Tumor Hypoxia and Gene Expression

Implications for Malignant Progression and Therapy

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Most histopathological classifications of human cancers include significant numbers of hypoxic cells. There is increasing evidence that, at least in certain types of human solid tumors, there is a positive relationship between the presence of hypoxia and poor outcome after radiation therapy alone or radiation combined with other therapies. Hypoxia appears to be an independent prognostic factor. There is evidence for enhanced malignant progression associated with hypoxia, including locoregional invasion and distant metastases. The presence of hypoxia may negatively affect outcome by induction of radiation resistance by the classical oxygen effect and/or by effects on gene expression and malignant progression, causing more aggressive locoregional and distant disease. It is now clear that hypoxia has the potential to influence expression of genes and activities of associated proteins that regulate growth and tissue homeostasis, resulting in cellular phenotypic heterogeneity. The molecular pathways involved in signaling and regulating changes in gene activities in response to external stresses such as hypoxia are becoming known. Identification of patients with hypoxic tumors will lead to improved selective therapy.

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The microenvironment in normal or malignant tissues may have a significant effect on responses to therapy. This is because the microenvironment contributes to regulation of cell phenotype. Recent discoveries have advanced our understanding of a number of important contributors to the microenvironment in tumors. These include the role of the pathophysiology of vasculature and blood flow causing hypoxia in tumors with associated changes in gene expression. Differences in responses of specific tissues may also occur due to contributions to local microenvironments from cytokines, cell–cell/extracellular matrix interactions, and the specific tissue cell type (cell context). The microenvironment may affect malignant progression by selecting for growth of genetic variants and by contributing to genetic instability. Understanding of the role of the microenvironment may provide molecular targets for tumor control therapies.

The role of hypoxia as a significant component of the microenvironment in human tumors is considered here from several perspectives: 1) evidence for hypoxia and consequences for responses to radiation therapy; 2) possible contributions of altered gene expression to malignant progression; and 3) molecular regulation mechanisms and signal transduction.

HYPOXIA IN HUMAN TUMORS

Many different histopathologic classifications of human solid tumors are now known to contain significant numbers of cells that exist at less than normal physiological oxygen levels mainly because of abnormal vascularization and blood flow (1). Both chronic and transient hypoxia (cyclic with reoxygenation) occur in tumors in animal models and both types of hypoxia probably occur in human tumors. Chronic hypoxia is caused by inadequate vascular supply in relation to the volume of oxygen-consuming tumor cells. Those cells at a distance from blood vessels are poorly oxygenated. Transient hypoxia results from temporary occlusion of vessels caused by alterations in pressure–perfusion relationships. It is important to recognize the presence of these two forms of hypoxia since intracellular stress responses and molecular pathways that are involved may be affected differently Furthermore, strategies for therapy to deal with resistant hypoxic cells may need to be optimized in order to deal with both types of hypoxia.

A variety of invasive and non-invasive methods have been developed to measure oxygenation of tumors in both animal models and humans (2, 3). Hypoxia in human tumors has been measured by oxygen sensitive electrodes

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and by hypoxia marker techniques using various labels that can be detected by different methods such as positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance spectroscopy (MRS), autoradiography, and immunohistochemistry. Most histopathologic classifications of human tumors are known to be heterogeneously oxygenated. Median pO_2 electrode measurements are greatly reduced compared to corresponding normal tissues (1). Significant fractions of the measurements in many of the tumors are severely or moderately hypoxic ($pO_2 < 15$ mmHg). Cells in such areas of the tumors would be resistant to radiation and have the potential to result in poor responses to fractionated radiation therapy. Furthermore, since we now know that expression of various genes and proteins is regulated by oxygen, other indirect mechanisms for hypoxic tumor cells to influence disease aggressiveness and outcome of therapy are possible. For all classifications of tumors where oxygenation has been assessed, hypoxia does not appear to correlate with pathological standards of stage, grade, and other similar parameters (4–9).

RELATIONSHIP OF HYPOXIA TO THERAPY OUTCOME

What is known about the relationship between hypoxia and therapeutic responsiveness or malignant progression in human tumors? Most published studies of human tumors demonstrate a positive relationship between the presence of hypoxia measured by oxygen-sensitive electrodes and poor outcome after radiation therapy alone or radiation combined with other therapies. The studies included patients who had advanced squamous cell carcinoma of the cervix (5–7, 10), head and neck (8, 11, 12), and soft tissue sarcomas (13, 14). Different oxygenation endpoints and different cut-off levels were used to stratify the hypoxic tumors. Outcome was assessed as locoregional tumor control, overall survival and/or disease-free survival, and freedom from distant failure.

Consistent with the early studies of Kolstad who measured pO_2 directly with polarographic electrodes (10), correlations between hemoglobin levels in patients with cervix carcinoma and the responses of their tumors to radiotherapy have been obtained (15). Subsequently, Gatenby et al. (11) reported oxygen tension measurements in lymph node metastases of head and neck cancers showing a significant relationship between low, mean tumor pO_2 and failure to respond to radiotherapy. In another study, 50% of patients with advanced carcinoma of the cervix with a median $pO_2 > 10$ mmHg exhibited recurrence-free survivals of greater than 3 years, whereas patients with median $pO_2 \leq 10$ mmHg had a 50% survival of only 8 months after treatment of both groups with radiotherapy with or without chemotherapy (5). Such differences could be related to therapeutic resistance caused by hypoxia or changes in malignant progression associated with hypoxia, e.g. more aggressive local disease or metastatic propensity. An extension of these studies (6) to include a total of 103 patients with advanced cancers of the cervix showed that 50% of the patients had hypoxic tumors as defined by median $pO_2 < 10$ mmHg. Therapy outcome was assessed in patients who received either radiation therapy or surgery. Patients with hypoxic tumors had significantly worse disease-free and overall survival probabilities compared with those with non-hypoxic tumors. The poorer outcome for the patients with hypoxic tumors was mainly due to locoregional failures with and without metastasis, irrespective of whether the therapy was surgery or radiation. Histopathology of the surgical specimens showed that tumors with low pO_2 exhibited larger tumor extensions and more frequent (occult) parametrial spread, as well as lymph-vascular space involvement, compared to well-oxygenated tumors of similar clinical stage and sizes. Cox regression analysis identified tumor oxygenation as an important independent prognostic factor.

Brizel et al. (13) showed that in high-grade soft tissue sarcomas there is a relationship between pretherapeutic tumor hypoxia and the development of distant metastasis as first site of failure following treatment with radiation, hyperthermia, and surgery. Accordingly, patients with hypoxic tumors had a poorer survival probability than those who had well-oxygenated tumors. Similar results have been reported recently by Nordsmark & Overgaard (14).

Bioluminescence detection measurements of lactate distribution in patients with cervical as well as head and neck carcinomas have shown a correlation: the primary tumors of patients with metastases contained about twice as much lactate as those of patients without metastases (16, 17). Lactate concentration is directly related to hypoxia, because more lactate is produced through anaerobic metabolism.

Recently, Brizel et al. (12) reported that tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. The disease-free survival was 78% for patients with median $pO_2 > 10$ mmHg but only 22% for median $pO_2 < 10$ mmHg. The average tumor median pO_2 in relapsing patients was 4.1 mmHg and 17.1 mmHg in non-relapsing patients.

These data indicate that the presence of hypoxia in human tumors may negatively affect outcome of radiotherapy by induction of radiation resistance by the classical 'oxygen effect' and/or by effects on gene expression and malignant progression causing more aggressive locoregional and distant disease.

MALIGNANT PROGRESSION AND GENE EXPRESSION

The most acceptable current model of the stages of increasing aggressiveness of cancer (malignant progression) is one that views cancer as an evolution of preclinical and clinical stages of disease. Many of the stages of cancer formally described by criteria of pathology, e.g. dysplasia, invasion, etc., can now also be associated with known molecular events. Thus, cancer progression involves accumulations of mutations involving oncogenes and suppressor genes which affect growth regulation and tissue homeostasis. A key element causing abnormal behavior of the cells is the disruption of signaling within and between cells associated with various molecular pathways that regulate activities for genes. Among these are important oncogenes and tumor suppressor genes. These changes in gene expression can also affect the properties and anatomical distribution of disseminated disease in the body.

It is now clear that hypoxia has the potential to influence the expression of genes that regulate many of these molecular pathways that would be expected to cause phenotypic heterogeneity and enhance malignant progression (Fig. 1). Thus, in addition to the well-known direct effects of hypoxia on responses of tumor cells to radiation and some drugs, selection for growth of genetic variants and enhanced genetic instability can occur in hypoxia. Severe hypoxia has been previously associated with the induction of an endonuclease activity that was postulated to contribute to the genomic instability of cancer cells (18). Another recent study directly tested the influence of tumor microenvironment on genetic instability (19). These investigators used a tumorigenic cell line that carried a recoverable, chromosomally based lambda phage shuttle vector designed to report mutations without the need for genetic selection of mutant cells. The cells were grown in parallel either in culture or as tumors in nude mice. The frequency of mutations arising in cells within the tumors was found to be five-fold higher than that in otherwise identical cells grown in culture. A distinct pattern of mutation was also seen, with more deletions and transversions in the tumors than in the cell culture. Furthermore, exposure of the cultured cells to hypoxia produced an elevated mutation frequency and a mutation pattern similar to those seen in tumors. These results indicate that the conditions within solid tumors are mutagenic and suggest that a fundamental mechanism of tumor progression in vivo is genetic instability induced by hypoxia in the tumor microenvironment.

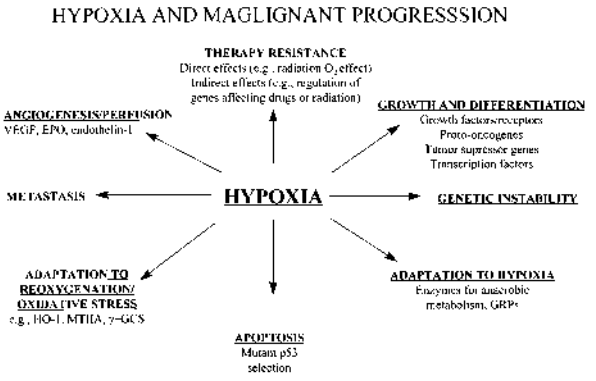

Fig. 1. Hypoxia contributes to malignant progression by modulating signal transduction and gene expression for many important properties of cancer cells.

Hypoxia also induces increased expression of proteins known as oxygen-regulated proteins. These can be classified to include a range of functions (Fig. 1): glucose-regulated proteins that act as molecular chaperones to maintain intracellular integrity of other peptides/proteins; redox stress enzymes/molecules to prepare cells to deal with reactive oxygen metabolises after oxidative stresses such as reoxygenation or reperfusion; metabolic enzymes such as those involved in anaerobic glycolysis to maintain energy or enzymes involved with polyamine biosynthesis; transcription factors and signaling molecules that regulate genes for proteins involved in growth control, apoptosis, differentiation, metastasis, and other functions; and growth factors, receptors, and cytokines that regulate processes such as angiogenesis, vascular permeability and inflammation. Details of the research up to 1996 on each of these have been reviewed (20). An update of research on the effects of hypoxia on expression of some of the more important genes involved with malignant progression is presented here.

Hypoxia has the potential to promote malignant progression by altering gene expression related to several important tumor phenotypic activities. Among all these known genes whose expression is affected by hypoxia, the transcription factors that are known to be oncogenes or suppressor genes may contribute most directly to malignant progression. For example, both the proto-oncogene c-jun in the AP-1 transcriptional complex (21, 22) and the tumor suppressor gene p53 (23) increase their expression in response to hypoxia. C-jun is an important effector of immediate-early signals associated with cellular growth, differentiation, and stress responses. The p53 protein plays a central role in regulating mitogenesis and growth arrest, especially in response to agents that induce DNA damage (24–27). In addition, p53 is a key regulator of apoptosis (27–29) and is involved in control of angiogenesis (30).

Hypoxic stress induces slow accumulation of p53 protein in the cell (23). Several different cell types that express wild-type p53 have been shown to undergo a greater frequency of apoptotic cell death than cells that express: mutant p53 (31). As a result, hypoxic stress, especially transient hypoxia, could lead to selection and eventual dominance of the tumor cell population by p53 mutant cells that are more malignant. Such a selection has been demonstrated both in vitro and in vivo in association with hypoxia regions in animal tumor cell models (31). Recently, hypoxia was also shown to stimulate both p53

induction and apoptosis in vitro in human cervical epithelial cells expressing human papilloma virus (HPV) oncogenes E6 and E7 but not in cervical fibroblasts infected with E6 and E7 (32). Cell lines derived from HPV-associated human cervical squamous cell carcinomas were less sensitive to apoptosis induced by hypoxia. It was suggested that this was due to the acquisition of additional genetic alterations that reduced their apoptotic sensitivity. Exposure of these cell lines to hypoxia in long-term cell culture accelerated the selection of subpopulations of cells with diminished apoptic potential. Thus, hypoxia-mediated selection of cells with diminished apoptic potential may contribute to malignant progression in human tumors and may in part explain why hypoxic cervical tumors are more aggressive.

Regulation of the growth of new blood vessels (angiogenesis) in primary and metastatic tumors is very important for malignant progression. Among the various factors that regulate angiogenesis, vascular endothelial growth factor (VEGF) is known to be strongly upregulated by hypoxia (33). VEGF expression is increased in regions near necrosis in tumors (33) and multicell spheroids in vitro (34, 35). Studies are ongoing in a number of research groups to assess the relationships between hypoxic regions, VEGF expression, and the development of new blood vessels. Such studies are especially important because of the apparent paradoxical results that both increased angiogenic index (36, 37) and increased hypoxia are predictive of poor prognosis.

Hypoxia has also been shown to increase the transient induction of the anti-angiogenic gene, thrombospondin-1 (TSP-1) in cells containing a wild-type p53 gene (38). On the other hand, the basal and hypoxia-inducible expression of TSP-1 was undetectable in cells with mutant p53. In contrast, VEGF was induced under hypoxic conditions regardless of the cellular p53 genotype (38, 39). These studies suggest that tumor oxygenation status together with tumor cell genotype play and important role in angiogenesis.

The hypoxia-specific transcription factor HIF-1 is best known for its role in regulating the activity of the erythropoetin (EPO) gene (40–42). This tetrameric transcriptional complex binds to a hypoxia response element (HRE) with known consensus sequence. HIF-1 is also the transcription factor for several glycolytic enzymes and VEGF (43–45).

Oncogenic transformation of cells by Ha-*ras* (46) or v-*src* (47) oncogenes enhances hypoxia-stimulated angiogenesis and this is associated with increased expression of VEGF which is mediated through the HIF-1 transcription factor. This provides an additional mechanism for transformed cells to survive the abnormal condition of the tumor microenvironment and favors malignant progression.

Several other growth factors and receptors are induced by hypoxia and may contribute to tumor progression and responsiveness to therapy. These include: platelet-derived growth factor β (PDGFβ), transforming growth factor β (TGFβ), endothelin-1, interleukin 1-$\propto$, Flt-1 receptor, and EGF receptor. In the case of EGF-R in human squamous carcinoma cells, reoxygenation after hypoxia produced a transient increase in active phosphorylated receptors on the cell surface, suggesting that such cells could conceivably have a growth advantage after reoxygenation during fractionated radiotherapy by stimulation with EGF (48).

Some oxygen-regulated proteins are also induced in response to other stresses that include radiation, chemicals, metals and drugs; e.g. glucose-regulated proteins (GRPs) (49), heme oxygenase, (HO-1) (50), DT-diaphorase (51), γ-glutamylcysteine synthetase (51), and metallothionein (human MTIIA and mouse MT-1) (52, 53). These proteins play an important role in sustaining cell survival during oxidative stress associated with reoxygenation or exposure to redox-cycling agents and in drug metabolism/detoxification (51, 54). Mutation/deletion analysis of the metallothionein proximal gene promoter sequence has shown that regulation of gene expression in response to hypoxia is mediated by metal response elements (MREs) in association with metal transcription factor-1 (MTF-1) (53).

HYPOXIA SENSING AND SIGNAL TRANSDUCTION

The rapid pace of gene discovery and increasing knowledge about their expression in relation to disease states have stimulated related research on the molecular pathways involved in signaling and regulating changes in gene activities in response to external factors such as hypoxia. In this regard, there has been a merging of research fields in cancer biology at the level of gene regulation. We now know that there are a large number of overlapping genes and regulatory signaling molecules and pathways for control of cell growth and differentiation, responses to stresses such as hypoxia or radiation damage, inflammatory responses, and regulation of programmed cell death (apoptosis). A schematic summary of the current status of knowledge of hypoxia-induced signal transduction pathways is presented in Fig. 2 and is described in more detail in what follows.

There are distinct pathways that transduce signals from cell-surface receptors or intracellular sensors of perturbations such as DNA damage to transcription factors that activate expression of specific genes. Growth factor receptors (GFR) are activated by dimerization and phosphorylation when bound by growth factors such as epidermal growth factor (EGF). Ligands for G-protein coupled receptors (GPR) include various cytokines and hormones. These receptors transmit signals mainly through phosphorylation cascades that initiate in association with members of the *Ras* superfamily and the cell membrane. Signal

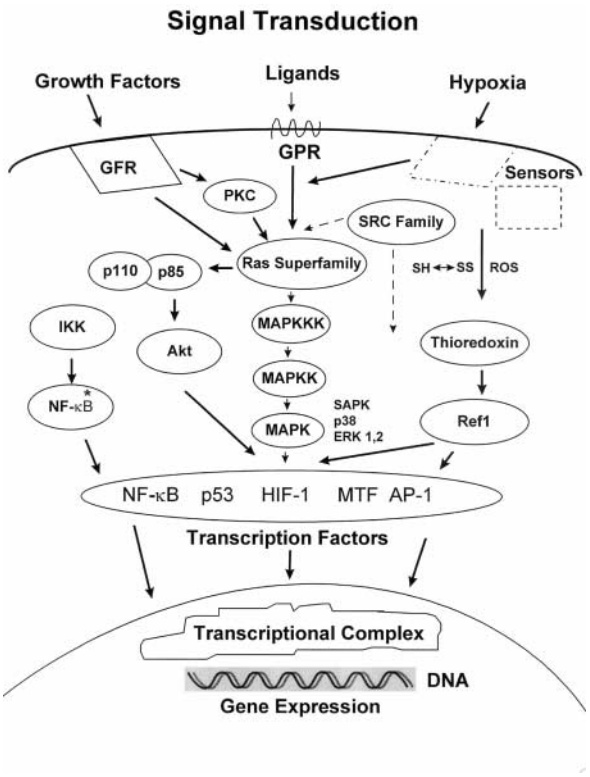

Fig. 2. The major hypoxia-induced signal transduction pathways. See text for abbreviations.

transduction for these known receptors, and those involved in response to hypoxia, occurs through cascades of molecular interactions activated through protein kinase (MAPK superfamily) and phosphatase enzymes. These cascades entail the targeted relocation of specific proteins in the cell and the reversible formation of protein complexes. The net effect of a given stimulus on the cell is determined by integration of the intensity and duration of activation of the individual pathways, and the particular signaling molecules expressed for specific cell types.

How are oxygen levels sensed and how might sensors interface with signal transduction pathways causing gene activation? One hypothesis that has been advanced is that the sensor is a heme protein (55). It has been suggested that this sensor would be located at or near the cell membrane and propagation of the signal would involve subsequent steps with reactive oxygen species (ROS) as second messengers, such as superoxide and hydrogen peroxide. These interface with cellular glutathione (GSH) regulation as well as hypoxia signal transduction pathways (56). Presumably an O_2 sensor and coupled signal transduction process would be highly conserved evolutionarily, because the ability of cells to sense and respond appropriately to changes in hypoxia/redox status must be a very central homeostatic process.

Signal transduction in response to hypoxia may be mediated in part through some growth factor kinase pathways. There may also be some overlap with pathways involved in response to other stresses, e.g. oxidative stress such as JNK/SAP kinase and p38 kinase. Membrane-associated Src kinase and protein kinase C (PKC) are involved since c-Src is phosphorylated early after hypoxia stress (57) and inhibitors of PKC decrease the activity of this pathway (58). The hypoxia activation of NF-κB proceeds through c-*Ras* and c-*Raf* and then diverges from the conventional growth factor pathway (57, 59).

PI 3-kinase, a lipid kinase consisting of an 85 kDa regulatory subunit (p85) and an 110 kDa catalytic subunit (p110), is a key component of growth factor signal cascades. Recently, it was shown that hypoxia modulates VEGF induction in *Ras*-transformed cells through this PI 3-kinase pathway involving activation of the proto-oncogene Akt and the HIF-1 transcription factor (60).

Because hypoxia affects the redox status of the cell, another level of regulation may occur through the reduction of thiol groups found in the DNA-binding domain of some transcription factors. For example, in vitro transactivation of AP-1 (61) and Sp-1 (62, 63) requires that thiols in the DNA-binding domain be maintained in the reduced state. A nuclear protein Ref-1 that maintains the reduced state of fos and jun at critical cysteines, and promotes AP-1 binding, is rapidly activated by hypoxia (22). Ref-1 is identical to HAP-1, an endonuclease involved in DNA repair activity. Regulation of this signaling pathway is modulated by the redox status of the cell. Enzyme activities and metabolic pathways that regulate thiol (SH↔SS) concentration and the concentration of ROS and reducing equivalents (NADPH) from metabolism are important contributors to redox homeostasis. The thioredoxin/thioredoxin reductase–redox system maintains Ref-1 in an active state (reduced cysteines) so that it, in turn, can promote transcription factor binding to DNA (64). Thioredoxin and thioredoxin reductase are also upregulated by hypoxia (65).

Since transcriptional activation involves the association of many different proteins with DNA, there may be other interactions in this complex that can be modulated by the redox status of the cell. There is also evidence to suggest that in vivo binding and transactivation by certain transcription factors (Sp-1, Egr-1, NF-KB) can be modulated by exposure of cells to thiol oxidizing or reducing agents (66). Because the stress of hypoxia alone and the combined stresses of hypoxia plus low glucose may significantly decrease GSH and total non-protein sulflhydryls (NPSH), it can be assumed that hypoxia may regulate some transcription complexes by this mechanism.

The proto-oncogene c-jun encodes a major component of the AP-1 transcriptional complex, and its expression can be influenced by hypoxia as well as growth factors and oxidative stresses. Since AP-1 consensus recognition sites are found in the regulatory regions of numerous genes, including for example, MT II A, VEGF, HO-1 which are

known to be induced by hypoxia (as discussed previously), we have investigated the mechanism of the signaling in this important pathway. Hypoxia alone appears to increase c-jun protein levels mainly by stabilization and extension of mRNA half-life (21) Combined hypoxia plus low glucose stress was regulated mainly by effects on transcription rather than message half-life.

Further investigation of the mechanism of induction of the c-jun protooncogene demonstrated that transcriptional activation of the c-jun promoter by hypoxia correlated with phosphorylation of the transactivation domain of the ATF2 transcription factor (67). Because ATF2 is a component of AP-1 complexes (c-jun/ATF2) that bind to the c-jun promoter region, this findings suggests that hypoxic signals transmitted to the c-jun promoter are mediated by protein kinases that target c-jun/AFT2 AP-1 complexes. ATF2 is a substrate of the p38 kinase and SAPK/JNK stress-activated members of the MAPK superfamily, whereas c-jun is a substrate of the SAPK/JNKs. Subsequent research in our laboratory has demonstrated that pathophysiological hypoxia induces p38 kinase and SAPK/JNK activities in human carcinoma cells (SiHa squamous carcinoma and $HepG_2$ adenocarcinoma) (68). The p38 kinase activity was persistently activated by hypoxia and was reversible on reoxygenation. SAPK/JNK was strongly induced but was attenuated by prolonged hypoxia, suggesting the operation of an inhibitory physiological mechanism. Analysis of potential signaling pathways upstream of hypoxia-inducible p38 kinase and SAPK/JNK activities suggested that tyrosine kinase activation is involved in these responses.

Although both p38 kinase and SAPK/JNK activities were enhanced in both carcinoma cell lines, there was no enhancement of apoptosis under these conditions of prolonged hypoxia, indicating that the induced kinase activities did not generate productive apoptic signals. It is possible that the transient induction of these activities would not lead to an apoptotic response (69) or that these specific cell types have escaped normal mechanisms for inducing apoptosis (70). A possible function of these hypoxia-inducible kinase activities is that they contribute to growth arrest in cycling cells, because hypoxia is growth inhibitory. Alternatively, these activities could regulate concerted gene expression required for adaptation to hypoxia or for responses to oxidative stress on reoxygenation.

IMPLICATIONS FOR TREATMENT

Since hypoxia is present in many solid tumors and not in normal tissues it represents an attractive target for selective tumor therapy. Better detection methods are needed to determine which tumors are likely to benefit from hypoxia-directed therapies. Therapy could be improved for patients with such tumors by addition of radiosensitizers or hypoxic cytotoxins (71). By imaging hypoxic regions of tumors and monitoring reoxygenation, it would be possible to optimize radiation treatment to change fractionation or include boost doses to radiation- resistant hypoxic fields. Information about which patients have hypoxic tumors may also be useful in decisions for more aggressive therapy since these patients may have a higher probability for metastases (at least for certain tumor types). It is also reasonable to consider exploiting tumor hypoxia as a selective mechanism for targeting gene expression for diagnostic and therapeutic purposes (72). Availability of methods to identify patients with hypoxic tumors would also be very useful for defining subpopulations of patients for clinical trials of new hypoxia-directed therapies.

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