Acute Tumor Lactate Perturbations as a Biomarker of Genotoxic Stress: Development of a Biochemical Model

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Abstract

Ionizing radiation is the primary nonsurgical treatment modality for solid tumors. Its effectiveness is impacted by temporal constraints such as fractionation, hypoxia, and development of radioresistant clones. Biomarkers of acute radiation response are essential to developing more effective clinical algorithms. We hypothesized that acute perturbations in tumor lactate levels act as a surrogate marker of radiation response. In vitro experiments were carried out using validated human-derived cell lines from three histologies: anaplastic thyroid carcinoma (ATC), head and neck squamous cell carcinoma (HNSCC), and papillary thyroid carcinoma (PTC). Cellular metabolic activity was measured using standard biochemical assays. In vivo validation was performed using both an orthotopic and a flank derivative of a previously established ATC xenograft murine model. Irradiation of cells and tumors triggered a rapid, dose-dependent, transient decrease in lactate levels that was reversed by free radical scavengers. Acute lactate perturbations following irradiation could identify hypoxic conditions and correlated with hypoxia-induced radioresistance. Mutant TP53 cells and cells in which p53 activity was abrogated (shRNA) demonstrated a blunted lactate response to irradiation, consistent with a radioresistant phenotype. Lactate measurements therefore rapidly detected both induced (i.e., hypoxia) and intrinsic (i.e., mutTP53-driven) radioresistance. We conclude that lactate is a quantitative biomarker of acute genotoxic stress, with a temporal resolution that can inform clinical decision making. Combined with the spatial resolution of newly developed metabolic imaging platforms, this biomarker could lead to the development of truly individualized treatment strategies. Mol Cancer Ther; 14(12); 2901–8. ©2015 AACR.

Introduction

Ionizing radiation (IR) is the most effective spatially distributed nonsurgical cancer treatment (1, 2). Through continued technical improvements in IR delivery, definitive treatment doses can now be delivered with decreased toxicity and improved efficacy (3). Continued improvement in the therapeutic index of IR requires approaches with sufficient temporal resolution to address optimal fractionation, adaptation to transient hypoxia, and synergistic incorporation of radiosensitizing agents (4).

It has been shown, by our group and others, that IR cytotoxicity is largely mediated through generation of reactive oxygen species (ROS) resulting in DNA damage (5–10). IR-induced ROS perturb cellular metabolism in a measurable and quantifiable manner that could theoretically be exploited for therapeutic purposes (5). Our group has demonstrated a proportional relationship between IR-induced ROS levels and cell death, and showed that this relationship at least partially accounts for some of the radioresistance encountered in solid tumors (6, 8). This relationship identifies an important therapeutic window to target radioresistant tumors, such as those driven by mutations in TP53 (6, 8).

Although high levels of ROS are essential to achieving a therapeutic effect, basal ROS play an important role in tumorigenesis and metastasis (11, 12). Because of this dual role, quantitative measurements of tumor cell and tumor ROS are important to maximize therapeutic efficacy and decrease normal tissue toxicity.

Lactate is a metabolite well known to correlate with changes in tissue ROS levels. This relationship has been shown to be important in both normal aging and oncogenic transformation (13). Brizel and colleagues showed that increased tumor lactate levels correlate with increased metastasis (13). Quennet and colleagues extended this initial work to demonstrate that lactate levels...
correlate with tumor cell-relative radiosensitivity (14). On the basis of this and other promising preclinical data, Le and colleagues conducted a potentially paradigm changing clinical trial, testing the potential of tumor lactate levels to predict treatment response in head and neck tumors (15, 16). Unfortunately, data generated by this trial failed to demonstrate a correlation between proton magnetic resonance spectroscopic measurements of lactate signal intensity and clinical outcomes (16).

These data generated a puzzling contradiction between the biochemical promise of lactate and the inability to successfully achieve clinical translation. It has been long established, in both preclinical (4, 17) and clinical series (18, 19) that hypoxia exerts a profound impact on in vivo radiation effects (20). Matsumoto and colleagues demonstrated convincingly that lactate perturbations identify hypoxic conditions, a known correlate with induced radioresistance (21). Furthermore, these metabolic alterations can be linked to genomic alterations such as TP53 (22). Because radiation effectiveness is also mediated by temporal constraints (e.g., fractionation, transient hypoxia, shifting tumor vascularity), we sought to focus on the high-temporal resolution detection of radiation-induced lactate perturbations. Specifically, as microenvironmental lactate levels have long been held as a harbinger of altered radioresistance (23), we hypothesized that IR-induced ROS perturbations are mirrored by rapid changes in tumor cell lactate levels in a measurable and quantifiable manner. This hypothesis is based on previous studies by our group that found that magnetic resonance spectroscopic imaging (MRSI) of hyperpolarized 13C pyruvate can detect acute changes in tumor lactate levels following metabolic inhibition and/or irradiation (5). In this study, we sought to develop and validate acute measurements of acute lactate changes provide a consistent and reliable means of detecting genotoxic stress with a temporal resolution suitable for clinical translation. To confirm translational relevance we aimed to demonstrate the following: (i) dose-dependent relationship between acute, transient changes in lactate levels, and IR exposure; (ii) correlation with acquired (i.e., hypoxia) and/or intrinsic (TP53 mutation) radioresistance; and (iii) generalizability across multiple histologies.

Materials and Methods

Cells

Previously described cell lines [anaplastic thyroid carcinoma (ATC), papillary thyroid carcinoma (PTC), head and neck squamous cell carcinoma (HNSCC)] were obtained from an established cell line bank in the laboratory of Dr. Jeffrey N. Myers under approved institutional protocols. All cell lines were tested and authenticated using short tandem repeat analysis (24). Cells were maintained in either RPMI or MEM growth media supplemented with glutamine, pyruvate, penicillin/streptomycin, and 10% FBS. Parental ATC, PTC, and HNSCC cell lines were obtained from the above investigator in the last 3 years and are routinely STR tested in our laboratory. HNSCC cell lines containing TP53 constructs have been previously described by our group and were obtained for the experiments detailed here within the last 4 months (6, 7).

Chemicals

2-deoxyglucose, cobalt (II) chloride, hydrogen peroxide, and N-acetyl cysteine (NAC) were purchased from Sigma-Aldrich.

Metabolic studies

For lactate, NAD+ and NADH measurements cells were harvested at various time points (0, 15, 30, 60, and 120 minutes) after irradiation (0, 2, and 5 Gy) and/or drug treatment using appropriate buffers and snap-frozen in liquid nitrogen. Lactate, NAD+, and NADH levels were analyzed by colorimetric assays using commercially available assays (BioVision), according to the manufacturer’s instructions.

Cytotoxicity studies

Drug and IR cytotoxicity were assayed using clonogenic assays. Cells were treated with 1 mmol/L 2-deoxyglucose for 24 hours, in normoxic (21% O2) or hypoxic (1% O2, achieved using hypoxia chamber or CoCl2 exposure) conditions, then irradiated using a high dose rate 137Cs unit (Mark I-68A,4.5 Gy/minute) to the indicated dose (0–6 Gy). Four hours after irradiation, fresh media were replaced and cells were incubated for colony formation for 10 to 14 days, then fixed and stained using a 1% formalin/crystal violet solution. Colonies were counted and surviving fractions were determined based upon the plating efficiency of the nonirradiated control group.

ATC tumors

Male athymic nude mice (8–12 weeks) were purchased from the National Cancer Institute (Bethesda, MD), maintained in a pathogen-free facility, and fed irradiated mouse chow and autoclaved, reverse osmosis-treated water. The animal facility was approved by the American Association for the Accreditation of Laboratory Animal Care and met all current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and the NIH. All procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas M.D. Anderson Cancer Center (Houston, TX). For orthotopic tumors, U-1HTH83 luciferase-expressing cells (2.5 × 106/mouse) were injected into the right thyroid lobe under direct visualization as previously described (5). For flank tumors, cells (2 × 106/mouse) were injected into both flanks of each animal. Tumor size was ascertained regularly throughout the experimental period using bioluminescence imaging and manual measurements as previously described (5, 7). Tumors were allowed to grow for 1 week before initiation of imaging experiments. Tumors were irradiated to indicated doses (5 Gy) using either a Co60 irradiator and custom lead blocks or an image-guided radiotherapy system (X-Rad 225Cs, Precision X-Ray Inc.). Both irradiation methods delivered the same targeted radiation dose to the same tumor volume. Tumors were harvested for either biochemical analysis or histologic and IHC analysis. The number of animals chosen for each in vivo experiment was based on previous experience with this animal model and the expected effect size. Statistical analysis of in vivo data was conducted as described below.

Statistical analysis

All in vitro experiments were carried out at least in triplicate (for each condition) and were repeated to ensure reproducibility. All statistical analysis for in vitro experiments was conducted using two tailed, Student t test analysis with a cutoff P value of 0.05 to demonstrate statistical significance. For all in vivo experiments, statistical significance was determined using Student t test analysis with a cutoff P value of 0.05 to demonstrate statistical significance.

Results

Oxidative stress perturbs ATC cellular reducing potential

ATC cells were grown under standard conditions and exposed to 2-DG, a known inhibitor of glycolysis and a compound
Ionizing radiation triggers acute perturbations in ATC cellular reducing potential

Ionizing radiation (IR) triggers a rapid, transient increase in cellular ROS that is rapidly scavenged by primary cellular reducing equivalents that are regenerated metabolically (Fig. 1A; refs. 12, 27, 28). Exposure of ATC cells to increasing doses of IR resulted in minimal perturbations in total cellular NADH levels (Supplementary Fig. S1). In contrast, NAD$^+$ levels increased within minutes of IR exposure and returned to baseline at 2 hours after irradiation (Supplementary Fig. S2). This resulted in a transient decrease in the NADH/NAD$^+$ cellular ratio that normalized over time (Fig. 1B). NAC reversed the effects of IR on the NADH/NAD$^+$ in a concentration-dependent manner, consistent with an ROS-driven mechanism (Fig. 1C).

Cellular lactate levels reflect IR-induced alterations in cellular reducing potential

The ultimate step in anaerobic glycolysis reduces pyruvate into lactate using electrons donated by NADH. As such, the conversion rate is an indirect reflection of cellular oxidative stress and the metabolic response to it (Fig. 2A). To further demonstrate this biochemical relationship, ATC cells were exposed to metabolic inhibition (2-DG) or oxidative stress ($\text{H}_2\text{O}_2$). Lactate production decreased in response to both agents, and effect that was reversed in a concentration-dependent manner by NAC (Fig. 2B). Consistent with effects on cellular reducing potential, IR exposure decreased lactate production acutely in a dose-dependent manner; this was reversed by NAC (Fig. 2C). This dose-dependent decrease in tumor cell lactate levels correlates with the dose-dependent increase in cell death following irradiation demonstrated using clonogenic survival (Fig. 2C). In order to further test the link between ROS and lactate levels following irradiation, $\text{H}_2\text{O}_2$ was used to increase tumor cell ROS levels. This resulted in a correspondent increase in the magnitude of tumor cell lactate changes following irradiation, in contrast with the effects of NAC, which restores lactate levels following irradiation (Supplementary Fig. S3).

IR exposure acutely decreases ATC tumor lactate levels

For orthotopic tumors, U-HTH83 luciferase-expressing cells ($2.5 \times 10^6$/mouse) were orthotopically injected into the right thyroid lobe under direct visualization as previously described (5, 29). Tumors were detected by bioluminescence activity using IVIS 200 imaging system (Xenogen Corp.). For flank ATC xenograft tumors, U-HTH83-lucif cells ($2 \times 10^6$/tumor) were injected subcutaneously into both flanks of each athymic nude mice. The subcutaneous tumors were allowed to grow for 10 to 12 days before irradiation. Four tumors were maintained as controls and four tumors were irradiated using a single fraction of 5 Gy. Tumors were harvested immediately after irradiation and tumor lactate levels were measured. Lactate levels in tumors exposed to IR were significantly lower than those in control tumors (Fig. 3A). The experiment was repeated using orthotopic ATC xenografts that more closely recapitulate human tumor growth and vascularity. As shown in Fig. 3B, irradiation (5 Gy) resulted in a significant, acute drop in tumor lactate levels compared with control tumors.

Cellular lactate levels reflect hypoxia-induced radioresistance

Hypoxic conditions can be reproduced using either a hypoxia chamber or chemical inhibition ($\text{CoCl}_2$; Fig. 4A). Although most tumor cells, including the ATC cell lines utilized here, are...
primarily glycolytic at baseline, this metabolic phenotype is exacerbated by either chamber or chemically induced hypoxia as demonstrated by increased baseline lactate generation (Supplementary Fig. S4). A similar shift in tumor metabolism is expected under in vivo conditions when transient alterations in vascularity induce hypoxic conditions of varying duration. Consistent with an increased glycolytic reliance, ATC cells become more sensitive to the cytotoxic effects of 2-DG under conditions of hypoxia (Fig. 4B). Under conditions of hypoxia (CoCl₂), the IR-induced transient drop in lactate levels persists, but the magnitude of the effect is reduced by approximately 50% compared with normoxic conditions (Fig. 4C). To evaluate the relationship between IR-induced lactate perturbations and IR effects on tumor cell death, we performed clonogenic survival assays in the presence and absence of hypoxia. ATC cells exposed to hypoxia (chamber) exhibited relative radioresistance compared with normoxic conditions at all tested IR doses (Fig. 4D). This effect was effectively reversed by the addition of 2-DG.

Cellular lactate levels reflect intrinsic tumor cell radioresistance/radiosensitivity

To test the generalizability of the above described relationship between IR, NADH/NAD⁺ ratio, and lactate levels, we evaluated the response using a pair of previously established human head and neck squamous cell carcinoma (HNSSC) cell lines that are isogenic with the exception for TP53 mutational status (6, 8). IR triggered a transient, reversible, dose-dependent drop in cellular NADH/NAD⁺ ratio and lactate levels (Fig. 5). Consistent with previously reported relative radioresistance, HN31 (p53 wt) cells demonstrated a blunted decrease in the NADH/NAD⁺ ratio and cellular lactate levels following irradiation as compared with HN30 (p53 mut) cells. In order to confirm that this differential response is driven by loss of p53 function, we tested the effect of IR on cellular lactate levels using a previously described pair of constructs. As shown in Fig. 5, HN30 cells in which p53 activity was decreased by shRNA interference demonstrated a blunted response to IR compared with lentiviral-vector-transfected HN30 control cells. At 5 Gy, the drop in lactate levels in shp53 cells was 49% at 15 minutes and 72% at 30 minutes after irradiation. These quantitative differences were reproducible across multiple replicates and experimental series as well as multiple IR doses (Supplementary Fig. S5). Specifically, at the lower dose of 2 Gy, the lactate drop in cells with suppressed p53 activity (shp53) was only 36% of that of lentiviral-transfected control cells at 15 minutes after irradiation and 59% at 30 minutes after irradiation. These data are consistent with our previously published data about the relative radioresistance/radiosensitivity of this pair of constructs (Supplementary Fig. S6; refs. 6, 8).

We further validated the relationship between cellular lactate levels and acute radiation exposure in multiple ATC and PTC cell lines. As shown in Fig. 6, multiple cell lines demonstrated a consistent drop in lactate levels following irradiation, with return to baseline within several hours after exposure.

Discussion

Improvements in dose delivery via intensity modulated radiotherapy have resulted in decreased normal tissue toxicity and
improved locoregional control through selective boosting of specific tumor regions in the clinical setting (3). Unfortunately, the lack of high-temporal resolution methods to interrogate the tumor microenvironment precludes selective targeting of tumor subvolumes at increased risk of local recurrence. This means that all volumes of a tumor are currently treated to a uniform volume, despite the fact that subregions of the tumor associated with hypoxia are the regions most likely to recur (30). Altered fractionation protocols and hypoxia modifiers can greatly affect effectiveness (31–33), yet the optimal regimen remains elusive (34). Although there are several direct imaging markers of hypoxia (35–37), their temporal resolution is relatively poor. Although previously identified genomic, epigenetic, and metabolic markers of relative radiosensitivity/radioresistance can provide prognostic information and may guide treatment decisions at a population level, they are less useful for tailoring treatment strategies to individual tumors (6, 8, 38). What is required is a real-time biomarker of radiation response with sufficient temporal resolution to inform clinical decision making.

In this study, we describe a biochemical model that can transform tumor lactate from an imperfect treatment correlate to an actionable indicator of acute radiation response. Our data.

Figure 3. IR exposure acutely decreases tumor lactate levels. A, bilateral flank tumors were created simultaneously. Tumors were allowed to grow. One set of tumors (n = 4) was maintained as a control and the other set of tumors (n = 4) irradiated to a total dose of 5 Gy. Immediately following irradiation, tumors were harvested. Tissue levels of lactate were measured and normalized to total protein content. Top panel illustrates paired tumor data, whereas bottom panel illustrates average data from control and irradiated tumor groups. B, control (n = 5) and irradiated (n = 5; 5 Gy) orthotopic xenograft tumors were harvested immediately after irradiation. Tissue levels of lactate were measured and normalized to total protein levels.

Figure 4. IR effects on lactate production persist in the presence of hypoxia and correspond to relative radiosensitivity/resistance. A, CoCl₂ exposure mimics hypoxia chamber exposure as demonstrated by HIF1α induction. B, hypoxia (chamber, 1% O₂) and CoCl₂ exposure increase sensitivity to 2-DG in a clonogenic survival assay. C, IR exposure triggers a transient, reversible drop in cellular lactate levels. This effect is diminished by approximately 50% following exposure to CoCl₂ for 24-hour after irradiation. All values are normalized to corresponding control condition (1.0). D, cells were exposed to increasing IR doses and effects of cell death ascertained using a clonogenic survival assay. 2-DG exposure radiosensitized tumors, while hypoxia (chamber, 1% O₂) resulted in increased relative radioresistance. *, P < 0.05, compared with corresponding control condition. Each experiment was carried out at least in triplicate, with values indicating means and error bars representing SD.
indicate that IR-induced ROS decrease tumor cell reducing equivalent levels, resulting in a transient drop in tumor cell lactate levels, consistent with classic historical radiobiological observations, from Tozer and colleagues (20), indicating lactate reduction after radiotherapy. It is essential to emphasize that this process occurs within minutes following irradiation. This is important for two reasons. First, this time frame is consistent with real-time decision making in the clinical setting. Second, it may at least partially explain previously published negative clinical data (16). Because lactate levels return to baseline within 2 hours after irradiation, delayed measurements, or measurements that are widely spaced out during treatment, are unlikely to capture the metabolic impact of treatment regimens. The ability to reproduce this effect through exogenous ROS, and reverse it through application of ROS scavengers conclusively links exogenous ROS, tumor-reducing potential and lactate levels. The dose dependence of IR-induced lactate perturbations further demonstrates that this is a quantitative not simply a qualitative relationship.

Utilization of acute lactate perturbations as a real-time biomarker of radiation response can potentially address an additional current clinical constraint: transient hypoxia assessment. Tumor hypoxia demonstrates not only spatial (39) but temporal heterogeneity as well (40), with acute and chronic components (41). IHC means of assessing tumor hypoxia are static, and the use of existing metabolic imaging methods are constrained by tumor size-associated spatial heterogeneity (42), variable tracer-specific time dependencies (43), and reported discordance with direct measurement in specific tumor histologies (44). Within a time frame of minutes, lactate levels can be used to distinguish radiation response in hypoxic conditions from its normoxic correlate in a manner consistent with acquired radioresistance. Dynamic integration of this approach via HP-MRSI (5) could allow for daily adaptive fractionation and dose painting designed to overcome hypoxia-induced radioresistance; conceivably, such data could be used to investigate biologically optimized radiotherapy schedules that might even occur at non-daily intervals or with varying fractionation, as suggested recently by Leder and colleagues (45).

Intrinsic radioresistance, driven by specific genomic or epigenetic events, has been shown to adversely affect treatment effectiveness (8, 38, 46). The most common oncogenic event in solid tumors, mutation of the TP53 gene, not only drives tumorigenesis and metastasis, but also contributes to radiation and chemotherapy resistance (6, 8, 38). Because not all mutations behave identically, and since the remainder of the genomic background can further modulate treatment responsiveness, a priori predictions based on TP53 mutational status cannot be used for individual tumor treatment planning. Our data demonstrate that, as in the case of acquired hypoxia, measurements of IR-induced...
Lactate Is a Marker of Genotoxic Stress

Lactate perturbations can distinguish between cells expressing wild-type TP53 and cells expressing mutant TP53 within minutes. It has been well established that solid tumors are heterogeneous with respect to genomic and epigenetic background. This becomes particularly important as clonal populations are obliterated or survive ongoing treatment, and as specific clones begin to repopulate solid tumors during disease recurrence. The ability to temporally resolve shifts in tumor cell populations during disease development and treatment can be a crucial asset to improved clinical effectiveness.

A translationally viable approach to lactate measurements in the clinical setting must be: (i) noninvasive, (ii) iterative, and (iii) capable of resolving biochemical data spatially and temporally. Previous attempts to measure tumor lactate levels using traditional proton magnetic resonance spectroscopy sequences may have been hampered by limited spatial and temporal resolution driven in part by the overlap of lactate (~1.3 ppm) and lipid (0.9–1.5 ppm) resonance (16, 47). We have recently published data demonstrating that MRI of hyperpolarized 13C pyruvate (HP-MRI) provides real-time measurements of tumor conversion of pyruvate into lactate with excellent spatial resolution (5, 7). HP-MRI provides a powerful imaging platform, with greatly improved signal-to-noise ratio compared with traditional proton magnetic resonance spectroscopy (16, 47, 48). Xu and colleagues utilized this imaging platform to differentiate between cell lines with high and low metastatic potential (15). Thind and colleagues demonstrated that HP-MRI can be used to detect acute normal tissue radiation toxicity in a lung model (49).

The biochemical model presented here demonstrates that lactate measurements possess the temporal resolution required for clinical translation. HP-MRI of 13C lactate has demonstrated, in both preclinical and clinical trials the spatial resolution required for treatment optimization. Together, these tools provide a unique avenue for development of truly individualized solid tumor treatment strategies (50). Specifically, we propose two overlapping approaches to improving outcomes following irradiation of solid tumors. First, lactate interrogation can be used in preclinical animal models to optimize the timing of radiosensitizing agents such as cisplatin in order to maximize oxidative stress at the moment of tumor irradiation. These data can be tested for increased treatment efficacy in the setting of a clinical trial.

Second, lactate interrogation via HP-MRI can be mated to newly available MRI-based irradiation platforms in order to develop adaptive irradiation algorithms that allow for dose adjustment throughout the radiation treatment course in order to maximize therapeutic index.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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