Radiotherapy and Oncology 106 (2013) 390–396

Contents lists available at SciVerse ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com

Experimental radiotherapy

Radiation induces aerobic glycolysis through reactive oxygen species

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ARTICLE INFO

Article history:
Received 9 October 2012
Received in revised form 6 February 2013
Accepted 12 February 2013
Available online 28 March 2013

Keywords:
Warburg effect
Radiation
Reoxygenation
Aerobic glycolysis
Reactive oxygen species

ABSTRACT

Background and purpose: Although radiation induced reoxygenation has been thought to increase radiosensitivity, we have shown that its associated oxidative stress can have radioprotective effects, including stabilization of the transcription factor hypoxia inducible factor 1 (HIF-1). HIF-1 is known to regulate many of the glycolytic enzymes, thereby promoting aerobic glycolysis, which is known to promote treatment resistance. Thus, we hypothesized that reoxygenation after radiation would increase glycolysis. We previously showed that blockade of oxidative stress using a superoxide dismutase (SOD) mimic during reoxygenation can downregulate HIF-1 activity. Here we tested whether concurrent use of this drug with radiotherapy would reduce the switch to a glycolytic phenotype.

Materials and methods: 40 mice with skin fold window chambers implanted with 4T1 mammary carcinomas were randomized into (1) no treatment, (2) radiation alone, (3) SOD mimic alone, and (4) SOD mimic with concurrent radiation. All mice were imaged on the ninth day following tumor implantation (30 h following radiation treatment) following injection of a fluorescent glucose analog, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose (2-NBDG). Hemoglobin saturation was measured by using hyperspectral imaging to quantify oxygenation state.

Results: Mice treated with radiation showed significantly higher 2-NBDG fluorescence compared to controls ($p = 0.007$). Hemoglobin saturation analysis demonstrated reoxygenation following radiation, coinciding with the observed increase in glycolysis. The concurrent use of the SOD mimic with radiation demonstrated a significant reduction in 2-NBDG fluorescence compared to effects seen after radiation alone, while having no effect on reoxygenation.

Conclusions: Radiation induces an increase in tumor glucose demand approximately 30 h following therapy during reoxygenation. The use of an SOD mimic can prevent the increase in aerobic glycolysis when used concurrently with radiation, without preventing reoxygenation.

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Ionizing radiation is used to treat cancer in over 500,000 patients annually in the United States. The efficacy of radiation therapy for solid tumors is limited by normal tissue toxicity and a variety of tumor characteristics that increase resistance to killing, such as hypoxia, autophagy and senescence. We have shown that radiation-induced reoxygenation also causes phenotypic changes that promote tumor and endothelial cell survival [1,2]. Importantly, we observed a post-radiation increase in the activity of the HIF-1 transcription factor, which regulates a plethora of genes involved in angiogenesis, metabolism, invasion and protection against oxidative stress [3]. Hypoxia upregulates the activity of HIF-1, but paradoxically, HIF-1 activity has also been shown to be induced by oxidative stress that occurs during reoxygenation after radiation therapy [1,4]. Since HIF-1 regulates the majority of glycolytic enzymes, including lactate dehydrogenase, its upregulation may stimulate cells toward glycolysis [3].

Substantial data have suggested that glycolysis under aerobic conditions, also known as the Warburg effect, provides a growth advantage for tumor cells and can lead to malignant progression [5,6]. More recent reports have identified the Warburg effect to be implicated in resistance to cytotoxic stress, including ionizing radiation as well as chemotherapy [7,8]. Therefore, treatment methods which block or reduce glycolytic metabolism after radiotherapy may increase tumor cell sensitivity to radiation and chemotherapeutic killing. In this work, we examined the hypothesis that radiation therapy promotes glycolytic metabolism post-radiation treatment, during the period of tumor reoxygenation.

Additionally, we examined whether reduction of oxidative stress after radiation reduces the glycolytic phenotype. We previously demonstrated that a manganese porphyrin-based superoxide

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http://dx.doi.org/10.1016/j.radonc.2013.02.013

dismutase (SOD) mimic could reduce oxidative stress after radiation therapy [1] as well as decrease tumor growth rate following treatment [9]. These radiosensitization effects are linked to inhibition of hypoxia-reoxygenation injury, which upregulates HIF-1 transcriptional activity following radiation. Here, we tested a newer, optimized SOD mimic (MnTnBuOE-2-PyP^{+5}) to investigate whether it can prevent or reduce upregulation of the glycolytic phenotype following fractionated radiation therapy. These results may have important clinical implications for trials aimed at preventing post-radiation metabolic changes and increasing the therapeutic index of radiation therapy.

Methods

Tumor treatments

40 athymic nu/nu female mice with skin fold window chambers containing 4T1 mammary carcinomas were randomized into four treatment groups: (1) no treatment, (2) radiation alone, (3) SOD mimic alone, and (4) SOD mimic with radiation treatment. Titanium window chambers were implanted into the skin-fold of the mice as previously described by Palmer et al. [10]. A 25 μL suspension (2.5×10^5 cells) of 4T1-RFP cells (4T1 mouse mammary tumor cells that constitutively express red fluorescent protein (RFP)) was injected into the dorsal skin fold and closed with a glass coverslip (12 mm diameter, No. 2, Erie Scientific, Portsmouth, New Hampshire). Radiation treatment began six days following tumor implantation, delivered in daily 5 Gy fractions × 3 days (15 Gy total), using a Mark IV cesium irradiator (dose = 702 cGy/min, JL Shepherd, San Fernando, CA). 11 cm lead blocks were used to isolate the skin flap for irradiation, while shielding the rest of the body. The radiation fractionation scheme was identical to that used previously by our group, when we evaluated an earlier analog of this drug with the 4T1 tumor line [1,11]. We rationalized that it would be best to keep with the same dose fractionation scheme to be consistent with the prior work.

The SOD mimic is a manganese-based porphyrin, MnTnBuOE-2-PyP5$^+$ [12]. This drug has been extensively characterized, and is known to effectively and catalytically inactivate several species associated with oxidative and nitrosative stress, including O_2^-, NO^-, $ONOO^-$ and OH^- [12,13]. Preliminary data have shown this compound to be distributed in the cell in a 3.2–1 mitochondrial to cytosolic ratio. Groups treated with the SOD mimic received subcutaneous MnTnBuOE-2-PyP5$^+$ injections starting four days after tumor cell implantation at a dose of 5 mg/kg, twice per day (12 h apart), for five days. In the treatment group receiving both SOD mimic injections and radiation, mice received their first drug injection two days before the start of radiation therapy. MnTnBuOE-2-PyP5$^+$ administration began 2 days prior to irradiation in order for drug levels to reach steady state concentration in tissues. Pharmacokinetic analyses have shown that steady state levels are reached after two days of treatment, using the regimen shown here (data not shown). The goal was to have adequate tissue levels of drug before radiation therapy began, to maximize the effect of the drug in reducing oxidative stress associated with reoxygenation. It should be noted that although this drug is a catalytic inactivator of reactive oxygen species, it has no function as a radioprotector in tumors. It accumulates in mitochondria [14], and its mechanism of action occurs in the period of reoxygenation after radiation, not during radiation. Prior studies have shown that at pharmacologically active doses, drugs of this class have no effect on clonogenic survival of the 4T1 tumor line [11]. We have recently verified that MnTnBuOE-2-PyP5$^+$ has no effect on clonogenic survival of several other tumor lines (data not shown). On radiation treatment days, mice received their first daily dose approximately 2 h prior to irradiation.

All mice were imaged on the ninth day following tumor implantation, which corresponds to approximately 30 h following the final radiation dose in the irradiated animals. Mice were imaged immediately after tail vein injection of a fluorescent glucose analog, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose (2-NBDG). This fluorescent glucose analog demonstrates rapid uptake into cells by GLUT receptors, is retained intracellularly without further metabolism, and mimics the pharmacodynamics of fluoro-deoxyglucose (FDG) used in PET [15–17]. Animals were housed in an on-site housing facility with access to food and water with standard 12 h light/dark cycles. All in vivo experiments were conducted according to a protocol approved by Duke University Institutional Animal Care and Use Committee.

In vivo microscopy

Mice were fasted (NPO) 6 h prior to imaging to control blood glucose concentration prior to 2-NBDG injections. Mice were anesthetized using isoflurane, 2% for induction and 1% for maintenance. Hyperspectral images of white light transmittance (520–620 nm) through the window were recorded for 15–30 min prior to 2-NBDG injection, in order to calculate vascular hemoglobin saturation, as described by Sorg et al. [18]. Animals were administered a 100 μL tail-vein injection of 2-NBDG (6 mM dissolved in sterile saline, Small Molecule Synthesis Facility, Duke University) and 2-NBDG fluorescence (525 nm) was recorded for 85 min at 1 (first 8 min), 30 (8–45 min) and 180 (45–85 min) second intervals.

All imaging was conducted using a Zeiss Axioskop 2 microscope. A 100 W halogen lamp was used for trans-illumination while a 100 W mercury lamp was used for epi-illumination fluorescence imaging of 2-NBDG and RFP expression. Optical filtering for the purpose of hyperspectral imaging was accomplished using a liquid crystal tunable filter (LCTF). Trans-illumination images were acquired from 520 to 620 nm in 10 nm increments. Epi-illumination 2-NBDG fluorescence images were acquired from 510–550 nm in 5 nm increments with a 470 nm bandpass excitation filter (40 nm bandwidth) and a 510 nm longpass dichroic beam splitter. RFP fluorescence was recorded from 610 to 690 nm using a 560 nm bandpass excitation filter (30 nm bandwidth) and a 600 nm longpass dichroic beam splitter. All images were collected with a 2.5× objective (NA = 0.075 and field of view = 10 mm) and a DVC 1412 CCD camera (DVC Company).

Statistical analysis and modeling

All calculations were conducted using MATLAB 2011b. Unless otherwise specified, data are reported as mean and standard error. Statistical significance was determined using Student's *t*-test or ANOVA where appropriate. The Kolmogorov–Smirnov test was used to test the significance of the signal enhancement ratio (SER) values and hemoglobin saturation. Only p values less than 0.05 were considered significant.

Results

Fasting affects 2-NBDG uptake

A quantification method for analyzing 2-NBDG fluorescence intensity over an 85 min interval that would allow for normalization for variations in cell density and background autofluorescence was required for this study. Signal enhancement ratio (SER) patterns have been previously used to quantitate the kinetics of contrast enhancement using dynamic contrast-enhanced MRI via three time points (Fig. 1) to characterize tumors in vivo [19–21]. Varying levels of SER have been shown to correlate with overall treatment outcome in these studies [20,21]. This method was used