Prognostic significance of tumor oxygenation in humans

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Abstract

Low tissue oxygen concentration has been shown to be important in the response of human tumors to radiation therapy, chemotherapy and other treatment modalities. Hypoxia is also known to be a prognostic indicator, as hypoxic human tumors are more biologically aggressive and are more likely to recur locally and metastasize. Herein, we discuss and summarize the various methods under investigation to directly or indirectly measure tissue oxygen in vivo. Secondly, we consider the advantages and disadvantages of each of these techniques. These considerations are made in light of our specific hypotheses that hypoxia should be measured as a continuum, not a binary measurement and that moderate, not severe hypoxia is of great biological consequence.
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1. Introduction

Hypoxia, i.e. low tissue oxygen concentration, has long been known to limit the response of tumor cells and animal tumors to radiation therapy (for review see [1,2]). Hypoxic cells are also resistant to chemotherapy both because of their relative isolation from the blood supply and that many of the drugs are only effective against dividing cells [3]. The importance of hypoxia as a prognostic and/or predictive factor for human tumors, however, was controversial for many years. This debate was fueled by the limited success of trials designed to sensitize hypoxic cells by hyperbaric oxygen [4,5] or nitroimidazole drugs [4–9]. The question was asked: if hypoxia is present and clinically relevant in human cancer, why have the agents and methods tested to target hypoxic tumor cells had so little success? Several possible answers to this question have surfaced over the intervening years. Examples include: (a) the administration of inadequate drug in order to limit patient toxicity [9]; (b) inability of the agent to access hypoxic tissues due to vascular insufficiency or acute changes in blood flow [10,11]; (c) drug hydrophilicity [12]; and/or (d) inability to compete against high tumor thiol levels [13–15]. Indeed, several of these factors may have confounded the results.

Two additional factors, the presence of intermediate hypoxia in tumors and the inclusion of patients in the trial who did not have hypoxic tumors are almost

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certainly important. 2-Nitroimidazoles are unable to sensitize cells that are only modestly hypoxic [16]. In Olive et al. [17], approximately 35% of the SiHa human cervical cancer xenografts had radiobiologic evidence of severe hypoxia (below 0.1% oxygen) and most of these tumors exhibited radioresistance consistent with a pO_2 of approximately 2% oxygen, e.g. modest hypoxia. Pimonidazole binding demonstrated that approximately 60% of the cells in these xenografts were intermediate in oxygenation or hypoxic, supporting the radiation response data. Using a modeling approach, Wouters and Brown have suggested that cells at intermediate oxygen levels could be more important than the 'hypoxic fraction', e.g. severely hypoxic cells, in determining tumor response to fractionated radiation therapy [18]. Employing quantitative fluorescence methods, we have been able to show the presence and importance of intermediately hypoxic cells in clinical and preclinical models [13,19–24]). We have defined physiologic oxygenation as $>10\%$ oxygen, modest hypoxia as approximately 2.5% oxygen, moderate hypoxia as approximately 0.5% and severe hypoxia as approximately 0.1% oxygen. Cells that are in the moderate–modest oxygen range would be considered intermediately hypoxic.

Clinical trials attempting to modify patient outcome after radiation therapy were planned on the assumption that all of the patients had radioresistant tumors due to poor tumor oxygenation. None of the small trials (median patient number was 97, range 17–620) showed a significant improvement in patient outcome. However, a meta-analysis performed by Overgaard et al. [7] on 7000 patients showed that drug modification of tumor resistance significantly improved the loco-regional tumor control after radiotherapy (odds ratio of 1.17) and the overall survival rate (odds ratio of 1.13). These observations suggest that identifying those patients with hypoxic tumors is critical for testing any anti-hypoxia therapy. The inclusion of patients without hypoxic tumors who would not be expected to benefit from improvement of tumor oxygenation would dilute the power of the clinical study and dramatically increase the number of experimental subjects required to identify a statistically significant therapeutic benefit [25,26]. Furthermore, the apparent benefit for any toxicity would be skewed [27].

2. Techniques to measure tumor oxygenation

In the 1980s, clinically relevant techniques were developed to assess the presence of hypoxia in individual human tumors. Recent data suggests that these methods can be used as prognostic markers to determine which patients could benefit from adjunctive anti-hypoxia therapy. Such hypoxia-specific therapies are available [22,28–30] and, in appropriately identified patients, these treatments can be effectively and safely tested. Clearly, the more sensitive and specific the association of a hypoxia measurement with outcome, the better this approach would be.

Several critical requirements to measure pO_2 in any (normal or abnormal) tissue have been proposed [24]:

(a) The measuring system should be *quantitative* both in terms of cellular pO_2 and tissue area involved.
(b) The dynamic range of the measurement should be large enough to include the entire pathological range of pO_2 values. Koch has previously summarized the pO_2 dependence of two clinically relevant hypoxia measuring techniques, nitroimidazole binding and needle electrodes [24,31]. 2-Nitroimidazole binding assays are most sensitive and accurate in the 0.02–2% oxygen range whereas needle electrodes are most sensitive at higher oxygen levels. Thus, these two methods may provide complementary information.
(c) If the administration of a drug is involved: its metabolism should exclude non-oxygen dependent binding and its pharmacokinetics and stability must be understood.
(d) In the presence of fluctuating tissue pO_2, the response of the sensing system must be characterized.

Techniques for measuring oxygen can be separated into those that are direct versus indirect. Direct oxygen measuring assays can be applied either in tissues (needle electrodes) or in blood (oxyhemoglobin saturation measurement [32], blood oxygen level diffusion imaging, BOLD [33]). Indirect measurements, where a reporter of oxygen level is the endpoint, are usually inverse, i.e. provide a positive

signal in the absence of oxygen. 2-Nitroimidazole binding, molecular markers such as hypoxia inducable factor (HIF), vascular endothelial growth factor (VEGF) and carbonic anhydrase 9 (CA9) [34–36], necrosis and lactate production [37–39] are all examples of indirect markers.

3. Invasive oxygen measurement techniques—needle electrodes

Polarographic needle electrodes provided the first evidence to conclusively identify the presence of hypoxia in human cancers. The early oxygen needle electrode studies by Kolstad, Wendling and Gatenby [40–42] demonstrated hypoxia in human rectal, cervix and head/neck tumors. However, these measurements were suspect due to the electrode's large diameter and its propensity to create tissue compression and bleeding. In the late 1980s, a smaller polarographic needle electrode made by the Eppendorf Company came into clinical use (KIMOC 6650, Sigma-pO$_2$-Histograph, Eppendorf, Hamburg, Germany). The electrode is mechanically moved progressively through tissue in a ratcheting motion. Measurements are made upon retraction of the tip in order to reduce artifacts caused by fluid tissue pressure and localized bleeding. The use of the electrode system is significantly limited by the difficulty of accessing tumors, cost, dependence on a technically-skilled user, inter-observer variability [43], failure to distinguish necrosis from hypoxia [44] and inability to provide information regarding patterns of hypoxia. Despite these limitations, its successful use in human tumors has resurrected research in the field of human tumor hypoxia.

Studies using the Eppendorf electrode illustrated the heterogeneous presence of hypoxia in uterine cervix, head/neck, sarcoma, brain, prostate, melanoma and pancreatic tumors [45–51]. Approximately 50% of uterine cervix cancers and nodal metastases from head and neck cancer contained regions of severe hypoxia (defined as less than 2.5 mm, 5 or 10 mmHg, depending on the report). The first data suggesting that hypoxia could be a predictive factor for patient outcome was published in 1993. Hockel et al. published an analysis of 31 cervix cancer patients treated with radiation, with or without chemotherapy [52]. After a median follow-up of 19 months (range 5–31 months), patients with hypoxic tumors (median pO$_2$ of $<$10 mmHg) had a significantly lower overall and recurrence-free survival. These observations were confirmed in a later study [53]. Tumor oxygenation was independent of pretreatment clinical tumor stage and size, histological type, or differentiation. Irrespective of whether surgery or radiation was employed as the primary treatment modality, patients with tumors having median pO$_2$ readings $<$10 mmHg were more likely to experience locoregional failures with or without distant metastases. Histopathological examination of the surgical specimens following radical tumor resection in 47 patients showed that low-pO$_2$ tumors exhibited more frequent (occult) parametrial spread and lymph-vascular space involvement, compared to well-oxygenated tumors of similar clinical stage and size. In 1996, Nordsmark reported the relationship between oxygenation and patient outcome in 35 patients with advanced head and neck cancer treated with 66–68 Gy external beam radiation therapy in 33–34 fractions [54]. Measurements were made in the nodal tissue of 34 patients and the primary tumor of one patient. The strongest independent variable in predicting radiation response was found to be the fraction of pO$_2$ values less than 2.5 mm Hg ($P = 0.018$). Brizel et al. reported the results on a group of 22 patients with non-metastatic, high-grade, soft tissue sarcomas undergoing preoperative irradiation and hyperthermia. The 18-month actuarial disease-free survival was 70% for patients with pO$_2$ values of $>$10 mmHg, but only 35% for those with median pO$_2$ values of $<$10 mm Hg ($P = 0.01$) [55]. There were eight treatment failures and lung was the first site of recurrence in all patients. The findings that patients with hypoxic cervix cancer treated with surgery alone were more likely to recur and that hypoxic sarcomas were more likely to metastasize were unexpected. Until these reports, the general consensus was that hypoxia was important in modulating radiation response, cell cycle regulated processes and bioreductively activated chemotherapy response. These new data supported the concept that hypoxic tumors were more biologically aggressive. Subsequent molecular investigations provided a rationale for such observations demonstrating that

hypoxia modulates cytokine regulation [34,56,57] and gene expression [58,59].

Since the initial studies documenting the presence and heterogeneity of hypoxia in human tumors, that hypoxia predicts disease-free or overall survival in patients with advanced cervix cancer and the site of hypoxia-dependent failure in these patients is distant metastasis (Table 1). Recent work from the Princess Margaret Hospital, Toronto, Canada suggests that hypoxia may only be an independent prognostic parameter in node negative patients. In the node-negative group, both tumor size ($P = 0.0012$; RR, 1.41) and percent of values less than 5 mmHg (HP5; $P = 0.007$; RR, 1.02) independently predicted outcome, whereas in the node-positive group ($n = 22$), neither tumor size ($P = 0.16$; RR, 1.18) nor HP5 ($P = 0.18$; RR, 0.99) were significant [60]. Hypoxic sarcomas also tend to metastasize, although the number of patients studied was small [55]. In head and neck cancer, hypoxia also predicts for outcome but the site of failure is local, suggesting hypoxia-based radiation resistance of the primary tumor (Table 1).

One of the unresolved issues is the determination of the appropriate endpoint for needle electrode studies. The above discussed studies variably chose 2.5, 5 or 10 mmHg, median pO_2 or hypoxic subvolume as the optimal endpoint. Since the endpoint chosen in each study was the median Eppendorf value of the tumors measured, this variability may reflect the heterogeneous biology between similar tumors at different institutions or variability of measurement techniques.

4. 'Inverse' hypoxia detection techniques—2-nitroimidazoles binding agents

The proposal to use 2-nitroimidazoles (originally developed as hypoxic-cell radiosensitizers) as hypoxia detection reagents was initially suggested in the late 1970s because these agents bind intracellularly in hypoxic cells [61,62]. Such compounds, e.g. misonidazole, form covalent bonds with intracellular macromolecules, identified primarily as protein thiols by Raleigh and Koch [63]. This binding of 2-nitroimidazoles is proportionately inhibited as a function of increasing oxygen concentration. The mechanism of this process involves cellular reductases which cause the formation of one-electron reduction products. The nitro-radical anions thus formed can be further reduced when oxygen is absent, and the higher reduction products (e.g. nitroso or hydroxylamine) become covalently bound to cellular macromolecules (adduct formation). However, in the presence of oxygen, the electron on the nitroimidazole radical is efficiently transferred to oxygen, resulting in the formation of the parent nitroheterocyclic and oxygen reduction products. These intermediates can efficiently be detoxified by superoxide dismutase and catalase [1,62,64,65]. The detection technique for bound adducts of 2-nitroimidazoles originally used liquid scintillation methods [66,67] and/or autoradiography [68,69]. More recently, antibody detection techniques have been developed. With such methods, detection of these bound adducts can provide information on the relative oxygenation of tissue at a cell-to-cell resolution [19,70].

At the current time, there are two 2-nitroimidazole agents being used in human clinical trials: pimonidazole (Ro 03-8799 (1-(2-nitro-1-imidazolyl)-3-*N*-piperidino-2-propanol)) [71,72] and EF5 (nitroimidazole [2-(2-nitro-1H-imidazol-1-yl)-*N*- (2,2,3,3,3-pentafluoropropyl) acetamide) [23,73–75]. These two agents are injected intravenously up to 48 h preceding surgical biopsy or excision and have the same mechanism of activation, but there are substantial differences in the chemical sidechain and therefore differences in their in vivo stability, pharmacokinetics, biochemistry and biodistribution.

Pimonidazole is a lipophilic 2-nitroimidazole with a basic side chain that, under physiological conditions exists as a racemic mixture of the *R*- and *S*-enantiomers. Pimonidazole is well distributed in the whole body, including the brain, and is excreted in part via the urinary tract (Table 2). The intracellular/extracellular concentration ratio and local concentration is pH dependent, leading to enhanced concentrations at acidic pH [76]. Pimonidazole is used in formalin fixed or frozen tissues with detection by either peroxidase [71] or fluorescence markers [72] conjugated to secondary monoclonal [77] or polyclonal antibodies [78]. Cells binding pimonidazole are interpreted as existing at a pO_2 of <10 mmHg (1% oxygen) based upon data showing that misonidazole binding in multicellular spheroids rises in a step-wise

Table 1
Significance of needle electrode-based pO_2 measurements for treatment outcome[a]

Author/ reference	pO_2 (mmHg)	Treatment	N	Univariate analysis			Multivariate analysis			
				DFS or OS	Distant spread	Loco-regional control	DFS or OS	Distant spread	Loco-regional control	Other significant parameters
Cervix cancer (measurement in primary)										
Hockel [52]	10	R, R + C	31	Y			Y			Figo stage
Hockel [53]	10	R or S	103	Y		N	Y			Figo stage (B)
Fyles [124]	5	R	74	Y						Size
Fyles [60]	5	R	106	Y	Y	N	N Y(NN)	Y	N	Tumor size, node status
Pitson [125]	5	R	128					Y (nodes)		Size
Sundfor [126]	5,10	NS	38		Y					
Sundfor [127]	5	R	40	Y		N	N		N	Hypoxic subvol.
Rofstad [128]	5	R/BR	32	N		N	N	Y	Y	
Knocke [129]	10	R	51	Y		Y				
Head and neck cancer (measurement in neck nodes and/or primary)										
Nordsmark [130]	2.5	R ± N	35	N		Y			Y	-
Brizel [131]	10	R ± S	28	Y		Y				
Brizel [132]	10	R, RC ± S	63	Y		Y	Y		Y	Radiation dose
Stadler [133]	2.5, 5	R,RC	59	Y			Y			Hypoxic subvol, Hb
Rudat [134]	2.5	R/A,H, RC	41	Y			Y			
Rudat [135]	2.5	R, RC	194				Y			Age, therapy
Terris [136]	Mean	R,RC	63	N		N				
Soft tissue sarcoma (measurement in primary)										
Brizel [55]	10	R,H,S	22	Y	Y					
Nordsmark [137]	Median	R,S	28	Y	Y					

[a] There may be some overlap in patients reported from the same institution.

DFS, disease free survival; OS, overall survival; R, radiation; S, surgery; RC, radiochemotherapy; H, hyperthermia; A,H, accelerated, hyperfractionated; BR, brachytherapy; N, nimorazole; NN, node negative patients; NS, not stated.

Table 2
Comparison of EF5 and pimonidazole

Parameter	Pimonidazole	EF5
Plasma half life (T 1/2)	5.1 h [138]	11.5 h [73]
Octanol/water coefficient	8.5 (Neutral)[a]	5.7
Renal excretion	≤31% at 24 h [139]	≤70% [73]
Known metabolites	N-oxide, O-conjugate	None
Biodistribution	Crosses BBB, pH dependent [140]	Crosses BBB, uniform [20]
Toxicity	None; 0.5 g/patient	None; 21 mg/kg
+ve associations	Comet [141]	Apoptosis (UD)
	Involucrun [71]	VEGF RNA [35]
	CA9 [34];	Electrode (Sarcoma) [75]
Inverse localization	Vessels [142]	Vessels [23]
	PCNA [143]	Ki67 [23]
No correlation	Electrode [141]	VEGF protein (UD)
	VEGF protein [101]	Electrode (brain) (UD)
	Metallothionine [71]	
Individual tumor response (rodent studies)		[21,22,144,145]
Association with patient outcome (treatment failure)	+(Head/neck) [81]	+(Brain) (Evans, 2002 NCI/EORTC meeting)

[a] Neutral pimonidazole is lipophilic but is in equilibrium with its charged basic form (very hydrophilic). This enhances renal excretion and shortens the plasma half life while promoting accumulation in acidic tissue. UD = unpublished data.

fashion at this pO_2 [79]. The area or number of pimonidazole-binding cells has been used as a semiquantitative marker for hypoxia.

EF5 is lipophilic and neutral, allowing even biodistribution to all organs, including the brain [20]. Its binding is unaffected by factors other than oxygen (pH, glucose, thiols; C. Koch, unpublished observations). Analysis of EF5 binding has been developed for both flow cytometric and immunohistochemical analysis, using a highly specific monoclonal antibody [31] for quantitative analysis of binding [23,74,75]. The analysis for each tissue section has been extended to include quantitation of the percentage of viable cells at each pO_2, reported as a cumulative frequency (Fig. 1). This analysis is possible because the monoclonal antibodies are directly conjugated to the fluorescent agents, for a one-step detection system. The resulting binding levels are calibrated to an absolute fluorescence standard and are corrected for the patient drug exposure [73] and the maximal tissue binding [23, 74,75]. Similar analyses can be performed using flow cytometry. Based on flow cytometric studies of human and rodent cells incubated with EF5 under various oxygen conditions, we have estimated that physiologic hypoxia (approx. 10% oxygen), results in approximately 1% of maximum EF5 binding, modest

hypoxia (approx. 2.5% oxygen) results in approximately 3% of maximum EF5 binding; moderate hypoxia (approx. 0.5% oxygen) results in approximately 10% of maximum EF5 binding and severe hypoxia (approx. 0.1% oxygen) results in approximately 30% of maximum EF5 binding (Koch and Evans, 2002 unpublished data).

Use of immunohistochemical markers for hypoxia detection has allowed the analyses of the relationships between, for example, hypoxia and other biological endpoints such as vessels (for other examples see Table 2). Such analyses are important for many reasons, including the future selection of hypoxia-directed therapies. The three-dimensional relationship between vessels and hypoxic regions should provide information regarding the type of hypoxia present. This is important because treatments directed at diffusion-limited hypoxia ('chronic') might not be successful in tumors that are hypoxic due to vascular changes ('acute' or perfusion-limited hypoxia). For example, the use of a therapy such as meta-iodobenzylguanidine (MIBG) [80] to decrease cellular oxygen utilization would only be effective in tumors with diffusion-limited hypoxia.

At this time, there are only minimal data regarding these agents as prognostic biomarkers. Small studies suggest that locoregional recurrence
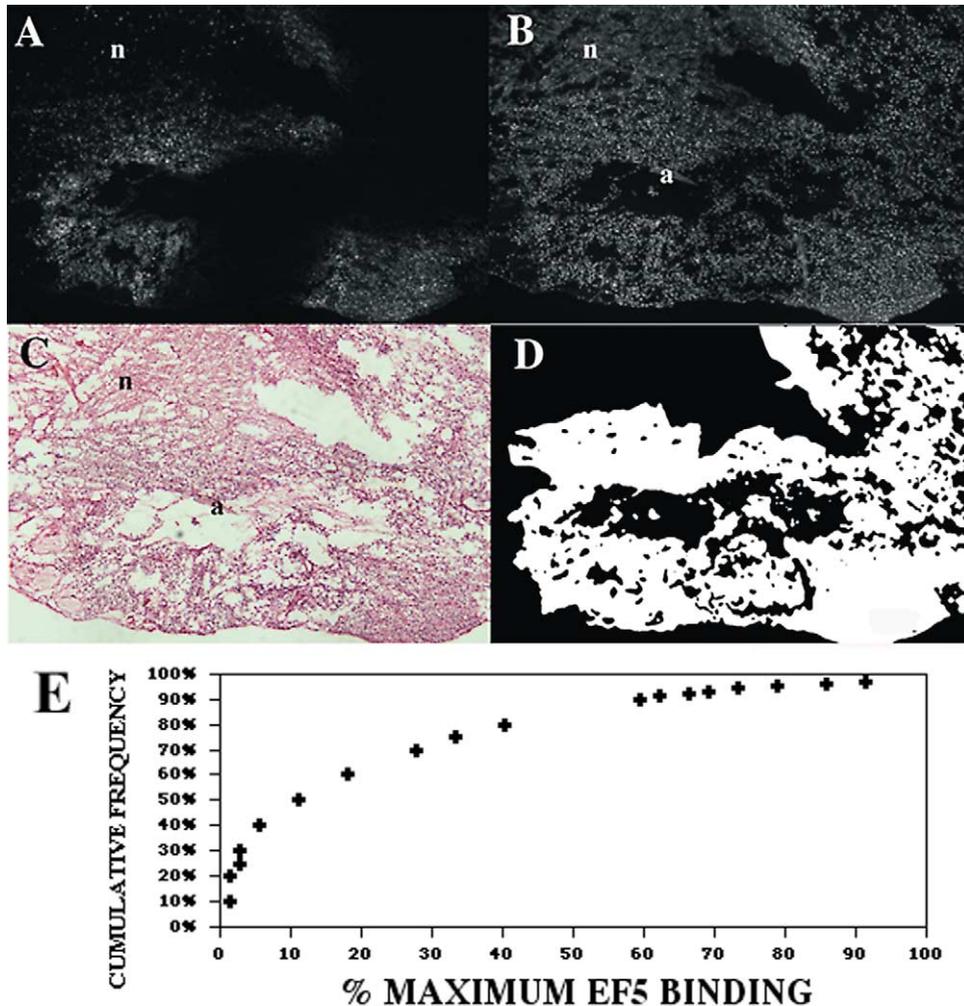
Fig. 1. Images of immunohistochemical sections and masks resulting from analysis of tissues from a patient with a WHO grade 4/4 glioma, glioblastoma multiforme. (A) EF5 binding is demonstrated in this image. Some regions of binding are adjacent to regions of necrosis (n). (B) represents the same tissue area as (A), counterstained with Hoescht 33342 to identify individual cell nuclei. This same slide is then counterstained with hematoxylin and eosin and this image is shown in (C). The Hoescht 33342 image is used to generate (in Photoshop 4.0®) the tissue mask shown in (D). After the mask is generated, regions of cellular necrosis, as identified by a neuropathologist are removed from further analysis (shown as black regions), as these cells are not viable and therefore not likely to determine treatment response. n = necrosis, a = artifact. Each image is 2.0 × 1.4 mm. (E) Cumulative frequency analyses based on the mask, (D) and the EF5 image, (A) is presented. Using Matlab®, a cumulative frequency histogram of the binding level for each relevant pixel was generated. Thus, 95% of the pixels in the image were at an EF5 binding of 79.8% of maximum EF5 binding.

was lower in head and neck patients with high pimonidazole binding [81] and that hypoxic brain tumors have a higher recursive partitioning classification [82] and more rapid tumor recurrence (S.M. Evans, 2002 Society of Neuro-oncology meeting, San Diego, CA).

5. 'Inverse' hypoxia detection techniques— endogenous markers

With the current interest in molecular biology and cellular signaling, there has been a quest to identify an intrinsic molecule whose presence and/or level

reflects tissue oxygenation. Oxygen levels have long been known to modulate the release of chemical messengers in the body; the classical example is the release of erythropoietin by the kidney under conditions of physiological or pathological hypoxia [83]. HIF is a key regulator maintaining oxygen homeostasis (for review see [57]). Many genes that are known to be induced by HIF activation are also expressed at higher levels in human cancers compared to normal tissue counterparts. Examples include VEGF [56,84–86], nitric oxide synthase-2 [87,88], insulin-like growth factor [89], and transforming growth factor [90]. Other factors that have been associated with hypoxia, although not necessarily via HIF regulation include carbonic anhydrase 9 (CA9 or CA IX) [91–93], thrombospondin [94,95] and osteopontin [96]. Several of these molecules, as well as HIF itself, have been evaluated as intrinsic markers of hypoxia both in serum and tissue samples.

The difficulty in using a measurement of tumor HIF as an intrinsic marker of hypoxia is that its activation can occur in the presence of oxygen. For example, immunohistochemical HIF staining has been demonstrated in renal cell tumors with extensive vascularization due to loss of pVHL [97]. In fact, the upregulation of HIF may be a general indicator of poor outcome, independent of hypoxia [98]. Since other cytokines such as VEGF and CA9 (see below) also have non-oxygen based regulation, it is critical that comparative studies with specific hypoxia markers be carried out.

The relationship between hypoxia, as measured by 2-nitroimidazole binding and other biologic endpoints has been studied (Table 2). In studies using hypoxia and EF5 in tumor cell spheroids [99] and pimonidazole in rat livers [100], VEGF is upregulated, consistent with previously observed predictions in vitro. However, studies in human squamous cell carcinomas [101] and glioma xenografts [102] suggested that VEGF *protein* was not regulated in concert with hypoxia, an unexpected conclusion. Subsequent studies using EF5 immunohistochemistry and VEGF mRNA in situ hybridization in several human tumor types detected VEGF mRNA co-localized with regional maxima of EF5 binding, which were often adjacent to regions of tissue necrosis. High EF5 binding occurred in tumor tissues corresponding to regions roughly less than 0.3%

oxygen [35]. Two explanations for the different findings in the human squamous cell and the glioma xenograft studies versus this latter report are (1) VEGF protein is secreted and may not stay 'localized' to hypoxic regions; and (2) the use of EF5 versus pimonidazole. The precise relationship between radiation response, pO_2, and VEGF regulation remains unclear. In vitro suspension culture studies of three cervix cancer cell lines suggest that the K_m of VEGF upregulation was between 1 and 3% oxygen, which is substantially more oxic than the K_m for radiation resistance and the above described regions of EF5 binding [35,103].

CA9 is a zinc metalloenzyme responsible for the reversible conversion of carbon dioxide to carbonic acid and water. The membrane-linked isoforms CA9 and CA12 were identified as genes that were down regulated by the von Hippel–Lindau protein (pVHL) [104]. As pVHL is a critical element in the regulation of HIF complex, it has been shown that the CA9 gene was hypoxia-inducible and dependent on HIF [34]. CA9 has been found to be a tumor marker in ovarian, endometrial and cervical cancer [105], an independent predictive factor for overall survival in invasive breast cancer [93] and an independent prognostic indicator of overall survival and metastasis-free survival for patients treated with radiation therapy for squamous cell cancer of the cervix [106]. CA9 staining is preferentially located in perinecrotic regions [91]. A significant positive correlation between tumor hypoxia (% of values less than 5 mmHg based on Eppendorf electrode studies) and the extent of CA9 expression has been shown in cervix cancers [106]. In a separate study, a substantial, although incomplete overlap with pimonidazole staining was shown [34]. Similarly, Olive et al. [107] demonstrated co-localization of CA9 and pimonidazole in cervix cancer, although the area of the tumor section that bound anti-CA9 antibodies represented double the number of cells that bound anti-pimonidazole antibodies. In studies of bladder and skin cancer, five of the 20 patients studied had more CA9 staining than pimonidazole staining [34]. Possible explanations for these findings include transient (acute) hypoxia or that pimonidazole only labels cells at less than 1.3% oxygen, whereas upregulation of cytokines and molecular markers occur at pO_2 values over a larger range, 0.2–2% oxygen [92].

6. Non-invasive hypoxia imaging

Non-invasive hypoxia imaging could be used as prognostic and predictive assays especially in patients where surgery is not clinically indicated. Several factors are important in determining the success or failure of a non-invasive imaging approach. Perhaps the most fundamental is the isotope half-life that should be tailored to the properties of the drug (Table 3). Isotopes with short half-lives have the advantage of reducing patient dose, but the disadvantage of drug clearance times which are longer than isotope decay times resulting in the presence of a substantial background of non-metabolized drug. Other factors being equal, positron emission tomography (PET) imaging is more accurate than single photon emission computed tomography (SPECT) imaging, but may require more radioactivity to capitalize on this advantage. The accuracy of PET imaging is inversely limited by the energy of the emitted positron, with fluorine and copper having relatively low energy positrons and hence higher resolution. The type of atom (metal vs. halogen) determines the type of possible ligand (chelate vs. covalent bond, respectively).

The various clinically available PET agents have been succinctly reviewed by Ballinger [108]. Most radiopharmaceuticals under development for hypoxia detection use 2-nitroimidazole as the targeting moiety and a nuclear medicine-imagable radioactive element, such as ^{18}F, ^{67}Cu, ^{64}Cu, (PET) or ^{123}I, ^{99m}Tc (SPECT) as the detection moiety (Table 1). Non-nitro-containing bioreductive complexes such as the ^{60}Cu-ATSM and ^{99m}Tc butylene aminooxime (BnAO or HL91) have also been evaluated.

The most widely studied imaging agent for hypoxia is fluoromisonidazole (^{18}F-miso). Data for ^{18}F-miso imaging in advanced head and neck cancer [109,110], lung cancer ([110], prostate cancer [110], nasopharyngeal carcinoma [111] and gliomas [112] have been published, demonstrating the feasibility of this technique. Virtually all of the patients in two studies [109,110] had hypoxic tumors based on ^{18}F-miso uptake. This was unexpected considering the results of compared to previous reports using the electrode technique [45–51,113,114]. Outcome analysis should be available on a much larger number of patients imaged by ^{18}F-miso [108].

^{123}I-labeled iodoazomycin arabinoside (^{123}I-IAZA; 1-(5-iodo-5-deoxy-beta-D-arabinofuranosyl) is an experimental radiopharmaceutical shown to have clinical utility for imaging regional tissue hypoxia. In 22 patients, a significant inverse correlation with the perfusion marker ^{99m}Tc-HMPAO was seen [115]. A study of ^{123}I-IAZA in 51 human patients with newly diagnosed malignancies demonstrated hypoxia in non-small cell lung cancer but not in malignant gliomas [116]. Since hypoxia has been demonstrated in human gliomas using the needle electrode technique [49,117,118] and EF5 binding

Table 3
Properties of representative imaging isotopes

| | PET | | | SPECT | |
	^{18}F	^{60}Cu	^{124}I	^{123}I	^{99m}Tc
Half-life	110 min	23 min	6006 min	786 min	360 min
Ligand stability	High	Chelate	Low	Low	Chelate
Isotope target	Bone	Various	Thyroid	Thyroid	Various
Linear resolution	2.0 mm	~2.5 mm	4.0 mm[a]	12–15 mm	8–12 mm
Voxel	8 mg	12 mg	>64 mg	2500 mg	1000 mg
Attenuation	Yes	Yes	Yes	Possible	Possible
Absolute DPM	Yes	Yes	Yes	No	No
Patient dose	Low	Low	High[a]	Medium	Low
Clearance	Renal possible	Variable	Gut	Gut	Gut
Labeling ease	Often difficult	Simple	Variable	Variable	Simple
Availability	Fair	Poor	Poor	Good	Excellent
Example	F-Miso, EF5	Cu-ATSM	IAZGP	IAZA	HL91

[a] Only 25% of the decays of ^{124}I result in positrons. The extra gamma emissions and high positron energy lower the possible image resolution and substantially increase the patient radiation dose.

[23], this finding suggests that the IAZA molecule may not access the brain even in the face of a tumor-induced break in the blood brain barrier. Preliminary observations suggested that patients with ^{123}I-IAZA-avid neck metastases from head/neck cancer had a decreased local control at 3 months, but follow-up data have not been published. Newer agents based on the azomycin-nucleoside structure (such as beta-D-iodinated azomycin galactopyranoside, IAZGP) have been developed [119], but have not yet been studied in humans.

[99m]Technecium (^{99m}Tc) is commonly used in nuclear medicine imaging because it is inexpensive, easy to chelate to various ligands and has a near-optimal half life (Table 3). In the 1990s, BMS181321 (Oxo [[3,3,9,9-tetramethyl1-1-(2-nitro-1H-imidazole-1-yl)-4,8-diaza) and butylene amine oxime (oxime (2,2′-[1,4-diaminobutane) bis [2-methyl-3-butanone] dioxime; Prognox, HL91) were developed as ^{99m}Tc-based hypoxia markers. Initial animal studies with HL91 as a hypoxia detection agent showed promise despite the absence of a rationally developed hypoxia-binding moiety. Although a number of Phase II studies have been performed with HL91, it is no longer in commercial development.

Fujibayashi and colleagues have developed a [62]Cu labeled diacetyl-bis (N^4-methylthiosemicarbazone) (Cu ATSM) for imaging hypoxic tissues [120]. Studies performed in the 9L gliosarcoma rat model compared the needle oxygen electrode and ^{64}Cu ATSM PET imaging before and after the tissue oxygen concentration was modulated by hydralazine or oxygen breathing. A correlation between low pO_2 and high ^{64}Cu-ATSM accumulation was observed [121]. Studies of [60]Cu ATSM have been performed in patients with lung cancer [122] and cervix cancer (Perry Grigsby, personal communication, 2002) with promising results.

^{18}F-EF5 is unique in that it is the only hypoxia-detection agent where the same molecule can be measured both invasively (flow cytometry and/or immunohistochemistry) and non-invasively (PET) in the same tumor by administering cold drug as a carrier for the radioactive agent. Studies in tumor bearing rats [123] and dogs (B. Kaser-Hotz, personal communication, 2002) support the concept that hypoxic regions can be identified with PET studies and confirmed with immunohistochemistry studies.

7. Conclusions

It is clear from published studies using each of the measuring techniques discussed that hypoxia is present in a subset of human tumors. However, the percentage of, for example, cervix cancer patients, having a 'hypoxic' tumor seems to be dependent on the technique used and how hypoxia is defined. All of these studies agree that there is substantial inter- and intra-tumoral heterogeneity within tumors of similar histology and site, emphasizing the importance of measuring hypoxia in individual patients. Although the published (Eppendorf needle electrode) results are statistically highly significant for hypoxia as a prognostic marker, the sensitivity and specificity of these assays are not optimal, e.g. there are substantial patients with 'hypoxic' tumors who do well and patients with 'oxic' tumors who do poorly. There are at least two approaches to improving these results, and both of these should be pursued.

Measurements and analysis of existing techniques need to be refined to accommodate the observation that hypoxia is continuous, not a binary process. The simple division of patients above and below a median value is likely to be artificial. As we are beginning to understand the actual pO_2 s at which pathophysiological processes occur, correlation of hypoxia measurements, biological processes and patient outcome should be possible. Measurement of the exact pO_2 values in tissues would allow a more reasoned classification of patients based on knowledge of the pO_2 where specific biological processes occur (such as radiation resistance, upregulation of cytokines). Studies discussed earlier in this review [17,18,20,23, 74,75] all support the idea that *moderately, not severely hypoxic* cells may be most important for determining biological resistance, perhaps because severely hypoxic cells are destined to die. Such analyses require that the hypoxia detection methods be calibrated to a known standard. This process is complex and tedious but may be initially necessary in order to correctly interpret data.

It is highly unlikely that only one factor is pivotal in determining therapy response. Multiple clinical and biological factors are apt to play important roles in patients' outcome. Thus, consideration of other factors, which, along with oxygen will be clinically important, is appropriate. One example of a critical

biological factor that must be considered along with oxygen content for radiation response is non-protein thiol concentration since tumor cells with moderate hypoxia but high non-protein thiols are as radiation resistant as cells with severe hypoxia [13].

8. Summary

A clinically relevant method for evaluating the presence and pattern of hypoxia in human tumors can improve patient prognosis and treatment planning. Intrinsic markers, such as VEGF, CA9 or HIF-1alpha have the potential advantage that patients would only require a biopsy. It is also possible that secreted markers of hypoxia could be monitored by a blood sample. In order for these markers to be optimized, a better understanding of the microenvironmental conditions modifying their regulation is required. At present, it appears that the endogenous markers are not solely regulated by oxygen (e.g. HIF-1alpha upregulation in patients with VHL syndrome). A more quantitative approach may be possible using hypoxia-detecting 2-nitroimidazoles and/or electrodes. These methods allow an understanding of the presence, level, and patterns of hypoxia as well as defining the biology of different disease sites. Labeled 2-nitroimidazoles and other redox sensitive compounds can monitor tissue hypoxia non-invasively. Such methods do not have the same resolution as the invasive approaches but have obvious advantages, particularly in their ability to monitor the whole tumor and its local or distant spread, should these regions also be hypoxic. Clearly, each technique has its advantages and disadvantages and we need to recognize and understand both. It may be possible that specific tumor types will be best monitored by a particular method and this may vary among different tumor types, grades or stages.

Acknowledgements

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