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In Vivo Imaging of Changes in Tumor Oxygenation During Growth and After Treatment

Anna Bratasz¹, Ramasamy P. Pandian¹, Yuanmu Deng¹, Sergey Petryakov¹, John C. Grecula³, Nilendu Gupta³, and Periannan Kuppusamy^{1,2,*}

¹ Center for Biomedical EPR Spectroscopy and Imaging, Ohio State University, Columbus, Ohio, USA

² Comprehensive Cancer Center, Davis Heart and Lung Research Institute, Department of Internal Medicine, Ohio State University, Columbus, Ohio, USA

³ Department of Radiology, Ohio State University, Columbus, Ohio, USA

Abstract

A novel procedure for in vivo imaging of the oxygen partial pressure (pO₂) in implanted tumors is reported. The procedure uses electron paramagnetic resonance imaging (EPRI) of oxygen-sensing nanoprobes embedded in the tumor cells. Unlike existing methods of pO₂ quantification, wherein the probes are physically inserted at the time of measurement, the new approach uses cells that are preinternalized (labeled) with the oxygen-sensing probes, which become permanently embedded in the developed tumor. Radiation-induced fibrosarcoma (RIF-1) cells, internalized with nanoprobes of lithium octa-*n*-butoxy-naphthalocyanine (LiNc-BuO), were allowed to grow as a solid tumor. In vivo imaging of the growing tumor showed a heterogeneous distribution of the spin probe, as well as oxygenation in the tumor volume. The pO₂ images obtained after the tumors were exposed to a single dose of 30-Gy X-radiation showed marked redistribution as well as an overall increase in tissue oxygenation, with a maximum increase 6 hr after irradiation. However, larger tumors with a poorly perfused core showed no significant changes in oxygenation. In summary, the use of in vivo EPR technology with embedded oxygen-sensitive nanoprobes enabled noninvasive visualization of dynamic changes in the intracellular pO₂ of growing and irradiated tumors.

Keywords

EPR oximetry; EPR imaging; LiNc-BuO; RIF-1 tumor; tumor oxygenation; radiation

Tumor hypoxia is strongly linked to poor treatment outcome from chemo- or radiotherapy in several human malignancies (1,2). Hypoxic tumors are biologically more aggressive, and it has been reported that hypoxic sarcoma (3) or cervical cancers (4) tend to metastasize. The fact that poorly oxygenated tumors are more aggressive and less susceptible to treatment suggests that the tumor oxygenation status is an important parameter for cancer treatment (5, 6). The observation of substantial inter- and intratumor heterogeneities among tumors of similar histology and sites further emphasizes the importance of the measurement of hypoxia in individual tumor/patients. The ability to monitor changes in the partial pressure of oxygen (pO₂) following treatment could have profound implications for the planning of effective therapeutic strategies (5,6). In particular, radiotherapy could benefit from modulated treatment based on regional variations in pO₂.

*Correspondence to: Periannan Kuppusamy, Ph.D., Ohio State University, 420 West 12th Ave., TMRF-114, Columbus, OH 43210. E-mail: kuppusamy.1@osu.edu.

The influence of tumor oxygenation on treatment outcome has stimulated the development of a variety of methods for measuring tumor oxygenation (7–9). These methods include paired-survival curve assays of hypoxic fractions (10), radiation-induced DNA damage measured by “Comet” assay (11), cryospectrophotometric measurements of hemoglobin oxygen saturation (12), immunohistochemical detection of nitroimidazole binding (13,14), polarographic oxygen electrodes (15), fluorescent and phosphorescent probes based on oxygen-quenching (16,17), and magnetic resonance (MR) methods (18–23). The polarographic electrode (or “pO₂ histograph”), although invasive, provides a direct measurement of the concentration of oxygen (pO₂), and is the only such device approved for use in patients. MR-based methods, such as nuclear MR (NMR) and electron paramagnetic resonance (EPR), have the advantage of enabling noninvasive measurement and imaging of the oxygen concentration in tissues (18, 24–26). The EPR-based method, known as “EPR oximetry,” uses oxygen-sensing spin probes whose EPR lines are broadened by molecular oxygen. The oxygen-induced line-broadening is usually linear with respect to the pO₂. The measured linewidth can be converted to the oxygen concentration using appropriate standard curves. This approach uses implants of particulate oximetry probes, such as lithium phthalocyanine (LiPc) or lithium octa-*n*-butoxy-naphthalocyanine (LiNc-BuO), whose EPR linewidths are highly sensitive to the local oxygen concentration (27,28). The probes can be implanted at the desired site (29) or internalized in cells, enabling highly accurate measurements of intracellular pO₂ (30). These probes are stable in tissues, nontoxic, and biocompatible (27). The measurements can be performed noninvasively and repeatedly over periods of months at the same site (29).

In the present work we used noninvasive EPR-based oximetry to determine the oxygen concentration in experimental tumors. Unlike the existing methods of oxygen measurement, wherein the probes (needle electrodes, optical probes, or EPR implants) are physically inserted during measurement, we used nanoprobe that were permanently embedded in the tumor. This was done by labeling the cancer cells with the oxygen-sensing nanoprobe at the time of implantation in the animal model. A particular advantage of this procedure is that it is noninvasive, in terms of both implanting the probe and obtaining readouts of oxygen concentration. Further, EPRI can be performed to map the spatial distribution of oxygen concentration in a tumor. Although the cellular labeling procedure is not applicable to preexisting tumors, it is useful for studying experimental tumors. The goal of the present study was to demonstrate the feasibility of noninvasively mapping changes in oxygen concentration in growing experimental tumors subjected to radiation treatment. The study was performed on implanted tumors in C3H mice using radiation-induced fibrosarcoma (RIF-1) cells labeled with oxygen-sensing nanoprobe. The results showed significant redistribution and reoxygenation in tumors as a function of growth, size, and radiation treatment.

MATERIALS AND METHODS

Reagents

Cell culture medium (RPMI 1640), fetal bovine serum, penicillin/streptomycin, sodium pyruvate, trypsin, and phosphate-buffered saline (PBS) were purchased from Gibco BRL (Grand Island, NY, USA). An in vitro 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) toxicology assay kit was obtained from Sigma (St. Louis, MO, USA). Lithium octa-*n*-butoxy-naphthalocyanine (LiNc-BuO) was synthesized as previously reported (27,28). Triarylmethyl radical (TAM, Ox063) was obtained from Nycomed Innovations (Malmö, Sweden).

Preparation of Oxygen-Sensitive Nanoprobes for Internalization Into Cells

Microcrystalline particulates of LiNc-BuO were suspended in complete RPMI medium 1640 (10 mg/0.5 ml) and sonicated for a total of 5 min on ice with a probe sonicator (Sonic

Bismembrator, model 100; Fisher Scientific), working at 20 kHz, at a setting of 7 W. At the end of sonication, the suspension was placed on ice for 1 min to allow the heavier particulates to settle down to the bottom of the tube. The supernatant liquid was then transferred to a separate tube for intracellular delivery. The supernatant contained particulates of LiNc-BuO in the form of a fine dispersion with a mean particle size of 270 ± 120 nm, as measured by a Zetasizer (Malvern Instruments, UK) particle size analyzer.

Internalization of LiNc-BuO Into Cells

RIF-1 cells were grown as a monolayer attached to the bottom of cell culture dish (10^5 cells/25-mm dish) with 2 ml of complete RPMI medium 1640. The cells were exposed to a 25 μ l of LiNc-BuO particulate suspension containing 20 μ g of particle. The cells were incubated at 37°C under a 95% air/5% CO₂ environment for 72 hr until they reached about 80% confluence. Following incubation, the cells were washed five times with PBS to remove the uninternalized particles, and then trypsinized and cultured in a new dish. When the cells became attached to the base of the dish, a fresh dose of probe was added and the cells were incubated until the next passage. This procedure was repeated for four cycles to optimize cellular uptake of the probe. At the end of the fourth passage, the cells were washed and assessed by light microscopy and Trypan blue assay. We estimated that about 70–90% of the cells were labeled with the probe. The uptake of particulates into the cells was quantified by EPR spectroscopy. The EPR measurements were performed using an X-band EPR spectrometer. The EPR spectra were measured using 5-mW microwave power, 10- μ T modulation amplitude, 100-kHz modulation frequency, 80-ms time constant, 0.5-mT scan, and 10-s scan time. The area under the EPR curve (double integral) of the cell suspension was compared with that of a trityl radical (triarylmethyl, TAM Ox063) standard to quantify the cellular uptake of the probe. A 20- μ l volume of the cell suspension containing 4000 cells and 10 μ M of TAM was drawn into a gas-permeable Teflon tube (ID: 0.82 mm; Zeus Industries, SC, USA) and then subjected to an EPR spectroscopy assay. The spin content was expressed as the number of spins per cell.

Cellular Membrane Integrity Assay

The cytotoxicity of the particulates to RIF-1 cells was studied using a cellular membrane integrity assay. Cells were grown in 60-mm Petri dishes containing RPMI 1640 medium supplemented with 10% FBS, 2% sodium pyruvate, and 1% penicillin/streptomycin. When the cells reached 60% confluence, the medium in each dish was replaced with fresh media containing 1 mg of intact (unsonicated) LiNc-BuO microcrystalline particulates. The cells were incubated for 24 hr and then rinsed five times with PBS to remove the noninternalized particulates. The cells were trypsinized, centrifuged, and stained with 1% Trypan blue to assay the integrity of the cell membrane.

Cell Toxicity Assay

The toxicity of nanocrystalline LiNc-BuO particulates on the of RIF-1 cells was established by MTT assay. After four cycles of passages in both the absence and presence of sonicated particulates of LiNc-BuO, the cells were washed five times with PBS to remove any externally attached particulates, trypsinized, and then suspended in media. The internalization of the particulates in the cells was confirmed by EPR spectroscopy, as described above. Then 100 μ l of RPMI medium 1640 media (without phenol red, and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin) containing about 5×10^3 cells per well were plated in 96-well dishes, incubated in an atmosphere of 95% air/5% CO₂ at 37°C for 24 hr, and assayed for cytotoxicity of probe on the cell using an MTT-based toxicology assay kit (Sigma). Measurements were taken using a Beckman Coulter AD 340 UV-VIS spectrophotometer set at a wavelength of 570 nm with a reference filter at 690 nm. The data were obtained as an average of three to five wells.

Animals

Female C3H mice were obtained from Charles River Laboratories (Wilmington, MA, USA). The animals were housed five per cage in a climate- and light-controlled room. Food and water were allowed ad libitum. The animals were 50 days old and weighed about 25 g at the time of the experiment. The animals used during the study were anesthetized with ketamine and xylazine (i.p.) and inhaled room air. During the measurements the body temperature of each animal was monitored using a rectal thermistor probe and maintained at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ using an infrared lamp placed just above the animal. All animals were used according to the Public Health Services Policy, the Federal Welfare Act, and Institutional Laboratory Animal Care and Use Committee procedures and guidelines.

Tumor Growth and Implantation of the Oxygen Probe

The oxygen-sensitive probes were introduced into the cells by coincubation as described in the Materials and Methods section ("Internalization of LiNc-BuO Into Cells"). The cells were trypsinized, centrifuged, and suspended in PBS (without calcium and magnesium ions). The uptake of the particulates into the cells was quantified by EPR spectroscopy before the cells were injected into the animals. The cells (1×10^6), internalized with the particulates, were injected subcutaneously into the upper portion of the right hind limb of C3H mice and grown as a solid tumor. The size of the tumor was measured daily using a digital Vernier caliper. The tumor volume was determined from the orthogonal dimensions (d_1, d_2, d_3) using the formula $(d_1 \cdot d_2 \cdot d_3) \cdot \pi/6$.

Histology

Twelve days after implantation of cells internalized with LiNc-BuO nanoparticulates, the nonirradiated tumor was isolated, fixed in paraformaldehyde, and then sectioned in paraffin (slice thickness = 4 μm) and stained with hematoxylin and eosin stain (H&E). Pictures of the slides were taken using an inverted light microscope (Nikon TE2000-U).

Tumor Perfusion

Tumor perfusion was imaged using Patent blue as previously described (31). The Patent blue solution (1.25%) was injected intravenously into the nonirradiated mice to measure tumor blood perfusion. One minute after injection the tumor was isolated, frozen, and sliced (into 2-mm slice thickness). Pictures of the slides were taken with a stereoscopic optical microscope (Nikon SMZ1000).

EPR Oximetry and Imaging Measurements

LiNc-BuO was used as the probe for EPR oximetry. The peak-to-peak linewidth of the EPR spectra of the probe in the tissue was used to determine $p\text{O}_2$. The value of $p\text{O}_2$ in the tissue was obtained from a standard curve of the EPR linewidth vs. the oxygen concentration, as reported previously (28). The in vivo EPR measurements were performed by using an L-band (1.2 GHz) EPR spectrometer with a bridged loop-gap resonator. Mapping of the particulates and oxygen concentration in the tumor was obtained by spatial and spectroscopic (spectral-spatial) EPRI methods that are well established in our laboratory (29,32,33). The principle of the EPR mapping of $p\text{O}_2$ is based on using the oxygen-dependent linewidth data from each voxel within the object. A notable caveat is that the $p\text{O}_2$ information can only be obtained from voxels that have a measurable EPR signal intensity. Voxels with undetermined $p\text{O}_2$ values are coded with black color. The following parameters were used for EPR image acquisition: sweep width = 1 mT, sweep time = 4 s, modulation amplitude = 18 μT , time constant = 0.04 s, microwave power = 5 mW, field of view (FOV) = 10 mm, and number of projections = 256. A program based on Matlab Software (The MathWorks, Inc.) was developed to compute $p\text{O}_2$ values from the image data. The software used an automated fitting of the line-shape data

to a Lorentzian function and converted the linewidth to pO_2 using a standard oximetry curve. The output of the result was a 256 color-coded 64×64 grid image of pO_2 . However, a linear interpolation was used in the hardcopy (high-resolution) representation of the images.

Tumor Irradiation

The animals were irradiated in two groups. The first group of animals had smaller tumors (mean volume = $114 \pm 14 \text{ mm}^3$, $N = 4$) and the second group had larger tumors (mean volume = $260 \pm 28 \text{ mm}^3$, $N = 5$) at the time of irradiation. The animals were under anesthesia during the irradiation procedure. The right hind limbs of the mice were arranged concentrically in an ellipsoid shape and secured to the treatment table with hypoallergenic tape. A Superflab bolus (1 cm) was placed over the hind limbs to increase the surface dose to 100%. Customized Cerrobend blocking was utilized to decrease the dose to the torso of the animals to less than 5%. A dose of 30 Gy was then delivered with 6 MeV electrons at a dose rate of 3 Gy/min by a Siemens linear accelerator. The source-to-surface distance was 100 cm. After completion of the radiation, the position of the hind limbs was verified with a light source, which indicated that the animals had not moved out of position.

Statistical Analysis

Data on graphs were taken from spectroscopy and expressed as the mean $\pm$ SEM. Comparisons among groups were performed using a Student's *t*-test with the level of significance (*P*) set at 0.05.

RESULTS

Characterization of Particulate Uptake by RIF-1 Cells

RIF-1 cells were incubated in the presence of nanoparticles of LiNc-BuO for up to four passages to establish optimal internalization. The uptake of the particulates in cells was visualized at the end of each passage by light microscopy. Figure 1a shows a typical image of cells in culture in the presence of particulates. The particulates taken up by the cells were observed to be clumped together rather than uniformly distributed within the cells. Large and uninternalized particulates could be easily removed from the culture by repeated washing. Additional washing of the cells or further incubation leading to proliferation did not show any unattached particulates in the medium, suggesting that the particulates were firmly internalized in cells. The mechanism of endocytosis and the subcellular distribution of the internalized particulates are not clearly understood. A detailed study of the cellular uptake of the particulates is currently under investigation. At the end of the fourth passage, the cells were washed with PBS to remove the uninternalized extracellular particulates, trypsinized, and suspended in medium. The cell suspension was then centrifuged and the cells and supernatant medium were subjected separately to EPR spectroscopy assays to characterize the presence of the internalized probes. No EPR signal was observed in the supernatant medium of cells cultured in the presence of the particulates or in control cells (not exposed to the particulates). On the other hand, a single EPR peak with a linewidth of $145.4 \pm 2.0 \text{ } \mu\text{T}$ was observed in cells equilibrated with room air. The oxygen sensitivity of the EPR linewidth ($0.761 \text{ } \mu\text{T/mmHg}$) was identical to that of uninternalized sonicated probes, suggesting that the oximetry properties of the probes were not modified upon internalization. The EPR spin density of the particulates internalized in cells was determined in comparison with a standard solution of TAM radical to be 6×10^{11} spins per cell. It should be noted that the amount of spin per cell is about one order of magnitude greater than the detection limit of the commercial EPR spectrometers.

Effect of Probe (LiNc-BuO) on Cell Metabolism and Tumor Growth

The cytotoxicity of LiNc-BuO particulates on the RIF-1 cells was established by cellular membrane integrity or MTT cell toxicity assay. As shown in the inset of Fig. 1b, the viability of the cells containing the LiNc-BuO particulates was not significantly different when compared to controls. The data suggested that incubation or internalization of the cells with LiNc-BuO particulates did not affect the viability of the cells in culture.

The effect of the internalized particulates on the growth rate of the RIF-1 tumor was studied using a tumor growth (volume) curve. Both untreated control and LiNc-BuO-internalized cells showed similar growth rates (Fig. 1b). There was no significant difference ($P > 0.05$) between the doubling times of control cells (2.8 ± 0.5 days) and cells internalized with the probe (2.4 ± 0.5 days).

Figure 1c shows the histology of an unirradiated tumor obtained 12 days after implantation of the cells. The tumor was grown from RIF-1 cells internalized with spin probes. No apparent differences could be observed in the tumor tissue as compared to untreated control (data not shown). The particulates were distributed fairly densely in the tissue. However, the images did not establish whether the spin probes remained internalized or were expelled and remained in the tissue interstitium.

In Vivo Imaging of Probe and pO_2 Distribution in Growing Tumor

Figure 2 shows typical images obtained from a tumor labeled with the spin probe at two different time points of tumor development, namely the fifth and ninth days, after inoculation. The image represents the distribution of the spin probe whose voxel intensity was above the detection threshold. It was observed that the area of distribution of the probe was smaller than the size of tumor. For example, on day 5 (Fig. 2), the probes were detected in about 62% of the tumor. The spectroscopic (spectral-spatial) EPR image, which was obtained from the same tumor at the same orientation, showed a very heterogeneous distribution of oxygen levels. The figure also shows the frequency plot (number of occurrences of a particular tumor pO_2 value in the image). The mean pO_2 value of the tumor on day 5 (volume = 86 mm^3) was 9.1 mmHg (median: 7.5 mmHg) and on day 9 (volume = 113 mm^3) was 7.4 mmHg (median: 4.9 mmHg). The data suggested a decrease in pO_2 during the tumor growth period. This was also evident from the left-shift of the values on day 9 (skew = 1.25) as compared to the distribution on day 5 (skew = 0.63). The images showed a considerable increase in the hypoxic fractions ($< 5 \text{ mmHg}$: 30.0 – 46.9%; $< 10 \text{ mmHg}$: 60.8 – 75.8%) during this period. It should be noted that the distribution of the probe within the tumor did not change significantly between the two time periods.

In Vivo Response of the Probe to Changes in pO_2

Carbogen (95% O_2 + 5% CO_2) breathing by mice is known to enhance blood flow and hemoglobin- O_2 saturation in tumors and hence increase tissue pO_2 . In order to map the spatially resolved changes in the tumor pO_2 , we performed additional experiments wherein the imaging measurements were repeated on the same tumor when the mice were breathing room air or carbogen. While the probe distribution (Fig. 3) was intact except for some minor degradation of image quality due to line-broadening, the oxygen map showed that carbogen breathing by the mice increased the overall tumor pO_2 , and that the oxygen distribution further showed a significant increase in the mean pO_2 value. The mean pO_2 values of the tumor before and after the mice breathed carbogen were 11.3 mmHg (median = 8.5 mmHg) and 15.1 mmHg (median = 12.6 mmHg), respectively. The increase in pO_2 after carbogen breathing by the mice was also evident from the right shift of the pO_2 histogram (Fig. 3). The data also showed a substantial reduction in the hypoxic fractions ($< 5 \text{ mmHg}$: 32.8 – 14.2%; $< 10 \text{ mmHg}$: 49.5 – 23.5%) upon carbogen breathing.

Mapping of pO₂ in the Tumor After Irradiation

In order to determine the response of pO₂ to tumor treatment, tumors were irradiated on the ninth or 11th day following inoculation of tumor cells internalized with the particulates, as described in the Materials and Methods section. Figure 4 shows the oxygen maps obtained from a small tumor (volume = 113 mm³) with internalized particulates before and 1 hr after 30-Gy irradiation. The mean and median pO₂ values of the tumor before treatment were 10.6 and 7.0 mmHg, respectively, and increased to 12.5 and 8.0 mmHg after irradiation. The increase was also evident from the right shift of the pO₂ values in the frequency plot (Fig. 4) before treatment (skew = 1.17) as compared to the distribution after treatment (skew = 0.82). The data showed a significant reduction in the hypoxic fractions (<5 mmHg: 32.5–28.1%; <10 mmHg: 59.1–53.8%) following irradiation. Figure 5 shows the distribution of oxygen obtained from a large tumor (volume = 327 mm³) at 1.5 hr and 7.2 hr after irradiation. The mean pO₂ value of the tumor before treatment was 6.8 mmHg (median = 6.7 mmHg). It showed a decrease (mean = 4.2 mmHg, median = 2.5 mmHg) at 1.5 h, followed by an increase (mean = 5.9 mmHg, median = 6.2 mmHg) at 7.2 h after irradiation. The images as well as the frequency plots clearly showed that there were substantial alterations in the oxygen concentration in the core region of the large tumor during the first several hours following irradiation. The data showed a substantially large increase in the “<5 mmHg” hypoxic fraction (26.8–70.8%) and a significant increase in the “<10 mmHg” fraction (78.0–91.5%) at 1.5 hr post-irradiation period. However, the values decreased (<5 mmHg: 36.2%; <10 mmHg: 84.1%) at 7.2 hr following irradiation.

The pO₂ levels in the pre- and post-irradiated tumor were calculated from two groups of animals: one group with smaller tumor volumes (average = 114 ± 14 mm³, *N* = 4) and another group with bigger tumors (average volume = 260 ± 28 mm³, *N* = 5). The measurements were made every few hours during the first 24 hr and daily for the next 3 days following treatment (Fig. 6). The mean pO₂ in the pre-irradiated smaller tumors was 10.0 ± 2.0 mmHg, which increased to 22.0 ± 1.7 mmHg at 6 hr after treatment. The pO₂ values slowly returned to baseline values in about 14 hr, and decreased to below baseline values (<7 mmHg) thereafter. In the case of larger tumors the initial pO₂ was 7.8 ± 0.2 mmHg, which decreased to <5 mmHg during 24 h post-irradiation.

In order to understand the effect of tumor size on the post-irradiation reoxygenation behavior, we determined the nature of blood perfusion in the core of the tumor, where the spin probes were maximally located. Following the imaging measurements, the mice were intravenously infused with a solution of Patent blue and the tumors were examined for blue staining, which is indicative of perfusion (31). As shown in Fig. 6, the small tumors showed relatively better blood perfusion compared to the large tumors. The perfusion data suggest that the pO₂ data obtained from the large tumors are from regions with a lack of perfusion, and that the observed oxygen dynamics in the smaller tumors following irradiation are due to changes that might occur in the surrounding viable tissue of the tumors.

DISCUSSION

A noninvasive method for in vivo mapping of oxygen concentration in a transplanted tumor is reported. The method uses paramagnetic detection and imaging of oxygen-sensing nanoprobe (LiNc-BuO nanoparticulates) that are incorporated into the growing tumor using cancer cells prelabeled with the probe. A particular advantage of the method is that it is noninvasive, in terms of both implanting the probe and obtaining readouts of intracellular oxygen concentration during tumor development. In contrast, established methods of oxygen measurements, such as Eppendorf electrode and OxyLite, require the insertion of a needle, catheter, or probe-head each time into the desired location, which can result in injury to the tissue and lead to artifacts in the measured pO₂ values (17,34,35). The present method avoids the problems associated with the recurrence of an invasive procedure for repetitive measurements of oxygen over a

period of time (from days to weeks). Although the present method is applicable only to experimental tumors, it can help to eliminate errors associated with fresh injury, such as disruption of capillary blood vessels and inflammation processes at the measurement site, which may lead to erroneous measurements of oxygen levels. In addition, the Eppendorf electrode and OxyLite methods are not suitable for repeated measurements as they do not allow the probe to be placed at exactly the same location each time (34).

The present method is similar to normal particulate-based EPR oximetry, wherein the probe is implanted at the desired site and noninvasive, repeated measurements of oxygen are made precisely thereafter (23). The advantages with the present method are that it is noninvasive with respect to implantation of the probe, and it enables intracellular measurements and imaging of pO_2 . A notable limitation of this method is that it is not suitable for clinical applications in its present form, since it would require prelabeling of cells in the tumor.

In our previously reported coembedded procedure (29), we injected the RIF-1 cells mixed (uninternalized) with large-sized (10 – 60 μm) LiPc particulates in subcutaneous tissue for tumor growth. In that case the probes were extracellular and clumped together, and thus did not significantly interfere with the tumor growth. However, in the present method the seeded RIF-1 cells are preinternalized (labeled) with submicron particulates of LiNc-BuO. Although this procedure showed some inhibitory effect on the tumor growth rate, it yielded a better “unclumped” distribution of the probe in the tumor, resulting in a wider distribution of the probe (42– 62% compared to 15–25% in the coembedded procedure). Thus, the present method has the ability to map intracellular pO_2 in a large portion of the tumor.

The results of the present study, obtained using a murine tumor prelabeled with oxygen-sensing paramagnetic nanoprobe, showed spatially resolved changes in oxygenation as a function of growth and in response to radiation treatment. In vivo imaging of the tumor showed a heterogeneous distribution of oxygen concentrations that exhibited substantial reoxygenation on hyperoxic challenge, as well as after treatment with ionizing radiation. In general, the measurements showed tissue-specific changes in oxygenation in large tumors with significantly compromised perfusion. There was considerable redistribution, as well as an overall increase in the tissue pO_2 in the core region of small tumors during the first 24 h post-irradiation, which can be correlated to increased perfusion. On the other hand, the necrotic regions showed no significant changes after irradiation.

We previously measured the pO_2 values in RIF-1 tumors of approximately 6 mm in diameter by implanting a lithium phthalocyanine (LiPc) oximetry probe directly into the tissue, and found that the tumors were significantly hypoxic and continued to be hypoxic thereafter during the growth phase (28). In a subsequent study we used LiPc coimplanted with the tumor cells, which enabled us to monitor the changes in the tissue oxygenation that occurred immediately after inoculation. It was observed that the pO_2 rapidly decreased from 14 ± 2 mmHg on the first day of injection to about 4 mmHg within 4 days, when the tumor volume was ~ 50 mm³ (29). Thus it appeared that tissue oxygenation fell off rapidly in the very early stages of solid tumor formation. This suggests that tumor hypoxia occurred very early in the tumor progression due to an insufficient supply of oxygen to the exponentially proliferating cells.

Several groups have reported a wide range of pO_2 values in RIF-1 tumors measured using microelectrodes. Adam et al. (36) reported a median pO_2 of 1.2 mmHg for tumor sizes of 52–924 mm³. Pogue et al. (37) reported a median pO_2 of 12.9 mmHg for a tumor volume of 634 ± 203 mm³. Kavanagh et al. (38) reported a median pO_2 of 1.2 mmHg and a mean of 4.5 ± 0.5 mmHg. Similarly, there are also many reports on the variable effects of carbogen breathing on oxygenation in RIF-1 tumors (39–41). The variation in regional pO_2 and its response to

carbogen breathing is largely attributed to the heterogeneity of tumor oxygenation, as well the method of measurement.

The results of the present study show some similarities to those previously obtained using other methods. Mason (42) used the ^{19}F NMR spin relaxation rate of a perfluorocarbon (PFC) emulsion to measure the pO_2 value in a Dunning prostate rat tumor. He reported a pO_2 value of 75 mmHg in a small tumor (~10 mm), which dropped to ~1 mmHg when the tumor size had doubled. Baldwin and Ng (43) used PFC emulsion and MRS/MRI to measure the pO_2 in KHT murine sarcomas. The median pO_2 of the tumor decreased from 60 mmHg to 0 mmHg corresponding to tumor masses of 0.2 g and 1.8 g, respectively. The overall tendency of extracellular pO_2 to change as a function of tumor growth in RIF-1 tumors observed in the present study is similar to that reported in the aforementioned articles. The differences in the observed oxygen levels can be attributed to the tumor models and methods used. The EPR method measures the pO_2 , while the MRI method detects the concentration of dissolved oxygen in the tissue.

In the present study, RIF-1 tumors with embedded oxygen sensors were irradiated with a dose of 30 Gy on the ninth or 11th day of tumor growth. The pO_2 levels were measured before treatment, every few hours post-irradiation for 24 h, and daily for the next 3 days. The pO_2 increased after irradiation in tumors with small volumes (average = $114 \pm 14 \text{ mm}^3$) to a maximum at 4 – 6 h after treatment. Oxygenation of bigger tumors (average = $250 \pm 28 \text{ mm}^3$) was very low and unresponsive to the treatment. An increased level of pO_2 was observed during the 24-h post-irradiation period, and then it returned to the base-line value. The early increase in pO_2 (4 – 6 h) was probably due to a decrease in oxygen consumption under post-irradiation stress, or to changes in the interstitial pressure of the tumor tissue upon irradiation. Crockart et al. (44) reported an increase in pO_2 in fibrosarcoma type II (FSaII) and hepatocarcinoma transplantable liver tumors at 3–4 h after irradiation, which was attributed to a decrease in global oxygen consumption and an increase in oxygen delivery. The increase in oxygen delivery was due to radiation-induced acute inflammation and a decrease in interstitial fluid pressure (44).

The frequency plot obtained from the image data showed that the mean pO_2 value of the tumor was 10.6 mmHg (median = 7.0 mmHg) before treatment and 12.5 mmHg (median = 8.0 mmHg) after irradiation. The data showed a significant increase in the tumor pO_2 after X-ray treatment. This was also evident from the right shift of the values before treatment (skew = 1.17) as compared to the distribution after treatment (skew = 0.82). These data look slightly different from those reported by Goda et al. (45). After a 20-Gy radiation dose, the initial pO_2 value of $8.7 \pm 1.1 \text{ mmHg}$ in RIF-1 tumor decreased in 24 hr to $5.4 \pm 1.2 \text{ mmHg}$, and increased in 72 h to $8.2 \pm 1.0 \text{ mmHg}$. The time course for these changes in pO_2 was found to be independent of the dose. In another work, the authors showed that an increase in the pO_2 of the tumor can be associated with increased tumor perfusion (46), which can be related to the increase in nitric oxide production (47). Olive (48) reported that 6 hr after a squamous cell carcinoma tumor (SCCVII) was irradiated with a 10-Gy dose, the hypoxic fraction decreased from 18% to 6%. She concluded that the rapid reoxygenation of the tumor following exposure to 10-Gy radiation could be attributed to a decrease in oxygen consumption and an increase in tumor perfusion, rather than to redistribution or a loss of cells. Knowledge of the changes in tumor pO_2 could be used as an indicator of the time to schedule multiple cycles of radiation to improve efficacy.

In summary, we have demonstrated, using permanently embedded oxygen-sensing nanoprobe in an experimental tumor, that accurate measurement and imaging of cellular pO_2 can be performed in a tumor in vivo. The method enables repeated measurements of tissue oxygenation to be obtained from the same site. The results show significant reoxygenation in tumors as a function of growth, size, and radiation treatment. The use of in vivo EPR technology

with embedded oxygen-sensing nanoprobe enabled non-invasive visualization of dynamic changes in pO_2 in tumors.

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FIG. 1.

Internalization of the oxygen-sensing probe particulates in RIF-1 cells, and their effect on cell viability and tumor growth. **a:** Phase-contrast microscopic image (1500× magnification) of RIF-1 cells internalized with probes. The nanoprobe is seen as a black aggregate in the cell. The arrow indicates a large (micron-size) uninternalized single crystal of the probe. **b:** Tumor growth curves are shown for tumors grown with cells untreated (control; $N = 5$) or internalized with LiNc-BuO probe ($N = 7$). The tumor volume was calculated using measurements of three orthogonal dimensions. The tumors, in general, showed similar growth patterns. However, the data from the internalized group showed an overall reduced growth rate as compared to the control group. Inset in b: Effect of internalized particulates on cell viability. RIF-1 cells were cultured in the presence of extracellular LiNc-BuO crystals (size $> 5 \mu\text{m}$) for 24 h or intracellular LiNc-BuO crystals (size $270 \pm 120 \text{ nm}$) and assayed at the end of a single or four passages. The results indicate that the particulates had no significant effect on the viability of

RIF-1 cells in culture. **c:** Histology image under a high color threshold showing the distribution of the oxygen-sensing probes (indicated by arrows) in nonirradiated tumor tissue. The histology was obtained 12 days after implantation of cells internalized with LiNc-BuO nanoparticulates.

FIG. 2.

Representative images (10 mm $\times$ 10 mm) of probe and oxygen distribution in a growing RIF-1 tumor. The images were obtained on day 5 (volume = 86 mm³) and day 9 (volume = 113 mm³) after the mouse was inoculated with RIF-1 cells internalized with the nanoparticulate spin probes. The probe is seen to be distributed in the core of the tumor, which is about 62% $\pm$ 9% of the tumor volume on day 5, and 42% $\pm$ 6% on day 9. Note that the oxygen information is obtained only from the regions where the particulates are present. The images show the presence of significantly hypoxic pockets in the region of the tumor under examination.

FIG. 3.

Images of tumor oxygenation (pO₂) in an RIF-1 tumor taken when the mice were breathing room air or carbogen. The images (10 mm × 10 mm) were obtained on day 12 after the mice were inoculated (tumor volume = 125 mm³) with RIF-1 cells internalized with the nanoparticulate spin probes. The left panels show the distribution of the probe in the core of the tumor. Oxygen information is obtained only from regions where the particulates are present. The pO₂ image of the air-breathing mouse shows the presence of pockets of hypoxic regions in the tumor, which become more oxygenated during carbogen breathing. The histograms reveal a significant increase in tumor oxygenation upon carbogen treatment.

FIG. 4.

Effect of X-ray irradiation on tumor oxygenation. Change and redistribution of tumor oxygen levels are shown for a small (volume = 113 mm³) RIF-1 tumor before (pre-) and 1 h after (post-) X-ray irradiation. The tumor was implanted with RIF-1 cells internalized with nanoparticulates of LiNc-BuO probe. A dose of 30 Gy was delivered with 6 MeV electrons at a dose rate of 3 Gy/min. The images were obtained on the day of irradiation. Data show a redistribution of pO₂ with a modest increase in the pO₂ values.

FIG. 5.

Effect of X-ray irradiation on the oxygenation of a large tumor. Redistribution of tumor oxygen concentration is shown for a large (volume = 327 mm³) RIF-1 tumor before (pre-) and 1.5 h and 7.2 h after X-ray irradiation. The tumor was implanted with RIF-1 cells internalized with nanoparticulates of LiNc-BuO probe. A dose of 30 Gy was delivered with 6 MeV electrons at a dose rate of 3 Gy/min. The images were obtained on the day of irradiation. The data show a redistribution of pO₂ in the central core of the tumor. The data also show a significant decrease in the first 1.0–1.5 h followed by an increase 7.2 hr after irradiation.

FIG. 6.

Changes in tumor oxygenation levels following irradiation. Two groups of tumors were studied: (●) small tumors with an average volume of $114 \pm 14 \text{ mm}^3$ ($N = 4$), and (○) large tumors with an average volume of $250 \pm 28 \text{ mm}^3$ ($N = 5$). The tumors were obtained by inoculating the mice with RIF-1 cells internalized with nanoparticulates of LiNc-BuO spin probe. The right hind limbs containing the tumor were irradiated with a single dose of 30 Gy at a dose rate of 3 Gy/min. Insets: Blood-perfusion images of a small and a large tumor. The mice were infused with a solution of Patent blue to visualize blood perfusion in the tumor. **a:** Perfusion image of a small tumor (volume = 133 mm^3). **b:** Perfusion image of a large tumor (volume = 413 mm^3). The intensity of the blue color in the image represents the extent of blood perfusion in the tissue. The dark blue color in the images corresponds to the highly perfused muscle tissues surrounding the tumor (indicated by contour). The core of the large tumor is mostly pink, suggesting that the region is poorly perfused.