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Biology Contribution

Oxygen and Perfusion Kinetics in Response to Fractionated Radiation Therapy in FaDu Head and Neck Cancer Xenografts Are Related to Treatment Outcome

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Summary

This work examines the kinetics of tumor oxygenation during and after fractionated radiation therapy. These oxygenation kinetics show differential associations with local tumor control outcome. Such relationships have not been described previously.

Purpose: To test whether oxygenation kinetics correlate with the likelihood for local tumor control after fractionated radiation therapy.

Methods and Materials: We used diffuse reflectance spectroscopy to noninvasively measure tumor vascular oxygenation and total hemoglobin concentration associated with radiation therapy of 5 daily fractions (7.5, 9, or 13.5 Gy/d) in FaDu xenografts. Spectroscopy measurements were obtained immediately before each daily radiation fraction and during the week after radiation therapy. Oxygen saturation and total hemoglobin concentration were computed using an inverse Monte Carlo model.

Results: First, oxygenation kinetics during and after radiation therapy, but before tumor volumes changed, were associated with local tumor control. Locally controlled tumors exhibited significantly faster increases in oxygenation after radiation therapy (days 12-15) compared with tumors that recurred locally. Second, within the group

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N.R. and M.W.D. contributed equally to this work.

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of tumors that recurred, faster increases in oxygenation during radiation therapy (day 3-5 interval) were correlated with earlier recurrence times. An area of 0.74 under the receiver operating characteristic curve was achieved when classifying the local control tumors from all irradiated tumors using the oxygen kinetics with a logistic regression model. Third, the rate of increase in oxygenation was radiation dose dependent. Radiation doses $\leq$9.5 Gy/d did not initiate an increase in oxygenation, whereas 13.5 Gy/d triggered significant increases in oxygenation during and after radiation therapy.
Conclusions: Additional confirmation is required in other tumor models, but these results suggest that monitoring tumor oxygenation kinetics could aid in the prediction of local tumor control after radiation therapy.

Introduction

Radiation therapy plays a significant role in the treatment of a wide variety of cancers (1), and particularly in the management of localized head and neck cancer (HNC) because it is a noninvasive and function-preserving modality (2). Tumor oxygenation is associated with tumor radiosensitivity, angiogenesis, and metabolism (3). Hypoxic tumor cells are 3 times more resistant to radiation therapy than aerobic cells and would dominate response if they persisted throughout a course of fractionated radiation therapy. Classic radiobiologic theory posits that once aerobic cancer cells are killed, a proportion of the remaining hypoxic cells in the tumor become reoxygenated, thereby regaining sensitivity to the next radiation fraction (4). The classic reoxygenation theory does not require a physical increase in pO_2 in the tumor.

However, increases in tumor oxygenation have been reported after fractionated radiation therapy. Several studies have observed tumor increases in oxygenation, detected by microelectrode and/or immunohistochemical techniques, induced by multifraction radiation therapy with nude mice bearing human HNC xenografts (5-7). Harriss et al (6) used an oxygen-sensitive probe to obtain tumor pO_2 values and found that the median pO_2 of irradiated tumors (10 $\times$ 4 Gy) increased with each successive radiation dose, relative to untreated controls measured at the same time (6). Maftei et al (7) reported decreases in hypoxic fraction assessed by pimonidazole staining, 24 hours after (2 $\times$ 10 Gy) irradiation in FaDu xenografts. Ressel et al (5) examined association between hypoxic fraction and treatment outcome using microelectrodes and demonstrated that median pO_2 values in squamous cell carcinoma xenografts increased over time after radiation therapy. Animals with complete tumor remission 60 days after treatment had the lowest fraction of median pO_2 $<$10 mm Hg 10 days after treatment (5).

The invasiveness of microelectrode techniques limits measurement frequency and total number. Further, tissue damage by the implanted sensors might interfere with tumor response to radiation. In this study, a noninvasive optical technique was used to serially measure changes in perfusion and oxygenation as assessed by total hemoglobin ([THb]) and hemoglobin saturation before, during, and after fractionated radiation therapy in mice with FaDu xenografts. Our primary hypothesis was that oxygenation kinetics would correlate with the likelihood for local tumor control after fractionated radiation therapy. Indeed our results confirm that hypothesis. Our findings provide a strong rationale for temporal monitoring of tumor oxygenation kinetics after radiation therapy and may identify optimal windows in which to assess the efficacy of radiation therapy, before discernable changes in tumor volume. To distinguish the kinetics of change in oxygenation during fractionated radiation therapy from classic reoxygenation theory, we use the term "oxygenation kinetics" throughout this article.

Methods and Materials

Mouse protocols were approved by the Institutional Animal Care and Use Committee. Figure 1 shows the study timeline for these studies.

Fractionated radiation therapy of FaDu HNSCCs xenografts

Approximately 1 $\times$ 10^6 FaDu cells were injected subcutaneously into the right flank of nude mice (nu/nu) to initiate tumor growth. Radiation therapy commenced when tumor volumes reached 100 to 400 mm^3. Thereafter, tumor volume was measured 2 to 3 times per week for the first 2 weeks after the start of radiation therapy, and then 1 to 2 times per week for up to 120 days after the first day of radiation therapy, or until the tumor volumes reached 5 times the volume measured on the first day of radiation therapy.

Mice were irradiated with 5 daily fractions of radiation from 7.5 to 13.5 Gy per fraction, using a commercial X-RAD320 irradiator (Precision X-Ray, Bradford, CT). The unit produced a collimated x-ray beam (with mean energy of 110 kV) at a dose rate of 0.64 Gy/min. Mice were anesthetized via isoflurane during irradiation, and only the tumor area was irradiated. In each experiment, mice were randomly assigned 3:1 to irradiated and nonirradiated control groups.

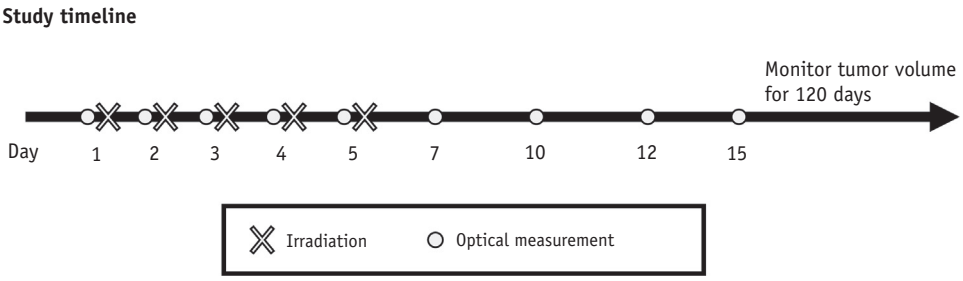

Fig. 1. Study timeline of the experiment. Optical measurements were obtained during and after radiation therapy. Tumor volumes were monitored for 120 days to confirm treatment outcomes.

Optical measurement schedule

Vascular oxygenation (SO_2%) and [THb] were computed from tissue diffuse reflectance spectra (DRS) collected on a portable optical instrument (8, 9). The sensing depth of the probe was determined to be 1.2 mm with a Monte Carlo simulation (10). Tissue DRS were obtained from all mice before each radiation fraction, during radiation therapy, and after radiation therapy, on days 7, 10, 12, and 15. (Fig. 1). Immediately before the measurements, DRS were obtained at 5 random sites on the tumor of each mouse. The mean DRS was analyzed using an inverse Monte Carlo model to compute SO_2% and [THb]. Follow-up values of SO_2% and [THb] were divided by their baseline to obtain baseline-corrected values. Change in the baseline-corrected SO_2% (f-SO_2) across an interval of time from t_1 to t_2 was defined as [f-$SO_2(t_2)$ − f-$SO_2(t_1)$], where t_1 and t_2 are 2 selected time points.

All mice that underwent radiation therapy were assessed for local tumor recurrence up to 120 days after treatment. Treated mice with no visible tumor for at least 50 days were classified as local control (LC); treated mice that showed local recurrence within the 50-day period were classified as local failure (LF). For LF mice, time to failure was defined as the earliest time at which the recurrent lesion had increasing volume for 2 consecutive observations. Nonirradiated tumor-bearing mice formed the control (CTL) group.

Statistical analysis

A repeated-measures model was used to test for a difference among CTL, LC, and LF groups on the quadratic trajectory of SO_2% across time. The Wilcoxon rank-sum test was used to test for group differences on the rate of change across various intervals of time (for example, day 3-5, day 7-10, and day 12-15). The Spearman correlation coefficient was used to assess the correlation between the rate of change in optical endpoints over various time intervals and time to failure in mice only in the LF group. To investigate the dose dependency of oxygenation kinetics, mice receiving 7.5 and 9.5 Gy were combined (low radiation dose group). The SO_2% of the low radiation dose group and the high radiation dose group (13.5 Gy) were compared with CTL using the Wilcoxon rank−sum test. All tests were two-tailed, with α of 0.05. Logistic regression models were built to predict LF within all treated mice. The models were built on the basis of the rate of changes in f-SO_2 across various time intervals. A leave-one-out cross-validation technique was used to generate receiver operating characteristic curves from which the area under the curve was computed. Data analysis was conducted using MATLAB (Mathworks, Natick, MA). Logistic regression models were computed with the SAS software (SAS Institute, Cary, NC).

Results

Oxygenation kinetics are associated with LC rate after radiation therapy

Table 1 summarizes the number of mice in LC (n = 10), LF (n = 31), and CTL groups (n = 17) according to dose of radiation received. Within the LF group, the longest lesion-free time was 35 days. Thus, we defined LC as any animal that remained disease free at 50 days after treatment.

Figure 2 demonstrates that oxygenation kinetics are associated with therapy outcomes. A radiation dose-effect curve for local tumor control was generated with data fitted to a Hill equation (Fig. 2A). The dose required to achieve LC in 50% of the animals was 38.5 Gy. Figure 2B and 2C shows the time to LF and tumor volumes for each group, respectively. Irradiation resulted in an overall increase in

Table 1 Outcome in each radiation dose group

	No. of mice per dose level			
Group	7.5 (37.5) Gy	9.5 (47.5) Gy	13.5 (67.5) Gy	Total
CTL	4	6	7	17
LF	6	2	2	10
LC	4	13	14	31
Total	14	21	23	58

Seventeen mice were in the control (CTL) group. A total of 10 and 31 mice achieved local control (LC) and local failure (LF), respectively. Radiation was administered in 5 daily fractions at the doses indicated. The dose per fraction and total dose, in parentheses, are shown.

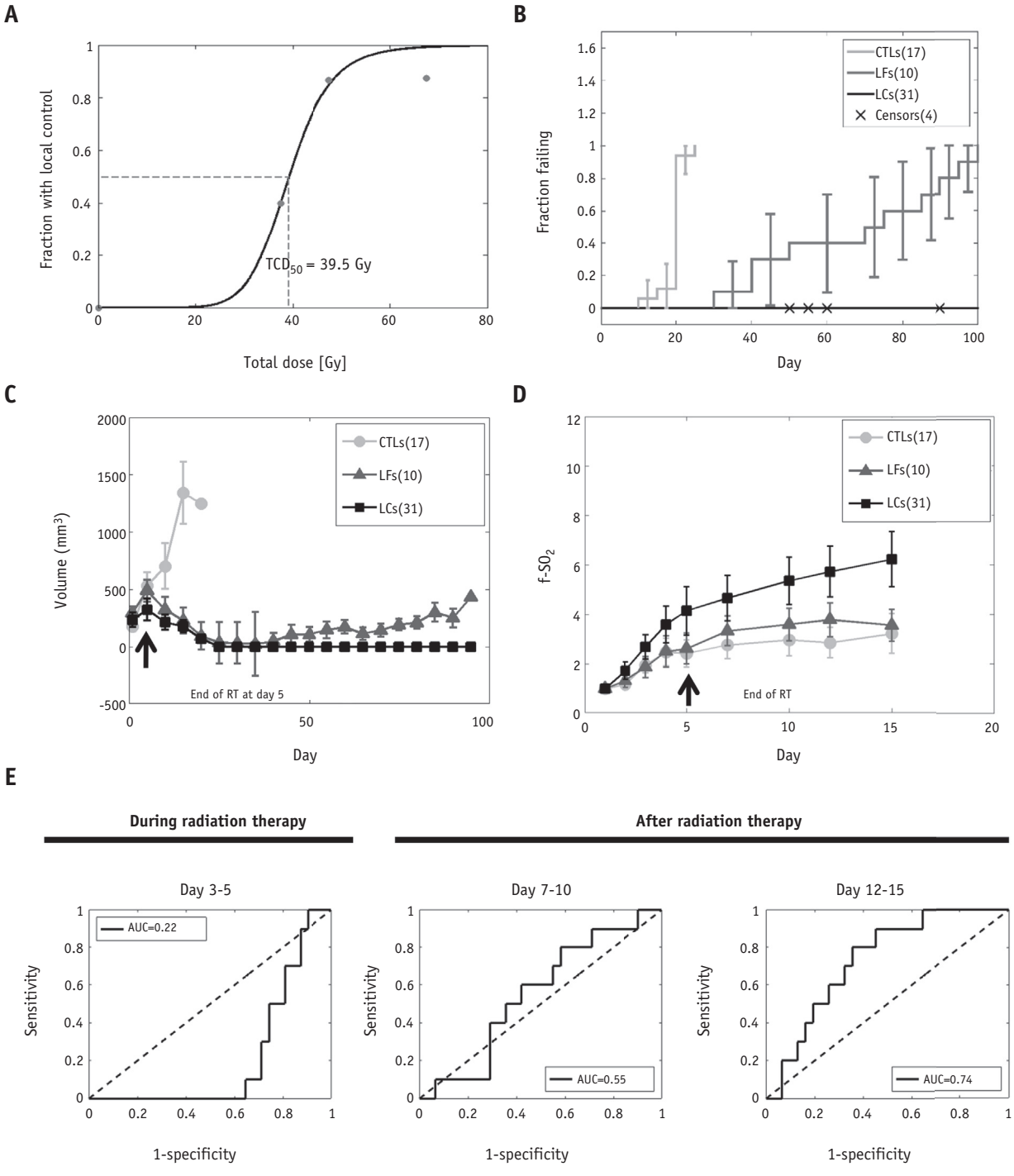

Fig. 2. Optical measurements of change in oxygenation kinetics before measureable volume changes differentiate tumors that achieve local control from those that fail. (A) Radiation dose-effect curve for local tumor control. The solid line was constructed by fitting the data to the Hill equation. TCD_{50} = dose required to achieve local control in 50% of the animals. (B) Time to local failure for local failure (LF), local control (LC), and control groups (CTL). Error bars show the 95% confidence intervals. (C) Differences in tumor volumes between LC and LF groups are visible 40 days after radiation therapy. (D) Optical measurements of tumor oxygenation kinetics during radiation therapy (RT) and after completion of radiation therapy. Tumor oxygenation kinetics were higher in the LC group than in the LF group starting 5 days after radiation therapy. At this time point there is no significant change in tumor volume. Error bars represent standard error of the mean. (E) Area under the receiver operating characteristic curve (AUC) computed from the logistic regression analysis for classifying the LF mice. The regression model was built on the change of baseline-corrected vascular oxygenation (f-SO_2) obtained from different time intervals. A leave-one-out cross-validation technique was used.

SO_2% for the LC and LF groups relative to the CTL group (Fig. 2D). Within irradiated mice, tumors in the LC group achieved higher SO_2% compared with the LF group, particularly after completion of the radiation therapy course. The 2-degree-of-freedom test showed a significant difference ($P<.001$) among groups in f-SO_2 trajectory across time. The LC group showed a moderate increase and then a decrease in [THb] across the 2-week period. Overall, an early increase in the mean f-[THb] for the LC and LF groups suggests an increase in the overall blood volume or perfusion as a result of radiation therapy (Fig. E1, available online at www.redjournal.org). A similar, latent increase of the mean f-[THb] in the CTL group may be related to tumor-directed angiogenesis. The 2-degree test for a difference among groups in trajectory across time had a P value of .02.

The rate of f-SO_2 change was evaluated over 3 specific time intervals relative to the onset of radiation therapy (day 3-5, day 7-10, and day 12-15). These intervals were chosen to represent time frames during radiation therapy, shortly after radiation therapy was completed, and an interval after radiation therapy but before any discernable change in tumor volume, respectively. Table 2 summarizes the rate of f-SO_2 changes over the 3 time intervals for LC, LF, and CTL groups. A negative rate of f-SO_2 change indicates a decrease in SO_2% during the time interval. The LC group showed a positive rate of change in f-SO_2 in all 3 time intervals. The rate of the f-SO_2 change in the LC group was significantly higher than in the CTL group in the day 7 to 10 interval ($P=.01$) and was significantly higher than in the LF group in the day 12 to 15 interval ($P<.01$). In addition, rate of the f-SO_2 change in the CTL group was significantly higher than in the LF group in the day 12 to 15 interval ($P=.05$). The cross-validated area under the curve computed from logistic regression models built from the rate of f-SO_2 changes in day 3 to 5, 7 to 10, and 12 to 15 intervals was 0.22, 0.55, and 0.74, respectively. Figure 2E shows the corresponding receiver operating characteristic curve computed from each interval.

Table 2 Oxygenation kinetics after radiation therapy is associated with tumor recurrence

	Rate of f-SO_2 change		
Group	Days 3-5	Days 7-10	Days 12-15
CTL (11)	0.238 (0.039)	0.039 (0.017)	−0.047* (0.022)
LF (10)	0.377 (0.048)	0.087 (0.019)	−0.076 (0.017)
LC (12)	0.727 (0.047)	0.23† (0.01)	0.169‡ (0.01)

The mean (standard error) of the rate of change in baseline-corrected vascular oxygenation (f- SO_2) across various time intervals per group is shown. The P values were computed from Wilcoxon rank−sum test for comparing the rate of change in f-SO_2.

* Rate of the f-SO_2 change in the control (CTL) group is significantly higher than in the local failure (LF) group in the interval from day 12-15 ($P=.05$).

† Rate of f-SO_2 change in the local control (LC) group is significantly higher than in the CTL group in the interval from day 7-10 ($P=.01$).

‡ Rate of the f-SO_2 change for LC is significantly higher than for LF in the interval from day 12-15 ($P=.01$).

There is a strong association between rate of change of SO_2% and time to tumor recurrence

The association between rates of change of f-SO_2 and tumor recurrence time was evaluated within the LF group. Of the 10 LFs, 6 were from the 7.5-Gy group, 2 from the 9.5-Gy group, and 2 from the 13.5-Gy group. The rate of change in f-SO_2 during radiation therapy from day 3 to day 5 was negatively correlated with time to recurrence, whereas the rate of change in f-SO_2 after radiation therapy from days 12 to day 15 was positively correlated with time to recurrence (Fig. 3). In other words, within the LF group, tumors with shorter recurrence times exhibited a faster increase in oxygenation during radiation therapy and a slower increase in oxygenation after radiation therapy. No significant correlation was found between the rate of change of f-SO_2 across days 7 to 10 and recurrence time. In the CTL group there were no significant correlations between changes in f-SO_2 and the rate of tumor growth across any time intervals (data not shown).

Low-dose/fraction radiation does not initiate an increase in oxygenation

Figure 4 shows oxygenation kinetics for mice irradiated with the lowest 2 radiation dose fraction sizes (7.5 Gy and 9.5 Gy combined) and highest radiation dose fraction size (13.5 Gy). The f-SO_2 of mice receiving high-dose radiation was significantly higher than in control mice after day 2 ($P<.05$ for day 3 and 15, $P<.01$ for day 3, 4, 5, 7, 10, and 12). Statistical significance was not observed when comparing oxygenation kinetics of tumors receiving 7.5 and 9 Gy to control tumors. The oxygenation kinetics are shown in Figure E2 (available online at www.redjournal.org).

Discussion

Tumor hypoxia is considered a major factor in predicting radiation therapy treatment outcome because hypoxic tumor cells are 3-fold more resistant to irradiation than aerobic cells (13). However, it is not clear whether kinetic changes in tumor oxygenation during or after radiation therapy are related to the probability of achieving local tumor control. Clinical studies using polarographic microelectrodes to measure tumor hypoxia show that the efficacy of radiation therapy is negatively influenced by the extent of pretreatment tumor hypoxia (14-18). Nevertheless, it has been difficult to evaluate the kinetics of oxygenation because other methods to measure tumor oxygenation kinetics are invasive (microelectrodes) or quite expensive (positron emission tomography). Reoxygenation during

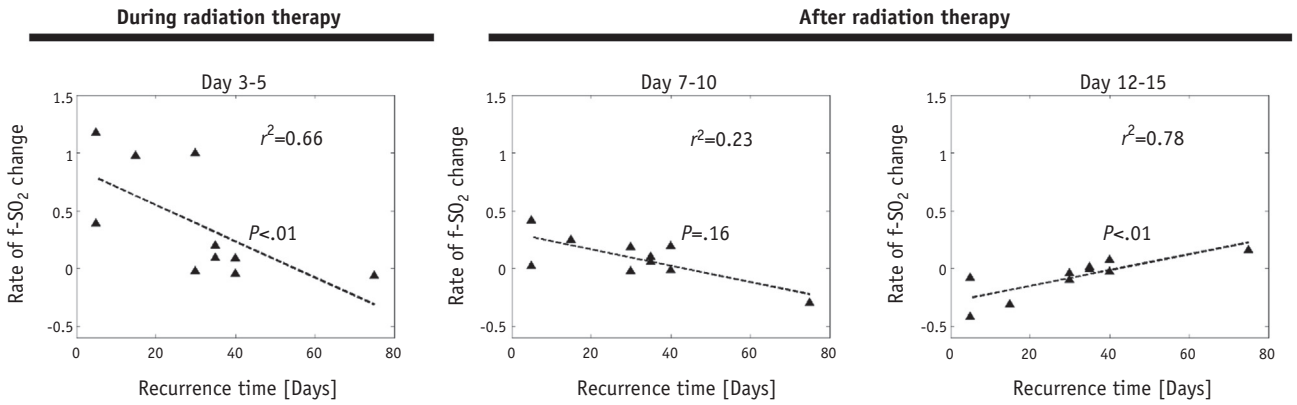

Fig. 3. Oxygenation rate is significantly correlated with recurrence time within the local failure group. The rate of change in baseline-corrected vascular oxygenation (f-SO_2) after radiation therapy from day 12 to day 15 was positively correlated with tumor recurrence time. The rate of change in f-SO_2 from day 3 to day 5 during radiation therapy was negatively correlated with time until tumor recurrence. Within the local failure mice, tumors that showed higher oxygenation profiles during radiation therapy tended to recur faster.

chemoradiotherapy was associated with treatment outcome in one study of HNC patients (11). Brizel et al (19) used microelectrodes to show that reoxygenation early in the course of thermoradiation therapy of soft-tissue sarcomas was associated with a favorable response to treatment. In another study, no evidence for reoxygenation after the first 10 to 15 Gy was seen in patients with HNC treated with fractionated chemoradiotherapy (18). Optical spectroscopy is relatively inexpensive and completely noninvasive. Using optical spectroscopy, this study, for the first time, reports daily serial tumor vascular oxygenation measurements in mice, during and after fractionated radiation therapy.

Local control and LF mice exhibited significantly different trajectories in oxygenation kinetics: LCs demonstrated significantly improved oxygenation compared with LFs, 12 to 15 days after the first day of a 5-day fractionated course of radiation therapy. These changes occurred at a time when tumor volumes between the LC and LF mice were not significantly different. The classification performance is better when the model was built on the parameters computed from day 12 to 15 than from day 3 to 5 and day 7 to 10 intervals. Local control mice showed improved oxygenation and blood perfusion, whereas LF demonstrated a decrease or no improvement in oxygenation and blood perfusion in the day 12 to 15 interval. The results are consistent with the concept that the improvement in oxygenation observed after radiation therapy may be the result of decreased oxygen consumption in the tumor due to tumor cell death. However, these effects occurred before measureable reduction in tumor volume. Secomb et al (20) previously demonstrated that relatively minor changes in oxygen consumption rate (10%-30% reduction), as could

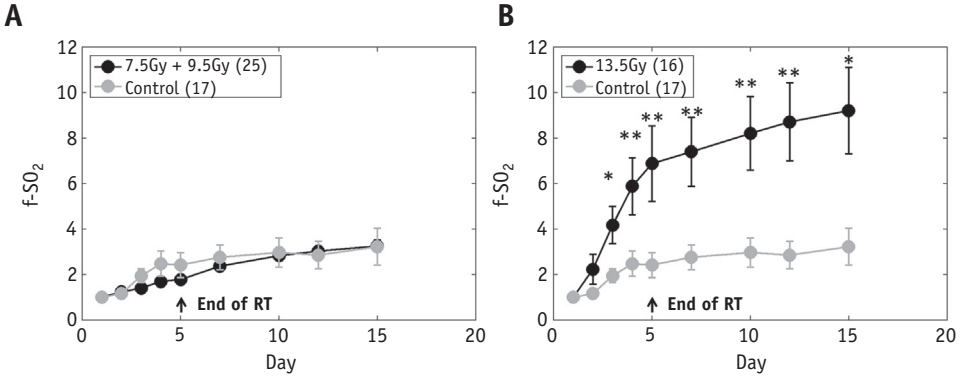

Fig. 4. Fractions of 7.5 Gy and 9.5 Gy cannot initiate an increase in oxygenation. (A) Baseline-corrected vascular oxygenation (f-SO_2) for tumors irradiated with 7.5 Gy and 9.5 Gy was not significantly different from the control tumors during and after radiation therapy (RT). (B) The f-SO_2 of tumors irradiated with 13.5 Gy was significantly higher than in control tumors after day 2 ($P<.05$ for day 3 and 15, $P<.01$ for day 3, 4, 5, 7, 10, and 12). P values were computed with Wilcoxon rank−sum test. Error bars show standard error. ($^{*}P<.05$, $^{**}P<.01$).

occur with a cell loss of equal magnitude, can dramatically reduce tumor hypoxia. Cell loss of this magnitude would not likely be detectable on a tumor volume measurement (20). There was no consistent relationship between [THb] and change in SO_2. These results suggest that perfusion change is not directly responsible for the changes in SO_2. This is further evidence that changes in oxygen consumption rate are likely influencing the oxygenation kinetics. Secomb et al (20) have shown previously that oxygen consumption rate has a more dynamic effect on oxygen transport than changes in perfusion.

The relationship between the observed oxygenation rates and time to failure from day 3 to day 5 during radiation therapy is strikingly different from day 12 to day 15 after radiation therapy for the mice in the LF group (Fig. 3). The negative relationship between the changes in tumor oxygenation during radiation therapy and time of tumor recurrence (days 3-5) might be explained by the upregulation of hypoxia-inducible factor 1 (HIF-1) (21). It was previously reported that an HIF-1 target gene, vascular endothelial growth factor, is upregulated 24 to 48 hours after radiation therapy. The upregulation of vascular endothelial growth factor protects endothelial cells from death (21, 22). The upregulation of HIF-1 may have mediated the switch from aerobic to anaerobic metabolism, which could have further protected tumor cells from death (23). Zhong et al (24) previously reported that upregulation of HIF-1 after radiation therapy protects tumor microvessels and promotes a switch to anaerobic metabolism.

When mice were stratified by radiation dose, mice that received the highest dose of radiation had significantly different tumor oxygenation kinetics than mice that received the lower doses of radiation (Fig. 4). These results suggest that a faster increase in oxygenation can be triggered by higher radiation doses. The difference in oxygenation kinetics may be related to sensitivity of endothelial cells to radiation (25). Garcia-Barros et al (25) showed that tumors in endothelial cell apoptosis−resistant mice were relatively radioresistant because their endothelial cells do not undergo apoptosis via activation of the acid sphingomyelinase pathway. Moreover, doses of radiation therapy <10 Gy did not induce endothelial cell apoptosis in wild-type mice (25-27). Below 10 Gy, a decrease in endothelial cell kill combined with the increase in HIF-1 expression, discussed above, may offer radioprotection to tumor cells. If tumor cells are radioprotected, cell mass would remain relatively large, and oxygen consumption rates would be maintained (28).

Diffuse reflectance spectra provides a label-free, noninvasive, simple, and cost-effective means to quantitatively and noninvasively measure and quantify tissue hypoxia in vivo (12, 29-31). It is ideal for serial assessments of tumor hypoxia/perfusion before, during, and after radiation therapy. Early prediction of treatment failure could lead to clinical decisions about more-aggressive treatments, thereby improving likelihood for a favorable treatment outcome.

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