. ROS measurements were conducted using DCFDA (dichlorofluorescein diacetate) dye which reacts with peroxyl radicals in solution to activate its fluorescent potential. MMP

measurements were conducted using TMRE (tetramethylrhodamine ester) dye. TMRE dye is sequestered by polarized mitochondria; however, in cells with depolarized mitochondria the TMRE dye is washed away. ROS levels were significantly heightened following irradiation in NOK cells, whereas for T24 cells ROS levels remained almost constant. MMP levels also slightly decreased following irradiation but remained above mitochondrial control levels. In contrast, T24 cells' MMP remained constant at mitochondrial control levels for both irradiation and no irradiation. These data indicate that NOK mitochondria are producing potentially cytotoxic ROS following irradiation not present in the T24 cells.

Because of the potential link between HIF-1, radioprotection and metabolism, expression of Hexokinase II (HK-II), a transcription target of HIF-1, was quantified using the Western Blot gel electrophoresis technique. Hexokinase is a unique isoform of the glycolytic, hexokinase family and is mediated by the HIF-1 complex. HK-II has been found to be over expressed in many cancer types. It was found that T24 cells had a higher concentration of HK-II than NOK cells. HKII has been demonstrated to have significant cellular protective effects against apoptotic and necrotic cell death as well as damage to organelles from ROS.

Together, these data indicate that following irradiation T24 cells have a capacity to increase levels of glycolysis as compared to NOK cells. We hypothesize that this capability may be driven by T24 cells' expression of HIF-1 to transcribe HK-II which has the highest capacity for glucose throughput. These metabolic fluxes resulting from radiation likely drive the interplay between radiation and radiosensitivity; moreover, these data show that the timescale of these fluxes may take place *during* a hypofraction. A thorough understanding of how these effects manifest in different cancer types as well as in higher order systems may unlock a new potential avenue for steering adaptive radiation therapy treatment.