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Quantitative Assessment of Tumor Oxygen Dynamics: Molecular Imaging for Prognostic Radiology

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Abstract One of the fundamental molecules governing the survival of mammalian cells is oxygen. Oxygen has gained particular significance in tumor developmental biology and oncology. An increasingly diverse array of methods is now available to characterize tumor oxygenation. This Prospect will consider a new method, Fluorocarbon Relaxometry using Echo planar imaging for Dynamic Oxygen Mapping (*FREDOM*), which we have recently developed for oximetry, examine application to a specific therapeutic example and place this technique in the context of other approaches. J. Cell. Biochem. Suppl. 39: 45–53, 2002. © 2002 Wiley-Liss, Inc.

Key words: magnetic resonance imaging; hexafluorobenzene; oxygen; tumor; tissue factor; antibody targeting

The human genome has been sequenced and it is now realized that cells are ultimately characterized by gene expression and proteomics, which govern phenotype. There is also an increasing realization that environmental factors (epigenetic) can strongly influence cell development and response to therapy. This is particularly relevant to oncology. It has long been appreciated that hypoxic tumor cells are more resistant to radiotherapy [Gray et al., 1953]. Indeed, a threefold increase in radio resistance may occur when cells are irradiated under hypoxic conditions compared with $pO_2 > 15$ Torr for a given single radiation dose. However, recent modeling has indicated that the proportion of cells in the range 0–20 Torr may be most significant in terms of surviving a course of fractionated radiotherapy [Wouters and Brown, 1997]. Increasingly, there is evidence that hypoxia also influences such critical characteristics as angiogenesis, tumor invasion, and metastasis [Höckel and Vaupel, 2001; Knowles and Harris, 2001]. Moreover, repeated bouts of intermittent hypoxic stress may be important in stimulating tumor progression [Cairns et al., 2001]. Thus, the ability to measure pO_2 noninvasively, and repeatedly, with respect to acute or chronic interventions becomes increasingly important.

Early work examined cells in vitro, where ambient oxygen concentrations are readily controlled. In vivo, hypoxia may be achieved by clamping the blood supply to a tumor, but other levels of oxygenation reflect the interplay of supply and consumption. Robust fine needle polarographic electrodes opened the possibility of measuring pO_2 in tumors in situ in vivo to define local pO_2 under baseline conditions or with respect to interventions. In early work, Cater and Silver [Cater and Silver, 1960] showed the ability to monitor pO_2 at individual locations in patient's tumors with respect to breathing oxygen. Later, Gatenby et al. [1988] showed that pO_2 in a tumor was correlated with clinical outcome. Tumor oximetry received its greatest boost with the development of the Eppendorf Histograph polarographic needle electrode system. This computer-controlled device equipped with a stepper motor can reveal distributions of tumor oxygenation and has

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been applied extensively to clinical trials. Many reports have now shown that tumors are highly heterogeneous and have extensive hypoxia: strong correlations have been shown in cervix and head and neck tumors between median pO_2 or hypoxic fraction and survival or disease free survival [Brizel et al., 1996; Höckel et al., 1996; Nordsmark et al., 1996; Fyles et al., 1998]. Thus, tumor oxygenation is now recognized as a strong prognostic indicator and this device has laid a convincing foundation for the value of measuring pO_2 in patients. However, the Histograph is highly invasive and it is not possible to make repeated measurements at individual locations, precluding dynamic studies to assess the influence of interventions on tumor pO_2.

Thus, many other techniques have been developed to assess tumor oxygenation more or less directly (Table I) [Stone et al., 1993]. Magnetic resonance methods are attractive, since MR is inherently non-invasive. The pioneering work of Thomas [1988] showed that tissue pO_2 could be imaged in various organs based on the ^{19}F NMR spin-lattice relaxation rate (R1) of perfluorocarbon reporter molecules following i.v. infusion. Prompted by these studies, we surveyed a number of PFCs and identified that hexafluorobenzene (HFB, Fig. 1) has many virtues as a pO_2 reporter [Mason et al., 1996]. Symmetry provides a single narrow ^{19}F NMR signal and the spin lattice relaxation rate is highly sensitive to changes in pO_2, yet minimally responsive to temperature. From a

TABLE I. Comparison of Tumor Oximetry Techniques

Technique	Reporter	Parameter measured	Invasiveness	Spatial characteristic	Temporal resolution	References
Oximetry						
FREDOM	Hexafluorobenzene (perfluorocarbon)	R1	Minimally 32 G needle	Map multiple locations each 4 mm^3	6.5 min	[Hunjan et al., 2001; Zhao et al., 2001a, 2002a,b]
^{19}F MRS	Perfluorocarbon	R1	i.v.	Perfused regions	1 s to 6.5 min	[Mason et al., 1994]
ESR/EPR	Charcoal	Linewidth	23 G needle i.t.	Single location	Seconds	[Stone et al., 1993]
	Nitroxides	Linewidth	i.v.	Global or map	Seconds	
PEDRI	Free radical	OverHauser Enhancement	i.v.	Cubic millimeter maps	10 min	
Needle electrode (Histograph)	Oxygen	Current	26 G needle	Single location	~1 s	[Cater and Silver, 1960; Gatenby et al., 1988; Zhao et al., 2002b; Mason et al., 1999a]
	Oxygen	Current	26 G needle	Multiple tracks	1 s per location	[Brizel et al., 1996; Höckel et al., 1996; Fyles et al., 1998; Mason et al., 1999a]
Optical probe (OxyLite™, FOXY™)	$RhCl_2$	Fluorescent lifetime	26 G needle	Typically 2–4 locations	Real time	[Zhao et al., 2001b]
Phosphorescence	PD complex	Lifetime	i.v.	Maps	<1 min	
Mass spectrometry	Oxygen[a]	Atoms	Needle	Single location		
Vascular oxygenation						
NIR	Hemoglobin[a]	HbO_2/Hb	Non-invasive	Global	Real time	[Liu et al., 2000]
BOLD	Hemoglobin[a]	HbO_2/Hb	Non-invasive	Maps	1–5 s	[Howe et al., 1999]
Cryospectro photometry	Hemoglobin[a]	HbO_2/Hb	Biopsy	Microscopic	Once	[Stone et al., 1993]
Hypoxia						
Histology	Nitroimidazoles					
	Pimonidazole	%Tissue stained	i.v. + biopsy	Microscopic	Once	[Stone et al., 1993]
	EF5	%Tissue stained	i.v. + biopsy	Microscopic	Once	[Stone et al., 1993]
SPECT	IAZA	Photon counts	i.v.	Maps		[Ballinger, 2001]
PET	EF5	Positron decays	i.v.	Maps		[Ballinger, 2001]
	Cu-ATSM	Positron decays	i.v.	Maps		[Ballinger, 2001]
Indirect methods						
^{31}P NMR	PCr, ATP, Pi[a]	Phosphorylation potential	Non-invasive	2–10 cc	> 10 min	[Stone et al., 1993]
COMET	DNA strand breaks[a]	Electrophoretic migration	Biopsy	Cellular	Once	[Stone et al., 1993]

[a]Endogenous.

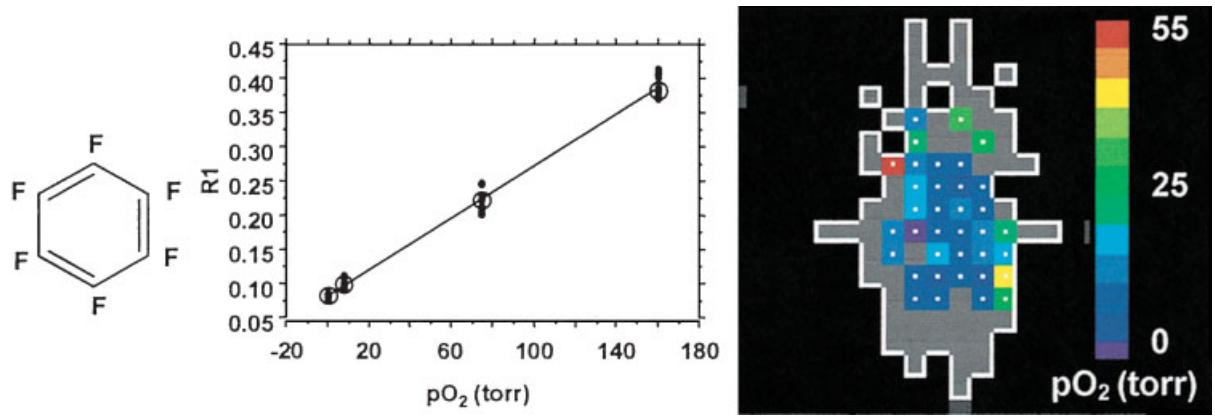

Fig. 1. Hexafluorobenzene, the linear oxygen dependence of the ^{19}F NMR spin-lattice relaxation rate (R1) and a typical pO_2 map from a Hodgkin's lymphoma xenograft in a SCID mouse.

practical perspective, HFB is readily available commercially in high purity, cheap and well characterized in terms of lack of toxicity [Zhao et al., 2001a]. Indeed, HFB exhibits no mutagenicity, no teratogenicity or fetotoxicity and the manufacturer's material data safety sheet indicates $LD_{50} > 25$ g/kg (oral-rat) and LC_{50} 95 g/m^3 per 2 h (inhalation-mouse).

Recognizing that tumors are heterogeneous and that pO_2 may fluctuate, we developed a procedure, which allows repeated quantitative maps of regional pO_2 to be achieved with multiple individual locations simultaneously in 6.5 min with a precision of 1–3 Torr when pO_2 is in the range 0–15 Torr [Hunjan et al., 2001]. We have applied *FREDOM* to diverse tumor types and interventions [Hunjan et al., 2001; Zhao et al., 2001a,b, 2002a,b; Song et al., 2002], as discussed in detail below, but here we describe the assessment of a human tumor xenograft with respect to respiratory challenge and administration of a vascular targeting agent.

METHODS

Human L540 Hodgkin's tumors were implanted subcutaneously in the flanks of male CB17 severe combined immunodeficient (SCID) mice and allowed to grow to ~1.5 cm diameter (3–6 mm thick). Mice were anesthetized using ketamine/xylazine. HFB (~40 μl, Lancaster) was injected directly in both central and peripheral regions of the disc-like tumors using a Hamilton syringe (Reno, NV) with a custom made fine sharp needle (32 G) using our standard procedure [Zhao et al., 2001b]. Each mouse was placed within a 2 cm single turn solenoid coil in an Omega CSI 4.7 T horizontal bore magnet system with actively shielded gradients. Proton and ^{19}F MR images were obtained to confirm the distribution of HFB. Tumor oxygenation was estimated on a regional basis using ^{19}F echo planar imaging (EPI) relaxometry of the HFB [Hunjan et al., 2001]. Briefly, following a pulse burst saturation recovery (PBSR) preparation sequence with a variable recovery time (τ), a single spin echo planar image was acquired. Applying the ARDVARC protocol (Alternated Relaxation Delays with Variable Acquisitions to Reduce Clearance effects) [Hunjan et al., 2001] enhanced the quality of the relaxation data, and typically a precision of 2–5 Torr at each of 20–50 voxels was achieved within a tumor in 6.5 min. The spin-lattice relaxation rate ($R1 = 1/T1$) was calculated for each voxel using a three-parameter fit of signal intensity $y_i = A(1 - (1 + W)\exp(-R1^*\tau_i))$ by the Levenberg–Marquardt least-squares algorithm. Selection criteria were applied to the data: data were accepted provided that the relative standard error of T1 ($\sigma T1/T1) < 25\%$ and $0 < \sigma T1 < 2.0$ s. Oxygen tension was estimated using the relationship pO_2 (Torr) $= 7.6 \times (R1 - 0.077 + 0.00009T)/(0.018 - 0.00017T)$, where T is rectal temperature in °C and R1 is the spin-lattice relaxation rate in s^{-1} [Le et al., 1997].

The inhaled gas could be manipulated via a nose cone from air to carbogen (95% O_2/5% CO2; 1 dm^3/min). In other cases, the air-breathing mice were injected i.v. with anti-VCAM1-tTF (truncated tissue factor) coaguligand (200 μl = 40 μg protein) to produce tumor-specific infarct [Huang et al., 1997]. The coaguligand was

constructed by conjugating the MK2.7 monoclonal antibody directed against mouse VCAM-1 to the extracellular domain of human tissue factor (TF), as described previously [Ran et al., 1998]. Thrombosis of tumor vessels was verified histologically in experiments parallel to NMR [Ran et al., 1998]. Briefly, mice were selected at specific time points before and after administration of coaguligand, anesthetized and perfused with heparinized saline. The tumors were removed, fixed in saline and paraffin embedded. Sections were cut, stained with hematoxylin-eosin and the number of thrombosed vessels counted.

Statistical significance of changes in oxygenation was assessed using analysis of variance (ANOVA) on the basis of Fisher PLSD and data are quoted as mean $\pm$ SE of the mean.

RESULTS

Typical baseline oxygenation ranged from hypoxia to 50 Torr with mean 9.7 ± 1.4 (SE) Torr and median 7.2 Torr (Fig. 2): no significant changes occurred during three repeat measurements while breathing air over a period of 24 min. Within 8 min of altering the inspired gas to carbogen, there was a change in oxygenation with elevation in mean ($P < 0.01$) and median pO_2, and decreased hypoxic fraction (Fig. 2). Oxygen tension continued to rise sometimes reaching a plateau after 16 min with mean pO_2 significantly above baseline ($P < 0.00001$). Upon returning the inhaled gas to air, tumor pO_2 gradually declined, but after 24 min was still significantly elevated compared with baseline ($P < 0.01$).

Administration of the anti-VCAM-1.TF coaguligand caused a rapid reduction in pO_2 in well-oxygenated tumor regions (initial $pO_2 > 10$ Torr) with mean declining from 15.5 ± 2.7 to 4.2 ± 2.4 Torr ($P < 0.01$) after 16 min and to -1.5 ± 2.4 Torr ($P < 0.001$) after 70 min. Little change was observed in regions, which were initially poorly oxygenated (initial $pO_2 < 10$ Torr), although some locations indicated increased oxygenation (Fig. 3). Histology showed open blood vessels in the untreated tumors, but substantial thromboses at 4 h.

DISCUSSION

In the brief experiments described here, we have demonstrated the ability to monitor both baseline pO_2 distributions in a tumor in vivo and dynamic changes with respect to interventions. This approach allows multiple specific locations to be interrogated simultaneously and sequentially. The data indicate the importance of temporal and spatial resolution in investigating tumor physiology. During the period of 6.5 min, considerable changes occurred in tumor oxygenation in response to respiratory challenge (Fig. 2). The mapping capability is critical, since it reveals distinct heterogeneity in tumor

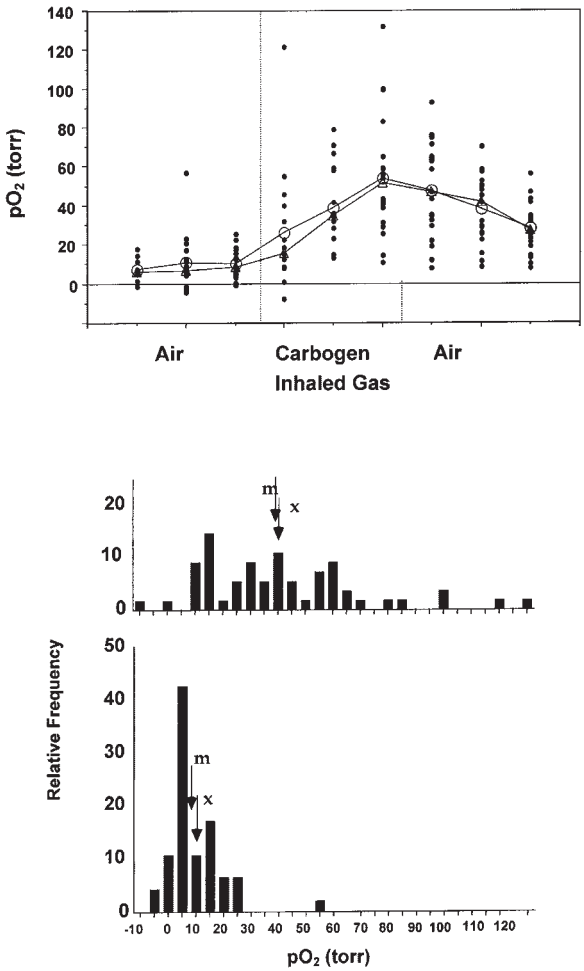

Fig. 2. Tumor oxygenation in response to respiratory challenge for human Hodgkin's tumors growing subcutaneously in mice: in each case data have been pooled from three repeat determinations. **Lower**: Histograms of baseline with mouse breathing air. Mean (x) $pO_2 = 9.7 \pm 1.4$ (SE) Torr, median pO_2 (m) = 7 Torr. **Center**: Mouse breathing carbogen (95% O_2/5% CO_2): mean $pO_2 = 41 \pm 3.9$ Torr, median 39 Torr. **Top**: Oxygenation of individual voxels from respective pO_2 maps. The general trend shows increased oxygenation with carbogen and a decline, albeit slower, upon return to breathing air. This emphasizes the need for rapid time resolution and danger of pooling data. Variation in mean (○) and median (Δ) pO_2 with respect to respiratory challenge is overlaid. Each measurement required 6.5 min.

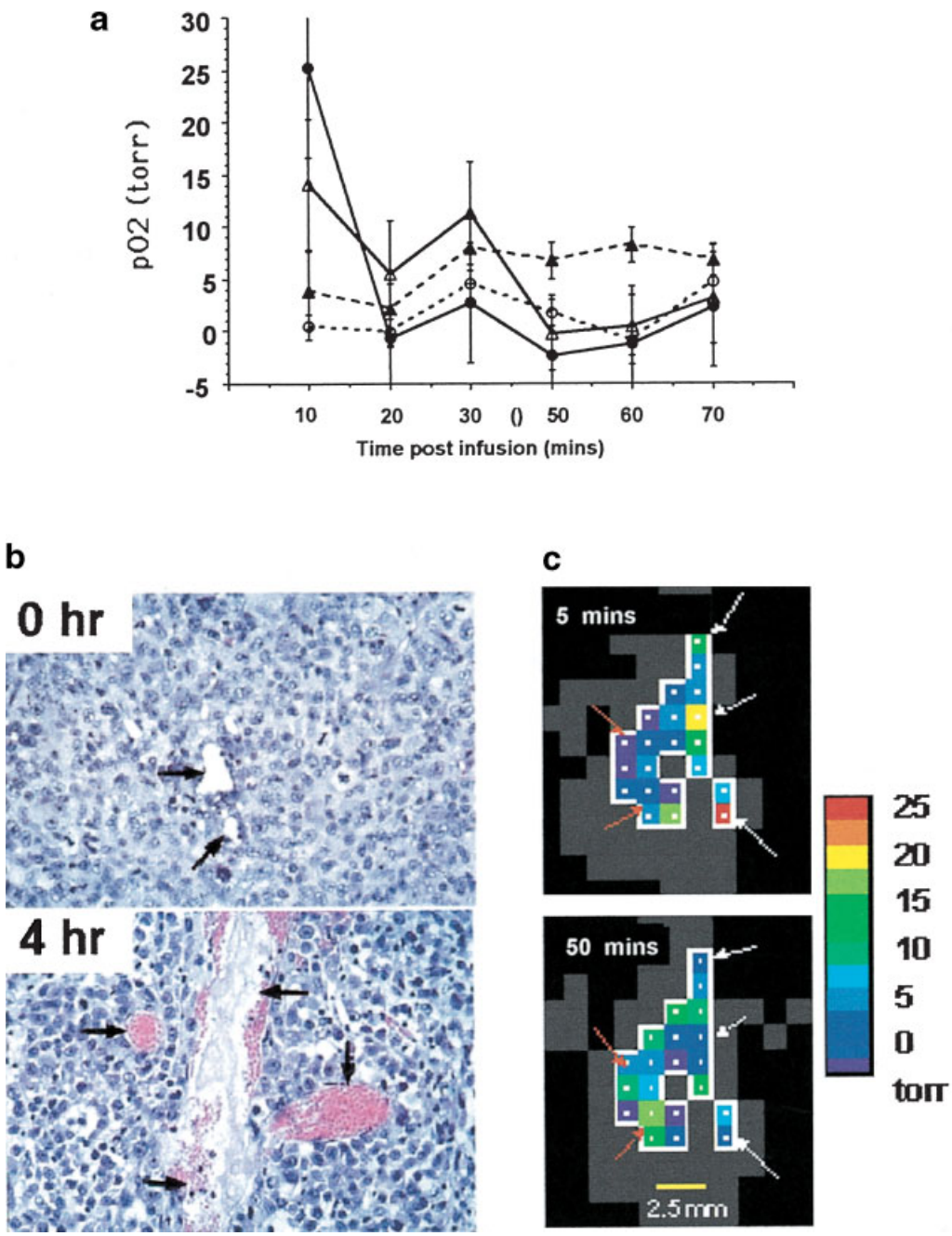

Fig. 3. **a**: Administration of the coaguligand caused a rapid reduction in pO_2 in well-oxygenated tumor regions (initial $pO_2 > 10$ Torr) with mean declining from 15.5 ± 2.7 to 4.2 ± 2.4 Torr ($P < 0.01$) after 16 min and to -1.5 ± 2.4 Torr ($P < 0.001$) after 70 min. Considering two representative individual tumor regions (voxels) ● and Δ with initially high pO_2, both showed a decline. By contrast, little change was observed in regions, which were initially poorly oxygenated (initial $pO_2 < 10$ Torr), for example, ○, although in some regions pO_2 was found to rise significantly ▲. **b**: H & E stained sections show open blood vessels in control tumor, but distinct thromboses 4 h following administration of coaguligand. **c**: Maps of pO_2 obtained using *FREDOM* 5 and 50 min after infusion of coaguligand. Arrows indicate regions showing particularly dramatic decrease or increase in pO_2.

oxygenation and differential response to intervention. In particular, the initially well-oxygenated regions of the tumor showed a significant decline in pO_2 (hypoxiation) following administration of the coaguligand, whereas the less well-oxygenated regions showed minimal response (Fig. 3). This could be attributed to differential vascular efficiency. One would expect

pO_2 to be highest in well-perfused regions and delivery of the coaguligand should also be most efficient there. Meanwhile, less well-oxygenated regions, and hence, by inference less well-perfused regions would tend to be less likely to be infarcted. Indeed, one region (Fig. 3) showed a significant increase ($P < 0.01$) in tumor oxygenation. This could imply diversion of blood to this region resulting from infarct elsewhere. In future, it will be important to correlate pO_2 measurements with blood flow as provided by such non-invasive techniques as ^{1}H Dynamic Contrast Enhanced (DCE) or Blood Oxygen Level Dependant (BOLD) contrast MRI. The ^{19}F NMR data are preliminary, but do coincide with histological investigations. Specifically, many, but not all, tumor capillaries were found to be occluded. The great potential advantage of FREDOM over traditional histology is that each tumor serves as its own control, and indeed, individual voxels reveal both baseline heterogeneity and differential response to interventions. Moreover, non-invasive procedures minimize the number of animals and quantity of agent required. Since antibodies may be costly, and difficult to create in large quantities, savings could greatly enhance the efficiency of agent development.

We have previously applied the *FREDOM* approach to various tumor types including syngeneic rat tumors [Mason et al., 1999b; Zhao et al., 2001a,b, 2002a,b]. In diverse sublines of the Dunning prostate R3327 tumor, we have shown distinctly different oxygenation patterns, which were related to tumor differentiation, vascularity, and growth rate [Zhao et al., 2000, 2002b]. Thus, the undifferentiated AT1 subline with volume doubling time of 5 days showed distinct heterogeneity with pO_2 values ranging from > 50 Torr to hypoxia when anesthetized rats breathed air. In response to breathing elevated oxygen (either oxygen or carbogen) those regions initially well oxygenated showed a rapid and significant response, while initially poorly oxygenated regions showed little, if any, response [Hunjan et al., 2001]. The fast growing MAT-Lu subline (VDT 2.7 days) had greater baseline hypoxia and similar response to breathing elevated oxygen [Zhao et al., 2001b, 2002b]. Meanwhile, the moderately well-differentiated HI subline (VDT 9 days) showed very different behavior. Baseline oxygenation was significantly higher, yet a substantial fraction of large tumors was hypoxic. Remarkably, all regions irrespective of initial pO_2 showed rapid and significant increase in pO_2 with respect to breathing elevated oxygen [Zhao et al., 2002b]. The highly differentiated H tumor (VDT 20 days) had significantly higher baseline oxygenation, but the response to increased oxygen breathing was sluggish compared to HI [Zhao et al., 2000]. Recently, we have also shown application of the technique to mammary 13762NF adenocarcinomas [Song et al., 2002]. Baseline pO_2 was similar to the Dunning prostate AT1, but all regions responded to oxygen intervention, though often slowly with continual increases over 45 min. Such data have suggested that a given tumor type has characteristic baseline pO_2 distributions and response to an intervention. However, baseline pO_2 alone has not been a good indicator of the potential response to an intervention between different tumor types. If such data are confirmed in the clinic, they indicate the potential importance of measuring pO_2 in the tumors of individual patients, so that therapy may be individualized and optimized with inclusion of appropriate adjuvant interventions.

Histograph data have shown value in evaluating baseline pO_2 measurements. In the future, patients may be stratified into well-oxygenated and poorly oxygenated groups for differential therapy. A next step should be the evaluation of the tumors, to see whether their pO_2 can be modulated by a relatively innocuous intervention, such as breathing oxygen. Elevation of tumor oxygenation might bring these hypoxic tumors into the well-oxygenated range and should show an improved response to radiation. Non-responders (persistently hypoxic tumors) could be considered for hypoxia selective cytotoxin therapy, such as tirapazamine [Brown, 1999] in an attempt to render them more sensitive to treatment.

Of greater relevance to the biochemical community is the ability to screen interventions in animals. With the increasing emphasis on speed of development for new therapeutic approaches, non-invasive techniques become increasingly important. As combinatorial approaches gain application, many more agents are available, but typically in small quantities. In the past, assessment of the efficacy of novel coaguligands has relied on histological endpoints (destructive) or gross anatomical evaluation (slow). The *FREDOM* procedure, described here, allows individual locations within a tumor to be followed

for a period of hours. These procedures can be used to rapidly screen new agents and suggest optimal time points for sacrifice and histological evaluation. This will both spare animals and, perhaps more significantly, reduce the amount of drug required. The non-destructive imaging technique will accelerate the discovery process for targets, efficacious conjugates, and successful development of drugs.

As shown in Table I, there are many alternative techniques available to investigate tumor oxygenation. Electrodes have been considered by some to be a "gold standard" and we have shown that pO_2 distributions assessed with the Eppendorf Histograph are commensurate with *FREDOM* [Mason et al., 1999a]. We have also shown similar dynamic response to interventions such as hyperoxic gas inhalation using fiber optic probes or electrodes or *FREDOM* [Zhao et al., 2001b, 2002b]. Electrodes, fiber optic probes, mass spectrometry probes, and many incarnations of ESR require insertion of a sizable needle into tumors and report limited regions raising issues of sampling. While our current implementation of *FREDOM* does require a needle for intratumoral (i.t.) injection, the mobile fluid (HFB) allows a very fine sharp needle (32 G). Moreover, we achieve maps of pO_2 at multiple locations simultaneously. Sampling remains an issue, since we typically interrogate 5–10% of a given tumor volume. However, the highly consistent intertumor behavior between multiple tumors of a given type (and size) suggests appropriate sampling. Moreover, since HFB is highly volatile and clears from tumors within 24 h, repeated measurements on subsequent days for chronic longitudinal investigations requires re-administration of the HFB: the highly consistent data achieved in tumors with such successive measurements indicates the effective representation of the true distribution of oxygen tensions within the tumors [Zhao et al., 2001b]. To avoid violating the tumor itself, reporter molecules can also be delivered i.v. (e.g., oxygen sensitive phosphorescent dyes, ESR sensitive agents, NMR sensitive fluorocarbon emulsions), but such an approach may bias measurements towards vascular oxygenation and particularly towards well-perfused regions of a tumor [Mason et al., 1994; McIntyre et al., 1999].

Specific classes of reporter molecule can reveal hypoxia as a surrogate for pO_2 (e.g., pimonidazole, EF5, CCI-103F) [Stone et al., 1993]. Following i.v. infusion these agents become reduced in tissues and are trapped. However, in the presence of oxygen, they are reoxidized and ultimately clear from the body. Histological assessment of the distribution of these agents provides microscopic indications of local hypoxia. Both EF5 and pimonidazole are currently being tested in clinical trials. In addition, various labeled derivatives have been developed which are NMR or PET active. Other nuclear imaging agents have been developed specifically to image hypoxia, for example, Cu-ATSM and the galactopyranoside IAZA [Ballinger, 2001].

While many successful reporter molecules have been developed to investigate tumor oxygenation, an alternative approach is to exploit endogenous indicators of oxygenation. Near infrared spectroscopy provides a direct measure of variation in tumor hematocrit (viz. blood volume) and relative hemoglobin oxygen saturation [Liu et al., 2000]. Cheap instrumentation can reveal rapid changes, but current technology provides very coarse spatial resolution, or indeed, global estimates. BOLD contrast MRI approaches provide high spatial and temporal resolution related to hemoglobin oxygen saturation, but signal also responds to variations in vascular volume, and flow [Howe et al., 1999]. Moreover, changes in vascular oxygenation may not coincide with pO_2, which is a balance between oxygen delivery and consumption and clearance. Ultimately, we must recognize that quantitative estimates of tumor tissue pO_2 have been shown to be directly related to clinical outcome in several tumor types, while other parameters such as vascular oxygenation require further validation in terms of their prognostic value.

Since many biochemical pathways are under oxygen regulation, they can provide an elegant window on hypoxia, for example, induction of Hif-1 and Glut-1 together with secondary responses such as increased production of VEGF, NIP3, and tumor associated macrophage activity [Knowles and Harris, 2001]. While such molecules could themselves indicate hypoxia, a more versatile approach is to adopt the hypoxic response elements as promoter sequences coupled to reporter genes such as GFP (green fluorescent protein) [Cao et al., 2001].

From a clinical perspective, ^{19}F MR remains somewhat esoteric, though the capability can be added to a clinical MR scanner for about $150k.

For preclinical biochemical studies, NMR systems have routine ^{19}F capabilities at 4.7, 7, or 9.4 T. Indeed, diverse reporter molecules have been created to access such diverse parameters as transmembrane pH, transmembrane chloride potential, [Ca^{2+}], temperature, and of course pharmacokinetics of drugs such as 5-FU [Mason, 1999]. ^{19}F NMR can also be applied to assess gene therapy, as demonstrated by others, for monitoring the conversions of 5-FC (fluorocytosine) to 5-FU (5-fluorouracil) and by us with the novel class of agent PFONPG as a substrate for β-galactosidase activity [Mason et al., 2002].

Ultimately, the value of a technique depends on its robustness, ease of use, and widespread implementation. To date, few labs had adopted the *FREDOM* approach because efficient investigation of HFB requires an unusual NMR pulse sequence. With the recent upgrade of our own instrumentation to the Varian Unity INOVA, the software is now available on this popular platform facilitating ready implementation elsewhere. While the *FREDOM* technique is currently limited to preclinical investigations, we are seeking Investigational New Drug (IND) approval for HFB to facilitate future clinical applications. As with electrode approaches, we foresee initial applications to readily accessible tumors, for example, head and neck, cervical, breast, and prostate. The ability to map pO_2 in < 8 min makes this technique a practical proposition for application to patients, and the value of monitoring dynamic changes in tumor oxygenation has the potential to influence treatment strategies.

In terms of research applications, it is known that tumor tissue pO_2 varies rapidly in response to many acute interventions ranging from irradiation to photodynamic therapy, various chemotherapies, and experimental new approaches such as vascular targeting agents shown here. We foresee *FREDOM* as a valuable tool for assessing the dynamic time course of such interventions to provide clear insight into the mode of action of therapeutic approaches and aid in the high throughput screening of new drugs such as vascular targeting and anti angiogenic agents.

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