Tumor Hypoxia: Definitions and Current Clinical, Biologic, and Molecular Aspects

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Tissue hypoxia results from an inadequate supply of oxygen (O_2) that compromises biologic functions. Evidence from experimental and clinical studies increasingly points to a fundamental role for hypoxia in solid tumors. Hypoxia in tumors is primarily a pathophysiologic consequence of structurally and functionally disturbed microcirculation and the deterioration of diffusion conditions. Tumor hypoxia appears to be strongly associated with tumor propagation, malignant progression, and resistance to therapy, and it has thus become a central issue in tumor physiology and cancer treatment. Biochemists and clinicians (as well as physiologists) define hypoxia differently; biochemists define it as O_2-limited electron transport, and physiologists and clinicians define it as a state of reduced O_2 availability or decreased O_2 partial pressure that restricts or even abolishes functions of organs, tissues, or cells. Because malignant tumors no longer execute functions necessary for homeostasis (such as the production of adequate amounts of adenosine triphosphate), the physiology-based definitions of the term "hypoxia" are not necessarily valid for malignant tumors. Instead, alternative definitions based on clinical, biologic, and molecular effects that are observed at O_2 partial pressures below a critical level have to be applied. [J Natl Cancer Inst 2001;93:266–76]

Traditionally, tumor hypoxia has been considered a potential therapeutic problem because it renders solid tumors more resistant to sparsely ionizing radiation *(1–3).* More recent experimental and clinical studies [reviewed in *(4–10)*] suggest that intratumoral oxygen levels may influence a series of biologic parameters that also affect the malignant potential of a neoplasm.

Sustained hypoxia in a growing tumor may cause cellular changes that can result in a more clinically aggressive phenotype *(11–15).* During the process of hypoxia-driven malignant progression, tumors may develop an increased potential for local invasive growth *(16,17),* perifocal tumor cell spreading *(11,18),* and regional and distant tumor cell spreading *(12,13,19–21).* Likewise, intrinsic resistance to radiation and other cancer treatments may be enhanced *(18,22–29).*

Hypoxia-induced or hypoxia-mediated changes of 1) the proteome (i.e., the complete set of proteins within a cell at a given time) of the neoplastic and stroma cells and 2) the genome of the genetically unstable neoplastic cells may explain the fact that tumor oxygenation is associated with disease progression, a link that has been demonstrated for a variety of human malignant tumor types *(11–15).* The first goal of this review is to compile current results from experimental and clinical studies, illustrating the interaction between hypoxia and the phenomena of malignant progression and resistance toward oncologic treatment.

In an increasing number of reports on tumor oxygenation, the term "hypoxia" has been used in a somewhat careless manner without due consideration of the clear definitions for certain experimental conditions and scientific questions. Different researchers discussing the problem of tumor hypoxia may use the term "hypoxia" in different ways, thus leading to a "Babylonian confusion." The second goal of this review is, therefore, to shed some light on the pitfalls of the casual use of the term "tumor hypoxia." Because evidence of the fundamental biologic and clinical importance of tumor hypoxia is increasing, molecular biologists, physiologists, and clinicians should take care to communicate on the same "wavelength" and clearly define what they mean when they use the term "tumor hypoxia."

Definition and Causative Mechanisms

Tissue hypoxia results from the inadequate supply of oxygen (O_2) that compromises biologic functions *(30).* Hypoxia can be caused by a number of factors, such as 1) low O_2 partial pressure (O_2 tension) in arterial blood due to, e.g., pulmonary diseases or high altitude (hypoxemic hypoxia); 2) reduced ability of blood to carry O_2 as a result of anemia, methemoglobin formation, or carbon monoxide poisoning (anemic hypoxia); 3) reduced tissue perfusion, generalized or local (circulatory or ischemic hypoxia); 4) deterioration of the diffusion geometry, e.g., increased diffusion distances, concurrent versus countercurrent blood flow within microvessels (diffusional hypoxia); or 5) inability of cells to use O_2 because of intoxication, as in cyanide poisoning (histotoxic or cytotoxic hypoxia). Because of finely tuned regulatory processes, increases in tissue O_2 consumption are generally matched by an increase in blood flow and, therefore, do not usually lead to hypoxia unless the system regulating blood flow fails to meet the increased O_2 demand of the tissue in question.

Biochemists usually define hypoxia as O_2-limited electron transport *(31).* Physiologists and clinicians define hypoxia as a state of reduced O_2 availability or decreased O_2 partial pressures below critical thresholds, thus restricting or even abolishing the function of organs, tissues, or cells *(32–34).* Anoxia describes the state where no O_2 is detected in the tissue (O_2 partial pressure = 0 mm of mercury [mmHg]).

In solid tumors, oxygen delivery to the respiring neoplastic and stromal cells is frequently reduced or even abolished by a deteriorating diffusion geometry, severe structural abnormalities of tumor microvessels, and disturbed microcirculation *(35).* In

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addition, anemia and the formation of methemoglobin or carboxyhemoglobin reduce the blood's capacity to transport O_2. As a result, areas with very low (down to zero) oxygen partial pressures exist in solid tumors, occurring either acutely or chronically. These microregions of very low or zero O_2 partial pressures are heterogeneously distributed within the tumor mass and may be located adjacent to regions with normal O_2 partial pressures. In contrast to normal tissue, neoplastic tissue can no longer fulfill physiologic functions. Thus, tumor hypoxia cannot be defined by functional deficits, although areas of necrosis, which are often found in tumor tissue on microscopic examination, indicate the loss of vital cellular functions.

Metabolic Hypoxia in Solid Tumors

When an unrestricted supply of oxygen is available, for most tumors, the rate of O_2 consumption (respiration rate) and adenosine triphosphate (ATP) production is comparable to that found in the corresponding normal tissue, despite the deregulated organization of cells in malignant tumors. To maintain a sufficient energy supply for membrane transport systems and synthesis of chemical compounds, an adequate supply of O_2 is required.

In hypoxia, the mitochondrial O_2 consumption rate and ATP production are reduced, which hinders *inter alia* active transport in tumor cells. Specifically, major effects of the reduced production of ATP are 1) collapse of Na^+ and K^+ gradients, 2) depolarization of membranes, 3) cellular uptake of Cl^-, 4) cell swelling, 5) increased cytosolic Ca^{2+} concentration, and finally, 6) decreased cytosolic pH, resulting in intracellular acidosis in tumor cells.

According to the definition given above, hypoxia is present in tumors when the O_2 partial pressure falls below a critical value causing the O_2 consumption rate or ATP production rate of a cell or a tissue to decrease progressively. On the basis of experimental results from isolated xenografted human breast cancer tissue *(36,37),* tumor tissue hypoxia with reduced O_2 consumption rates is expected when the O_2 partial pressure in the blood at the venous end of the capillaries (end-capillary blood) falls below 45–50 mmHg (Table 1). This critical threshold, however, has been validated only under the following boundary conditions: a tumor blood flow rate of 1 mL/g per minute, a hemoglobin concentration of 140 g/L, and an arterial O_2 partial pressure of 90–100 mmHg. Reducing the perfusion rate to 0.3 mL/g per minute yields an hypoxic tissue fraction of approximately 20% *(48).* When the hemoglobin concentration falls below 100 g/L or the normal O_2 content of arterial blood decreases (hypoxemia), the relative proportion of hypoxic tissue substantially increases in the experimental tumor system described.

On a global tissue level, the critical O_2 partial pressure in tumors, below which the detrimental changes associated with reduced O_2 consumption have been observed, is 8–10 mmHg (Table 1). Measurements of the microregional distributions of ATP by quantitative bioluminescence and photon imaging in rodent tumors have shown that the concentration of ATP is relatively constant (1.0–1.8 m*M*) as long as an adequate supply of oxygen (i.e., comparable to that of normal tissues or organs) can be maintained *(49,50).* In FSaII murine fibrosarcomas growing subcutaneously in mice, relatively constant ATP levels were present as long as the median O_2 partial pressure was 10 mmHg or higher [Fig. 1 and *(38)*]. Similar results were obtained in rat tumors when the global ATP content was evaluated with high-performance liquid chromatography *(39,40).* Median O_2 partial pressures of approximately 10 mmHg thus appear to represent a critical threshold for energy metabolism in FSaII tumors. At higher median O_2 tensions, the levels of ATP, phosphomonoesters, and total inorganic phosphate were relatively constant, coinciding with intracellular alkalosis or neutrality and a stable ATP/inorganic phosphate ratio, energy charge, and phosphorylation potential. Median O_2 partial pressures of less than 10 mmHg result in intracellular acidosis, ATP depletion, a drop in the energy supply, and increasing levels of inorganic phosphate (Fig. 1).

Oxidative phosphorylation for ATP formation will continue to a cellular O_2 partial pressure of 0.5–10 mmHg [Table 1 and *(42–45)*]. Certainly, the threshold O_2 partial pressure below which oxidative phosphorylation ceases is dependent on the cell line investigated and its respiratory capacity, the type of medium and substrate chosen, the temperature and pH of the suspending medium, and even the type and accuracy of the setup used to measure O_2 consumption rates.

Mitochondrial oxidative phosphorylation is limited at O_2 partial pressures of less than approximately 0.5 mmHg [Table 1 and *(32,45)*]. Above this threshold, mitochondria should function physiologically. Again, this critical threshold depends on the actual substrate supply, on the pH of the suspending medium, and on the technique used to measure O_2. Cytochromes aa_3 and *c* in ascites cells require O_2 partial pressures of greater than 0.02–0.07 mmHg [Table 1 and *(32,46,47)*] to maintain respiration. At O_2 partial pressures above this range, cytochromes are

Table 1. Critical O_2 partial pressures below which adequate metabolic functions in solid tumors (metabolic hypoxia) cannot be maintained*

Critical pO_2, mmHg	Entity measured	Experimental conditions	Parameter of interest	Reference(s)
45–50	End-capillary blood	Normoxemia, Hb content = 140 g/L; MRO_2 = 30 μL/g per min; TBF = 1 mL/g per min	O_2 consumption rate	(*36,37*)
8–10†	Tissue (global)	*In vivo*	ATP levels	(*38–40*)
≈5	CHO cells	*In vitro* (absence of glucose)	ATP per cell	(*41*)
0.5–1	CHO cells	*In vitro*	O_2 consumption rate	(*42*)
0.5–2	Ehrlich ascites cells	*In vitro* (succinate as substrate)	O_2 consumption rate	(*43,44*)
8–10	Neuroblastoma cells	*In vitro* (Hanks' medium supplemented with bovine serum albumin)	O_2 consumption rate	(*45*)
≈0.5	Isolated mitochondria	*In vitro*	O_2 consumption rate	(*32,45*)
0.02–0.07	Cytochromes	*In vitro*	Oxidation status	(*32,46,47*)

*pO_2 = O_2 partial pressure; mmHg = millimeters of mercury; Hb = hemoglobin; MRO_2 = O_2 consumption rate; TBF = tumor blood flow; ATP = adenosine triphosphate; CHO = Chinese hamster ovary.

†Median pO_2.

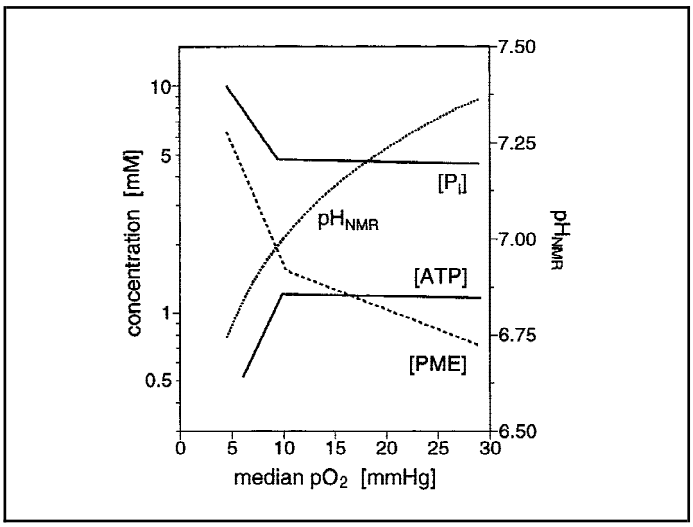

Fig. 1. Mean concentrations of adenosine triphosphate (ATP), phosphomonoesters (PME), and total inorganic phosphate (P_i) (**left ordinate**) and nominal intracellular pH (pH_{NMR}, measured by ^{31}P nuclear magnetic resonance) in FSaII tumors (**right ordinate**) as a function of median tumor O_2 partial pressure (pO_2) values in mmHg (millimeters of mercury) [redrawn from *(38)*].

fully oxidized. Spectrophotometric measurements on living and rapidly deep-frozen tissues indicate that the same is true *in vivo.*

From this rather rudimentary summary of critical O_2 partial pressures for metabolic hypoxia, there does not appear to be a single hypoxic threshold that is generally applicable. Hypoxic thresholds range from 45–50 mmHg in end-capillary blood to 0.02 mmHg in cytochromes (*see* Fig. 4). Furthermore, such data on hypoxic thresholds in a given tissue do not take into consideration the existence of severe heterogeneities even on a microscopic level related to variable O_2 demands and O_2 supply.

In this discussion of hypoxic thresholds, it is important to note that, for any functional parameter, a sharp threshold between hypoxia and normoxia does not exist and should not be expected. This review deals with the problem of hypoxia as a whole, encompassing mild, moderate, and severe hypoxia (divisions that are not well defined). Approaches in which oxygen effects have been defined under *in vitro* conditions by using half-maximum values (e.g., in ionizing radiation) have proven useful in some instances, such as comparing radiosensitivity of different cell lines under identical boundary conditions. However, use of half-maximal values in a more general discussion of hypoxia is not very informative because these values do not give the O_2 levels at which hypoxia starts and becomes a biologic problem.

Methods for Detection of Tumor Hypoxia

During the past decade, the oxygenation status of solid tumors has been evaluated by investigators in many specialized centers. Despite various limitations of the techniques used, a number of key findings have been described as follows: 1) Most tumors have lower median O_2 partial pressures than their tissue of origin; 2) many solid tumors contain areas of low O_2 partial pressure that cannot be predicted by clinical size, stage, grade, histology, and site; 3) tumor-to-tumor variability in oxygenation is usually greater than intratumor variability in oxygenation; and 4) recurring tumors have a poorer oxygenation status than the corresponding primary tumors [reviewed in *(4)*].

Assessment of the tumor oxygenation status by invasive and noninvasive procedures has been reviewed [*(51–54)* and Table 2]. Many methods can detect tumor hypoxia. Which method is most appropriate for a particular experimental or clinical need will depend on the feasibility of the approaches available in terms of invasiveness and the degree of resolution required, on whether measurement of a direct or indirect parameter is necessary, and, of course, on financial considerations.

The present "gold standard" is intratumor polarographic measurement of O_2 partial pressures by using microsensor techniques that adhere to the systematic random sampling principle *(53).* However, no single method will probably be suitable for all situations; therefore, where possible, use of more than one technique may be advisable. In all instances, careful interpretation of the data obtained is paramount, and researchers should bear in mind the exact parameters measured and the limitations of the specific methods used.

Hypoxia-Mediated Proteome Changes and Tumor Propagation

The intrinsic ability of a tumor to propagate by means of local destructive growth and dissemination is the hallmark of malignant disease. The hypoxic microenvironment in solid tumors, which affects neoplastic cells and non-neoplastic stromal cells such as macrophages and fibroblasts, should have profound effects on tumor propagation if one considers the proteome changes of cells demonstrated under hypoxic conditions *in vitro.* The proteome changes may result from the stimulation or inhibition of gene expression and from posttranscriptional and posttranslational effects induced by hypoxia or anoxia (Fig. 2).

Anoxia/hypoxia-induced proteome changes in neoplastic and stromal cells may lead to the arrest or impairment of neoplastic growth through molecular mechanisms, resulting in cellular quiescence, differentiation, apoptosis, and necrosis *(97–101).* Cells exposed to hypoxia are generally arrested at the G_1/S-phase boundary *(97).* O_2 partial pressures of 0.2–1 mmHg dispropor-

Table 2. Methods currently available or under development for detection of tumor hypoxia (selection)*

1) Invasive microsensor techniques for direct tissue pO_2 measurements
   - Polarographic O_2 sensors [e.g., (*55–57*)]
   - Luminescence-based optical sensors [e.g., (*58,59*)]
2) Electron paramagnetic resonance oximetry (*60,61*)
3) Techniques for intravascular O_2 detection
   - Cryospectrophotometry [HbO_2 saturation (*62–65*)]
   - Near-infrared spectroscopy [HbO_2 saturation (*66, 67*)]
   - Phosphorescence imaging (*68*)
4) Nuclear magnetic resonance spectroscopy and imaging techniques
   - ^{1}H-MRI, BOLD effect (*69–71*)
   - ^{19}F-magnetic resonance relaxometry (*72–76*)
5) Noninvasive detection of sensitizer adducts
   - [^{18}F]Fluoromisonidazole [PET (*77–80*)]
   - [^{123}I]Iodoazomycin-arabinoside [SPECT (*81–83*)]
6) Invasive immunohistochemical hypoxia marker techniques
   - Misonidazole [^{3}H-labeled (*52,84–87*)]
   - Pimonidazole (*88–90*)
   - Etanidazole (*91–95*)
   - Nitroimidazole-theophylline (*96*)

*Histomorphometric and DNA strand break (after 2–4 Gy) assays, as indirect measures of tumor hypoxia, are not listed. pO_2 = oxygen partial pressure; HbO_2 = oxyhemoglobin saturation; MRI = nuclear magnetic resonance imaging; BOLD = blood oxygen level dependent; PET = positron emission tomography; SPECT = single photon emission computed tomography.

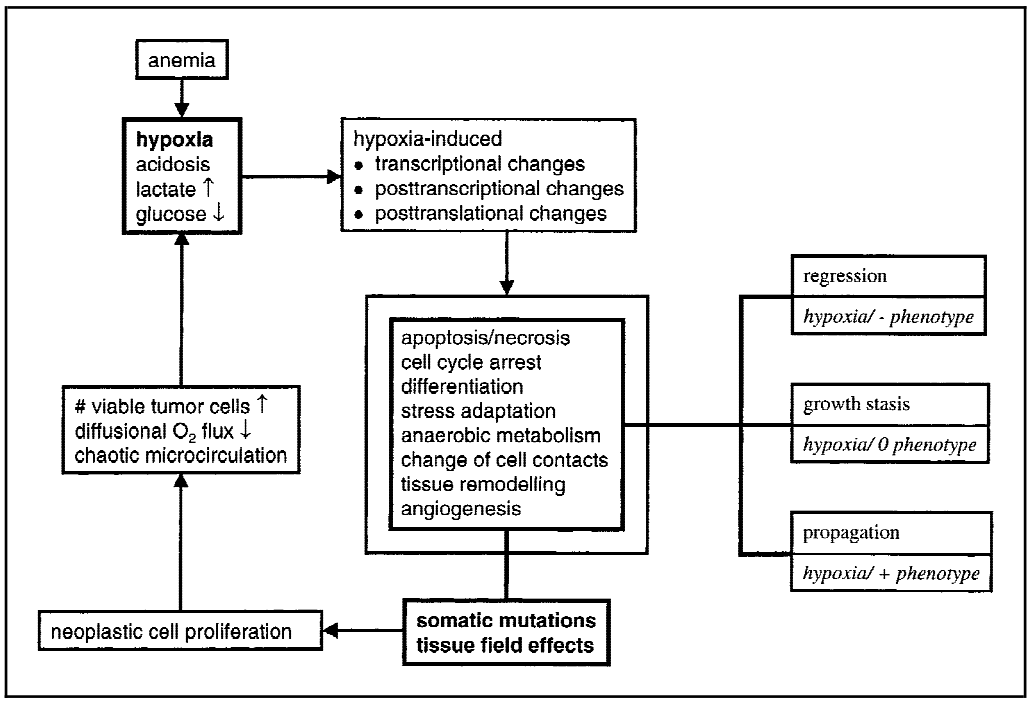

Fig. 2. Hypoxia-induced proteome changes (i.e., changes in the set of proteins within a cell at a given time) in the neoplastic and stromal cells influence tumor propagation. The phenotypic consequences of the hypoxic proteome changes in the neoplastic cells are modulated by genomic variation and microenvironmental factors besides hypoxia (tissue field effects). The net result within a tumor microregion can be regression (hypoxia/– phenotype), growth stasis (hypoxia/0 phenotype), or growth promotion and tumor dissemination (hypoxia/+ phenotype).

tionately lengthen the G_1 phase or arrest cells in the G_1 phase *(102–104).* Above this hypoxic threshold, variations in O_2 partial pressure should have only negligible effects on the proliferation rate. Under anoxia, most cells are arrested immediately, regardless of their position in the cell cycle.

Binding of the hypoxic marker pimonidazole to suprabasal cells in epithelia supports the hypothesis that hypoxia may act as a morphogen to induce the terminal differentiation of cells *(85,100).* This observation appears to have a counterpart in well-differentiated squamous cell cancer, where squamous cell differentiation is consistently observed in tumor areas several cell layers away from the nearest blood vessels (Höckel M: unpublished observations). The molecular mechanisms of hypoxia-induced terminal differentiation are largely unknown.

Hypoxia can induce programmed (apoptotic) cell death in normal and neoplastic cells *(101).* Indeed, oncogenic transformation of cells (e.g., transfection with human papillomavirus E6/E7 genes or c-myc genes) increases their susceptibility to hypoxia-induced apoptosis *(23).* The level of p53 in cells increases under hypoxic conditions, and the increased level of p53 induces apoptosis by a pathway involving Apaf-1 and caspase-9 as downstream effectors *(105).* However, hypoxia also initiates p53-independent apoptosis pathways involving hypoxia-inducible factor-1 (HIF-1), genes of the BCL-2 family, and other unidentified genes *(106,107).* Below a critical energy state, hypoxia/anoxia may result in necrotic cell death, a phenomenon seen in many human tumors and experimental systems. Hypoxia-induced proteome changes, leading to cell cycle arrest, differentiation, apoptosis, and necrosis, may explain delayed recurrences, dormant micrometastases *(108,109),* and growth retardation in large tumor masses *(110).*

In contrast, hypoxia-induced proteome changes in tumor and/or stromal cells may promote tumor propagation by enabling the cells to adapt to nutritional deprivation or to escape their hostile environment. Hypoxia stimulates the transcription of glycolytic enzymes, glucose transporters (GLUT1 and GLUT3), angiogenic molecules, survival and growth factors (e.g., vascular endothelial growth factor [VEGF], angiogenin, platelet-derived growth factor-β, transforming growth factor-β, and insulin-like growth factor-II), enzymes, proteins involved in tumor invasiveness (e.g., urokinase-type plasminogen activator), chaperones, and other resistance-related proteins *(8,17,29,97,106,111–119).* At the same time, hypoxia-induced inhibition of gene expression has been demonstrated for cell-surface integrins facilitating tumor cell detachment *(120).*

Many hypoxia-inducible genes are controlled by a common transcription factor, HIF-1, composed of two subunits, HIF-1α and HIF-1β *(121).* Increased concentrations of HIF-1 in the proteome of a hypoxic cell result from increased transcription of HIF-1α and HIF-1β genes and decreased HIF-1α protein degradation, an example of hypoxia-mediated posttranslational control *(122–124).* Jiang et al. *(111)* exposed human HeLa cells to concentrations of O_2 between 0.125% and 20% (with 5% CO_2 added and the remainder N_2) and then analyzed HIF-1 expression as a function of intracellular O_2 concentration. HIF-1 DNA-binding activity and the concentrations of HIF-1α protein and HIF-1β protein increased exponentially as cells were subjected to decreasing concentrations of O_2, with a half-maximal response at about 10 mmHg. Hypoxia-induced HIF-1 activation can also result in an increased production of VEGF. To determine the reduced O_2 concentration required to stimulate increased levels of VEGF messenger RNA (mRNA), Chiarotto and Hill *(125)* determined O_2 concentrations in culture medium from cervical cancer cell lines SiHa, ME-180, or HeLa cells, under distinct boundary conditions, and defined the threshold for increasing the level of VEGF mRNA above baseline as O_2 pressure of approximately 1 mmHg in the gassing mixture.

Nuclear factor κB (NFκB) is another transcriptional factor that can be activated by hypoxia *(126).* The threshold for activation of NFκB in AG1522 cells occurs after 3 hours at an O_2 partial pressure of about 15 mmHg *(127).* Thus, the critical O_2 levels necessary for hypoxia-induced gene expression are probably in the range of 1–15 mmHg (Table 3 and *see* Fig. 4). Below

Table 3. Critical O_2 tensions below which typical cellular functions in solid tumors progressively cease or anticancer treatments are impaired as a result of an inadequate O_2 availability

Critical O_2 tension, mmHg*	Function or parameter observed	Selective reference(s)
30–35	Effectiveness of certain (passive) immunotherapies	(*128*)
15–35	Cell death with photodynamic therapy	(*129–132*)
25–30	Cell death on exposure to x- and γ-radiation	(*2,3*)
10–20	Binding of hypoxia markers	(*87,95*)
1–15†	Proteome changes	(*22,114,121, 125,127*)
0.2–1	Genome changes	(*19,22,23,29, 97,133,134*)

*mmHg = millimeters of mercury.

†Experiments were partly performed with monitoring of the ambient gas mixture only. Pericellular O_2 partial pressures in the medium can be substantially lower, depending on number of cells, stirring procedures, and the cell line investigated.

these levels, mRNA levels often rise almost exponentially to a maximum value.

Whether the net phenotypic result of hypoxia-induced proteome changes of the tumor and stromal cells is neoplastic growth arrest (hypoxia/0 phenotype), growth impairment (hypoxia/– phenotype), or promotion through local, perifocal, regional, or distant tumor propagation (hypoxia/+ phenotype) may be determined by the genomic state, the degree of hypoxia, and microenvironmental epigenetic factors, in addition to hypoxia. Genomic changes in neoplastic cells that reduce the potential for cell cycle arrest, differentiation, and apoptosis may favor hypoxia-associated mechanisms that promote tumor growth and dissemination, such as stress adaptation, anaerobic metabolism, angiogenesis, tumor cell detachment and subsequent adhesion, tissue remodeling, and migration (Fig. 2).

Hypoxia-Mediated Malignant Progression

By clinical definition, malignant progression of a neoplasm means the increasing probability of local spread (through direct invasion of neighboring tissues and organs), perifocal spread (through migration of single neoplastic cells and microfoci into the interstitial space, lymphatic space involvement, and perineural invasion), regional spread (through metastases to the lymph nodes), and distant spread (through hematogenous metastases or dissemination into body cavities, such as the peritoneum and pleura), as well as the increasing resistance toward nonsurgical therapy concomitant with primary tumor growth. Clinical progression of a malignant tumor is a consequence of 1) an increasing neoplastic cell load, 2) microenvironment-induced (epigenetic) phenotypic changes in neoplastic and stromal cells, and 3) genotypic changes and clonal selection of neoplastic cells *(135).* Evidence is accumulating that hypoxia not only induces proteome changes influencing tumor propagation but also drives malignant progression through transient and persistent genomic changes in neoplastic cells *(18,19,29,133–136).* Hypoxia (with or without reoxygenation) promotes genomic instability (through point mutations, gene amplification, and chromosomal rearrangements) and may unveil pre-existing cryptic genetic variations (which are suppressed by chaperones in cells not experiencing permanent stress), thus increasing the number of genetic variants. Concomitantly, hypoxia exerts a strong selection pressure (Fig. 3).

The importance of the hypoxia-mediated clonal expansion of tumor cells with diminished apoptotic potential has been demonstrated both experimentally and clinically *(18,22,23).* The increasing inability of tumor cells to activate apoptotic pathways can explain many of the clinical consequences of malignant progression, such as locoregional and distant tumor propagation and resistance to nonsurgical therapy. Survival and proliferation of occult perifocal tumor cells with diminished apoptotic potential, located in hypoxic surgical scars, appear to be major pathogenetic events in the formation of local recurrences, despite complete surgical resection of solid neoplasms with microscopically tumor-free resection margins (R_0 resection; Höckel M: unpublished results).

In most investigations of hypoxia-induced genomic changes, transformed cells were incubated at almost zero O_2 partial pressure and then reoxygenated at atmospheric O_2 partial pressure. Rice et al. *(29)* demonstrated that, after incubating Chinese hamster ovary cells for up to 72 hours in less than 10 parts per million (ppm) of O_2, the dihydrofolate reductase gene was amplified, which led to increased methotrexate resistance. By incubating murine cells under similar conditions, Young et al. *(19)*

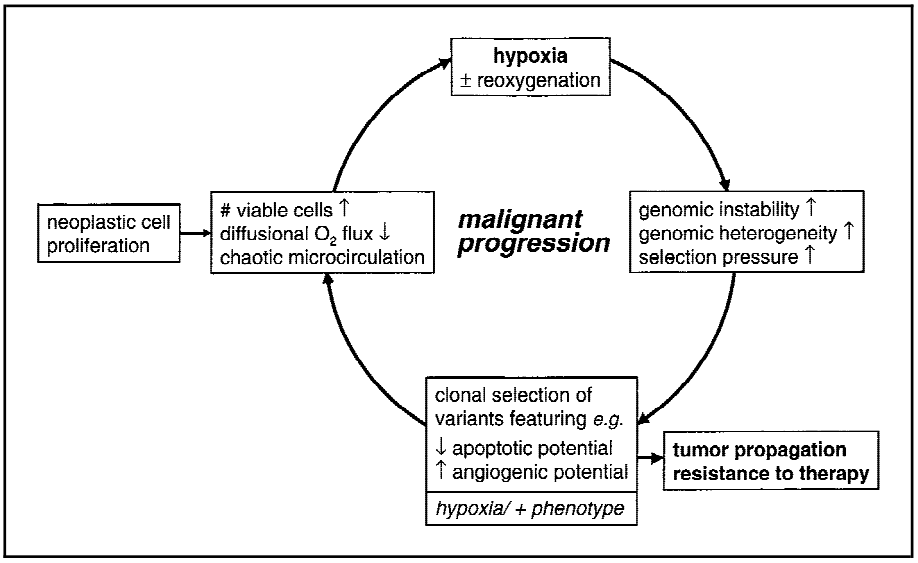

Fig. 3. Schematic representation of the paramount importance of hypoxia in the malignant progression of solid tumors through progressive genome changes and clonal selection of hypoxia/+ phenotypes. Tumor hypoxia is a consequence of the deregulated proliferation of malignant cells and an insufficient supply of oxygen [and other nutrients; also recently reviewed in *(161,162)*]. Sustained hypoxia (and intermittent reoxygenation) increases genomic instability and genomic heterogeneity. New variants adapted to survive and to proliferate under reduced O_2 partial pressures within the tumor (hypoxia/+ phenotypes) are selected through clonal expansion and aggravate tumor hypoxia. Thus, a vicious circle is established, leading to the dominance of hypoxia/+ phenotypes that are resistant to therapy and are able to survive and proliferate at various sites remote from the primary tumor.

observed that DNA overreplication transiently enhanced the formation of experimental metastases. To induce the transformation of a benign B16 melanoma cell phenotype to a malignant phenotype, Stackpole et al. *(137)* incubated monolayers of cells for 48 hours in an O_2-depleted medium. After 24 hours, these cultures were severely hypoxic (<50 ppm of O_2). Russo and coworkers *(134,136)* observed DNA breakage resulting from activation of an endogenous endonuclease in immortal rat embryo fibroblasts cultured under anoxic conditions for up to 24 hours. Reynolds et al. *(133)* used a mouse tumor cell line carrying a chromosomally based λ phage shuttle vector for reporting mutations. After exposing these cells to an O_2 partial pressure of less than 1 mmHg for 4 hours, they detected a mutation rate that was 3.4-fold higher than the rate in similar cells cultured under standard atmospheric conditions. Giaccia and colleagues *(22,23,97)* used an elegant procedure to select transformed cells with reduced apoptotic potential. Specifically, they cultured transformed mouse embryonic fibroblasts and transformed human cervical epithelial cells under a reduced O_2 partial pressure of less than 1 mmHg for 48 hours, followed by up to seven reoxygenation treatments. As a rule, hypoxia-induced genomic changes are detectable at an O_2 partial pressure of less than 1 mmHg, which is approximately one order of magnitude lower than the O_2 partial pressures associated with proteome changes (Table 3 and Fig. 4).

Hypoxia-mediated clonal selection of neoplastic cells with persistent genomic changes leading, *inter alia,* to apoptotic insensitivity and increased angiogenic potential stabilizes and further aggravates tumor hypoxia, which in turn promotes malignant progression. Thus, hypoxia is involved in a vicious circle that is regarded as a fundamental biologic mechanism of the malignant disease, once cellular proliferation has been deregulated [*(11,14)* and Fig. 3].

Tumor Hypoxia and Treatment Resistance

Tumor hypoxia may present a severe problem for radiation therapy (x- and γ-radiation), because radiosensitivity is progressively limited when the O_2 partial pressure in a tumor is less than 25–30 mmHg (Table 3 and Fig. 4). Hypoxia-associated resistance to photon radiotherapy is multifactorial. The presence of molecular oxygen increases DNA damage through the formation of oxygen free radicals, which occurs primarily after the interaction of radiation with intracellular water *(2).* Thus, because of this so-called "oxygen enhancement effect," the radiation dose required to achieve the same biologic effect is three times higher in the absence of oxygen than in the presence of normal levels of oxygen *(1–3).* Evidence suggests that hypoxia-induced proteome and genome changes may also have a substantial impact on radioresistance by increasing the levels of heat shock proteins or by increasing the number of cells in a tumor with diminished apoptotic potential or increased proliferation potential *(18,22,23),* both of which have been linked to radioresistance *(24,25).*

Oxygen dependency has been documented for a number of anticancer agents (e.g., cyclophosphamide, carboplatin, and doxorubicin) under *in vitro* and *in vivo* conditions *(138,139).* However, these investigations have been qualitative, and clear hypoxic thresholds for O_2-dependent anticancer agents are still not available, although they presumably exist for each agent. Thus, additional research is necessary to provide quantitative data on hypoxia-induced chemoresistance, although this information may be difficult to obtain under *in vivo* conditions. Multiple mechanisms are probably also involved in the hypoxia-induced resistance to chemotherapeutic agents, including an inhibition of cell proliferation *(140),* a hypoxia-induced decreased cytotoxicity of some agents *(98,138,141),* and tissue acidosis, which is often observed in hypoxic tumors with a high glycolytic rate *(142).* Furthermore, hypoxic stress proteins and the loss of apoptotic potential can impart resistance to certain chemotherapeutic drugs *(26–29,112,114,143).*

Photodynamic therapy-mediated cell death requires the presence of oxygen, a photosensitizing drug, and light of the appropriate wavelength, both *in vitro* and *in vivo* [for a review, *see (144)*]. Reports *(129,145),* however, vary greatly on the extent to which photodynamic therapy with hematoporphyrin derivatives is dependent on oxygen. Cells were not killed under anoxic conditions. The critical threshold below which progressively reduced cell death was observed varied from 15 to 35 mmHg

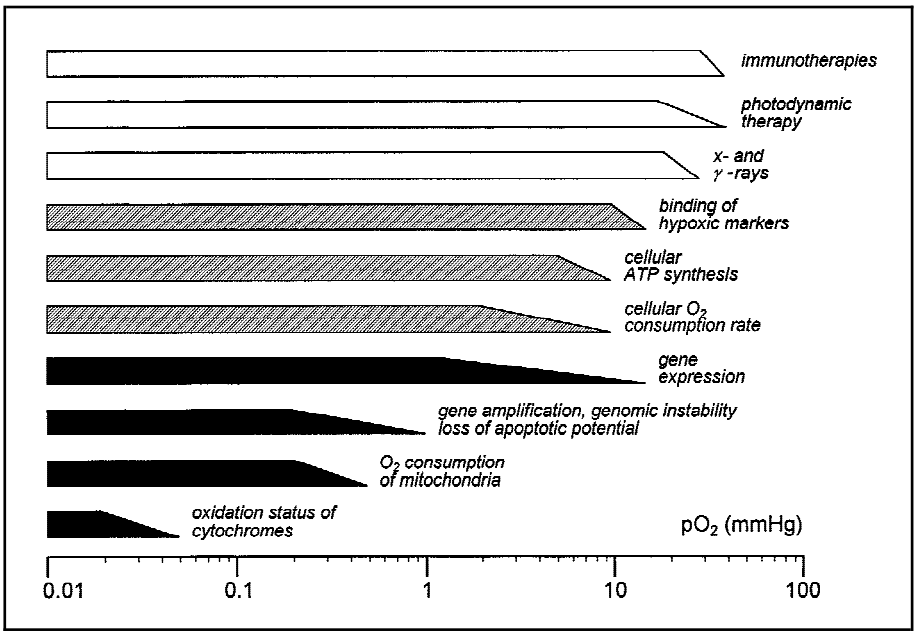

Fig. 4. Critical O_2 levels that characterize the upper limit of the hypoxic range, below which activities or specific functions of tumor cells progressively change. **Open bars** = therapy forms; **hatched bars** = cellular functions; **solid bars** = mechanisms at the subcellular and molecular levels. The **bars** indicate the respective hypoxic ranges, with the lengths of the bevels showing the variation in threshold values as found by various authors for different end points. pO_2 = O_2 partial pressure; mmHg = millimeters of mercury.

(129–131), probably because of the reduced production of singlet oxygen ($^1O_2$) species and different sensitivities to the treatment in different cell lines (Table 3 and Fig. 4). Considering the reduced effectiveness of photodynamic agents at lower O_2 partial pressures, the rapid induction of tumor hypoxia by photodynamic therapy itself—either as a consequence of a photodynamic therapy-induced decrease in blood flow or as a result of oxygen consumption by the photodynamic therapy process itself—has to be considered under *in vivo* conditions, since it may mean that this therapy is self-limiting *(129,132,146).* Photodynamic therapy involving prodrugs, such as aminolevulinic acid, may be further limited because conversion of the prodrug to the active photosensitizer appears to be less effective under hypoxic conditions.

Studies of cells *in vitro* have identified several factors that can influence the effect of hyperthermia on cell survival. Cell lines can vary substantially in their intrinsic heat sensitivity. In addition, cell cycle position, intracellular pH, nutrient deprivation, and ATP depletion can affect cell survival after a heat treatment *(147,148).* At 43 °C hyperthermia, hypoxia *per se* may not cause cell death as long as concomitant changes in the nutritional and/or bioenergetic status of the cells do not occur *(149).*

Finally, tumor hypoxia can dramatically alter the potency of cytokines (interferon gamma and tumor necrosis factor-α) and alter interleukin 2-induced activation of lymphokine-activated killer cells [reviewed in *(128)*]. The potency of treatment started to decrease at O_2 partial pressures of less than approximately 35 mmHg (Table 3 and Fig. 4).

Tumor Hypoxia as an Adverse Prognostic Factor

Gatenby et al. *(150)* used polarographic electrodes to investigate the oxygenation status of advanced head and neck carcinomas and, thus, to our knowledge, were the first to report substantial differences in O_2 partial pressures in tumors of patients who responded to radiotherapy versus tumors of those who did not. After a new generation of polarographic O_2 sensors was introduced in 1989, a prospective clinical trial was initiated in which the prognostic relevance of O_2 partial pressures in advanced cancers of the uterine cervix was investigated *(11,14,151,152).* An interim evaluation in 1991 found that a median O_2 partial pressure of 10 mmHg appeared to be a cutoff level to distinguish between hypoxic cervical cancers with poor prognosis and less hypoxic cervical cancers with statistically significantly better prognosis. The study also demonstrated that tumor oxygenation was independent of various patient and tumor characteristics, including patient age, menopausal status and parity, International Federation of Gynecology and Obstetrics (FIGO) stage, clinical tumor size, histopathology, and grade of malignancy. A Kaplan–Meier life-table analysis showed statistically significantly shorter survival and recurrence-free survival for patients with hypoxic tumors. The results were consistent with the hypothesis that radiobiologically hypoxic tumors (i.e., tumors with a reduced radiosensitivity at critically low O_2 levels) are less curable. However, other mechanisms of treatment failure, such as increased locoregional and distant tumor propagation, could not be excluded *(151).* By the end of 1995, 103 patients with advanced cancers of the uterine cervix had entered the study. From a histopathologic examination of the surgical specimens obtained from 47 patients during radical tumor resection, hypoxic tumors had larger extensions, more frequent (occult) parametrial spread, and more lymph-vascular space involvement than nonhypoxic tumors of the same clinical stage and size. Probabilities of 5-year overall and disease-free survival calculated for patients who underwent standard primary treatment for cure were again statistically significantly lower for those with hypoxic tumors than for those with nonhypoxic tumors of similar clinical stage and size. Cox regression analysis revealed tumor oxygenation as the strongest independent prognostic factor, followed by FIGO stage *(11).* Of special interest was the fact that the disadvantage in outcome for patients with hypoxic tumors was independent of the mode of primary treatment (radiation therapy or radical surgery). This finding led to the hypothesis that tumor hypoxia was associated with malignant progression in advanced cancer of the uterine cervix and that hypoxia not only may counteract O_2-dependent forms of therapy but also may advance tumor progression *per se* irrespective of treatment [*(11)* and Table 4].

More recently, Fyles et al. *(157)* also determined whether the pretreatment oxygenation status of tumors could predict disease-free survival by assessing data for 74 patients with cervical cancer who were treated with radiation. There was clear evidence that hypoxia (defined as the fraction of measured O_2 partial pressures of <5 mmHg) is a statistically significant adverse prognostic factor of disease-free survival. Furthermore, Knocke et al. *(154)* confirmed the prognostic relevance of pretreatment tumor oxygenation status by studying 51 patients with cancer of the uterine cervix who were treated with primary radiation (Table 4). Recently, Sundfor et al. *(158)* also reported a poor outcome associated with low oxygen tensions in 40 advanced squamous cell carcinomas of the uterine cervix.

In local recurrences of cervical cancer, oxygenation levels of

Table 4. Prognostic significance of tumor hypoxia*

Oxygenation parameters	End points	Tumor site	Reference(s)
Median pO_2 of ⩽22 mmHg	Disease-free survival, overall survival	Primary soft-tissue sarcomas	(*153*)
Median pO_2 of <10 mmHg	Disease-free survival, overall survival	Locally advanced primary cancers of the uterine cervix	(*11,14,151,154*)
	Disease-free survival, overall survival	Primary soft-tissue sarcomas	(*12,155*)
	Disease-free survival, overall survival	Primary head and neck cancers	(*20,155*)
	Incidence of metastases	Locally advanced primary cancers of the uterine cervix	(*13*)
Median pO_2 of <4 mmHg	Disease-free survival, overall survival	Locally recurrent cancers of the uterine cervix	(*14*)
Fraction of pO_2 values ⩽2.5 mmHg	Locoregional control, overall survival	Head and neck cancers	(*156*)
Fraction of pO_2 values ⩽5 mmHg	Disease-free survival, overall survival	Locally advanced primary cancers of the uterine cervix	(*157,158*)

*pO_2 = O_2 partial pressure; mmHg = millimeters of mercury.

the recurrent tumor were generally lower than levels in the primary tumors of comparable mass. In the cohort of patients with recurrent tumors, oxygenation measurements provided additional prognostic information. Patients with tumors that had a median O_2 partial pressure of less than 4 mmHg had a statistically significantly shorter median survival time than those with median O_2 partial pressure of 4 mmHg or more [Table 4 and *(14)*].

The pretreatment tumor oxygenation status was also assessed in patients with soft-tissue sarcomas *(12,153,155,159,160).* In these patients, the more hypoxic tumors were associated with a poorer survival when compared with normoxic tumors resulting from local treatment failure or distant metastases (Table 4).

In a study on the association between the tissue oxygenation status and the radiation response in lymph node metastases of squamous cell carcinomas of the head and neck, Nordsmark et al. *(156)* showed that the most hypoxic tumors had statistically significantly lower locoregional tumor control than well-oxygenated tumors. Cox multiple regression analysis found that an O_2 partial pressure of 2.5 mmHg or less was the strongest independent variable for prediction of a response to radiation therapy, when the end point was tumor control at the site where the O_2 partial pressure was measured. Brizel et al. *(20,155)* also showed that tumor hypoxia appears to adversely affect the prognosis of patients with primary and metastatic squamous cell carcinomas of the head and neck (Table 4).

How Can the Term "Tumor Hypoxia" Be Used Appropriately?

By the definition and criteria presented in this review, there is clear evidence that the range of hypoxia in malignant tumors can vary widely. Critical O_2 levels (hypoxic thresholds) characterize the upper limit of the hypoxic range below which activities and functions progressively become restricted. These O_2 levels can encompass O_2 partial pressures from 35 mmHg (start of reduced cell death in conventional photodynamic therapy or restricted efficacy of some immunotherapies) to 0.02 mmHg (below this level, cytochromes aa_3 and *c* are no longer fully oxidized) with all other critical O_2 levels for specific cellular functions or activities distributed in between (Fig. 4).

We, therefore, recommend that only data describing defined functions or activities of tumor cells or characteristics of tumors be compared. Because critical O_2 levels of different parameters or reactions can vary substantially, we recommend that only results from assays describing the same biologic parameter (e.g., radiation sensitivity) be tested for correlation. Otherwise, pseudocorrelations may be misinterpreted as real biologic interrelationships.

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