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Targeting the molecular effects of a hypoxic tumor microenvironment

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1. ABSTRACT

Tumor hypoxia is a serious and enduring problem for traditional solid tumor therapies. Many scientists continue to explore methods to improve or exploit tumor oxygenation; more recently, scientists have also focused on altering the molecular effects of hypoxia. These cellular responses to hypoxia and the resulting physiological effects, with a focus on angiogenesis, invasion/metastases, apoptosis, and metabolism, are examined. Recent efforts to mitigate or exploit these molecular pathways alone and in conjunction with traditional therapies are also explored. Current experimental results suggest that targeting multiple downstream molecular pathways of hypoxia will be more effective than targeting a single molecular pathway of hypoxia, and careful planning is necessary in scheduling these new therapies to optimize their effects in combination with traditional therapies.

2. INTRODUCTION

2.1. Clinical Implications of Tumor Hypoxia

Tumor hypoxia has become one of the most studied physiological phenomena in cancer research due to its complexity and pervasiveness in solid tumors. Tumor hypoxia has been shown to be prognostically significant in many clinical studies, independent of treatment type (1-6). Patients with hypoxic tumors have lower overall survival, decreased response rates, and higher rates of tumor reoccurrence and metasases (1-7).

The causes for poorer prognosis are multi-fold. Initially, hypoxia-related resistance in tumors was believed to be primarily a problem for radiotherapy, because hypoxic cells have increased radio-resistance compared with normoxic cells (8). At O_2 concentrations below 10 mmHg, radiosensitivity of cells decreases. Under anoxic

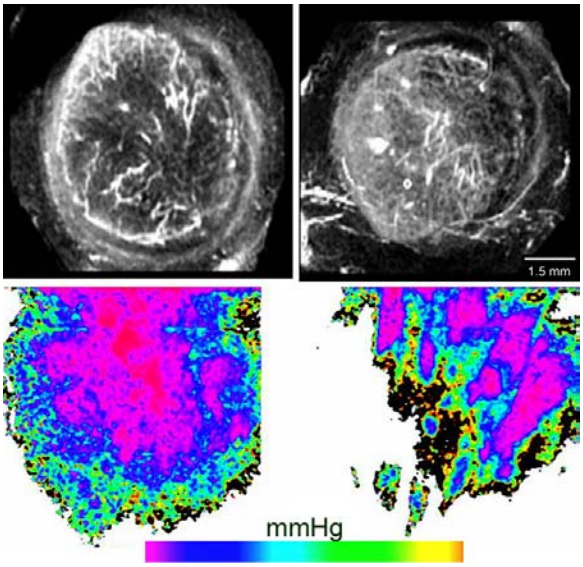

Figure 1. Longitudinal gradients in tumor oxygenation. A) Tumor and fascial images of high resolution magnetic resonance GdAlbumin angiography in tumor bearing dorsal skin-fold window chamber. Vessels on fascial side are larger, easily visible, and evenly distributed and perfused. Vessels on tumor side (which are fed from vessels on fascial side) are disproportionately distributed along edges, and unevenly perfused. B) Tumor and fascial phosphorescence lifetime images of pO_2. The tumor surface is significantly more hypoxic than the fascial surface, reflecting the longitudinal gradient which occurs along afferent blood supply. This dramatic drop in vascular oxygenation occurs over very short distance, in this Figure the distance between the fascial and tumor surface images is only 250 µm. Figure adapted from (214) with permission of the author and publisher.

conditions, a 3-fold higher dose of radiation is necessary to kill the same fraction of cells compared with irradiating them under normoxic conditions (8). In the clinical setting, fewer tumor cells in hypoxic regions are killed than in normoxic regions when given the same dose. The increased sensitivity of cells to radiation in the presence of oxygen occurs because molecular oxygen reacts with radiation induced damage in DNA, rendering changes in DNA base structure that is difficult for cells to repair (8).

Hypoxic tumor cells are also chemoresistant. Several factors contribute. First, the decreased rate of proliferation of hypoxic cells causes resistance to drugs that are cell cycle specific (9-11). Second, the same deficiencies in perfusion that lead to hypoxia contribute to inefficient drug transport to hypoxic cells (11). Third, hypoxic cells often have set up defenses to protect them from their environment, such as elevations in GSH, that lead to multidrug resistance (11).

In addition to treatment resistance, it has been shown that hypoxic cells also demonstrate other adaptations. Hypoxia leads to increased tumor cell invasiveness and increased angiogenesis. Both of these adaptations contribute to increased propensity for metastasis. Additionally, tumor cells are also known to develop a glycolytic phenotype, often altering their metabolism to utilize glucose when low oxygen levels are limiting aerobic respiration. While not contributing directly to treatment resistance, these alterations in metabolism favor tumor cell survival under noxious environmental conditions.

In summary, the plethora of effects caused by tumor hypoxia provide a strong rationale for trying to understand and potentially exploit this feature of tumors.

2.2. Definition of hypoxia

Before going into detail on the causes and consequences of tumor hypoxia it is necessary to define what is meant by this term. For the purposes of this review, we will define this as a threshold of 10 mmHg. We chose this value because it is the threshold below which radioresistance increases (8). Second, below this value, changes in tumor cellular function tend to occur (12), leading to the phenotypic changes described above. Additionally, below this value there are changes in red cell fluidity that increase blood viscosity, leading to alterations in tumor perfusion (13).

2.3. Determinants of tumor hypoxia

O_2 concentration in tumor tissue, as in all tissues, is the result of a balance between O_2 delivery and consumption. In normal tissues, this balance is tightly regulated to prevent hypoxia, even at times of peak O_2 metabolism. This balance is largely controlled by evenly distributed arteriolar-capillary networks. In the event that hypoxia does occur in normal tissues, balanced signaling cascades lead to vascular remodeling, or angioadaptation, until the tissue pO_2 is back within its normal range (14, 15). In normal tissues, the supply of oxygen is sufficient to meet the demands of the tissue.

Tumors are unable to regulate their O_2 levels because they are not able to strictly control O_2 delivery or consumption. This results in regions of hypoxia within the tumor which are spatially and temporally variable. No standard treatment has been developed which successfully and significantly decreases tumor hypoxia, although many have been proposed. To understand the difficulty behind alleviating tumor hypoxia, a clear portrayal of the physiologic and metabolic characteristics of tumors is necessary.

There are two types of oxygen gradients in tumors: (1) radial gradients, or decline in oxygen concentration as one moves radially away from a microvessel and (2) longitudinal gradients, which are defined as decline in vascular oxygen concentration when moving afferently along the vasculature (Figure 1). These two features are not independent. The lower the vascular oxygen concentration, the shorter the radial oxygen diffusion distance is. These same types of gradients can be found in normal tissues, but in the case of normal tissues, one rarely observes hypoxia. Thus, the gradients are much more subtle in magnitude.

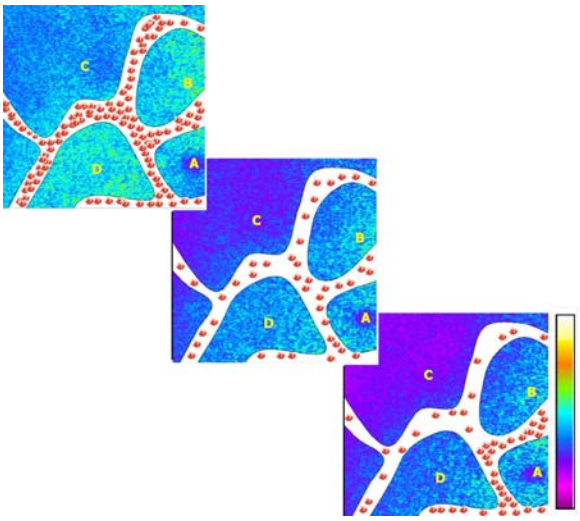

Figure 2. Diagrammatic illustration of fluctuations in tumor hypoxia. Each image represents a different time point. At location A, the tumor tissue is undergoing diffusion limited hypoxia at all time points, the red cell flux in nearby vessels has a negligible effect at this location. At location B, the tumor tissue is well-oxygenated at all time points, although increased red cell flux at the first and last time points do have a minor effect on the oxygen concentration. At location C, the tumor tissue is undergoing fluctuations in tumor pO_2. As the red cell flux decreases in nearby vessels at different time points, less O_2 is available to diffuse out to this location and it becomes hypoxic, even though the tissue immediately near this same vessel stays normoxic. At location D, the O_2 concentration fluctuates with time, but ranges from normoxic to intermediately hypoxic as the red cell flux in several nearby vessels changes.

O_2 availability in tumors is limited due to physiological constraints, particularly irregularities in tumor vasculature. The irregularities are caused by imbalances in angiogenic cytokines that regulate angiogenesis and vascular maturation. Four unique traits of tumor blood vessels have been described: abnormal branching structures and uneven distribution of microvessels; steep longitudinal oxygen gradients along afferent vasculature; decreased quantity of arterioles; and unsteady red cell distribution at bifurcations, leading to unstable red cell flux (number of cells passing through a microvessel per unit time). Abnormalities in the shape and distribution of microvessels clearly results in regions in which there are overabundances of microvessels, which can be connected by short shunts, and regions in which there is a scarcity of microvessels. In the regions in which microvessels are scarce, large inter-microvessel distances result in regions of tumor tissue which are beyond the radial diffusion distance of O_2, and are chronically hypoxic (remain below 10 mmHg for long periods of time).

Steep longitudinal oxygen gradients can also result in large areas of chronically hypoxic tumor tissue, although this is not due to the limited radial diffusion of O_2. Rather, axial O_2 gradients are seen in afferent flow. This phenomenon is further perpetuated by the reduced number of arterioles in tumors, as compared with comparable normal tissue. Lack of sufficient arteriolar supply and steep longitudinal gradients can result in tumor vessels which are themselves hypoxic, even when they are perfused. Consequently regions of tumor tissue adjacent to blood vessels can also be hypoxic.

Oxygen consumption rates of tumors are not exceedingly high compared with most normal tissues (16). This leads one to the conclusion that it is the deficiencies in oxygen delivery that are most responsible for hypoxia, as opposed to oxygen consumption rates. Nevertheless, at the microregional level, variations in oxygen consumption rate could contribute to hypoxia. For example, the oxygen consumption rate of proliferating cells averages 3-5 times that of G_o cells (17). Additionally, activated macrophages, which can be found in tumors, have very high oxygen consumption rates during periods of production of reactive oxygen species.

In addition to the deficiencies in oxygenation caused by vascular architecture, it is now well established that the oxygenation state of tumors is not stable (Figure 2). Unsteady red cell flux through the entire vascular structure of a tumor creates temporal instability in oxygenation state that extends from the microvascular supply vessels to the regions most distant from that supply (18, 19). The instability in oxygenation has implications for radiotherapy fractionation and may also influence gene expression in ways that are independent of hypoxia itself.

3. MODIFYING DOWNSTREAM EFFECTS OF TUMOR HYPOXIA

Since the discovery that tumor hypoxia alters the efficacy of therapy, many clinical and pre-clinical studies have been published, examining a plethora of means to reduce tumor hypoxia.. Hyperbaric chambers and hyperoxic gas breathing with or without radiosensitizers have shown limited success in some tumors (20-22). A number of other approaches have been attempted, including administration of agents to right shift the hemoglobin saturation curve, cell free hemoglobin, and artificial blood substitutes (23, 24). Hyperthermia treatment has been shown to decrease tumor hypoxia in murine, canine and human tumors (25, 26). When combined with hyperoxic gases hyperthermia has been shown to virtually eliminate hypoxia and increase radiation response in pre-clinical models. To date, however, there is no accepted clinical standard for eliminating tumor hypoxia (27-29). Aside from trials of hyperbaric oxygen, there is no level-one clinical evidence that modification of hypoxia improves local control when modified during radiotherapy treatment.

Without a reliable method to significantly reduce tumor hypoxia some investigators have instead focused on specifically targeting hypoxic cells (30-33). Hypoxic cytotoxins are now in clinical trials. Other approaches that have been tested pre-clinically include anaerobic bacteria and adenoviral vectors that proliferate and kill tumor cells specifically under hypoxic conditions (33).

An alternative strategy to directly killing hypoxic cells is to target molecular consequences of hypoxia. Some of the molecular effects can be mitigated or exploited either as direct therapies or as methods of improving the effects of standard therapies.

3.1. HIF

The central connection between physiological hypoxia and the cellular response is mediated by hypoxia-inducible transcription factors, or HIFs. Three HIF isoforms have been reported in human and rodent cells, with oxygen-dependent HIF-α subunits HIF-1α, HIF-2α, and HIF-3α.

These HIF-α subunits have been shown to heterodimerize with the oxygen-independent HIF-1β, or aryl hydrocarbon receptor nuclear translocator (ARNT) (34). The literature has mainly focused on the effects of HIF-1α, which will be the focus of this review, although more recently HIF-2α (35, 36) and HIF-3α activities in tumors have also been investigated (37).

HIF-1 regulates over one hundred genes, including those involved in angiogenesis, invasion/metastasis, apoptosis, and metabolism.

Both HIF-1α and HIF-1β are constitutively expressed in all cells (12, 38, 39). The O_2 sensors that control expression of HIF-1α are a family prolyl hydroxylases (PHD 1-3) (40, 41). Using O_2 as a limiting substrate, these Fe (II)-dependent PHDs catalyze the hydroxylation of prolyl residues within the oxygen-dependent degradation domain (ODD) of HIF-1α under normoxia (39). The hydroxylated HIF-1α subunit binds to the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex, resulting in degradation of the hydroxylated HIF-1α at high pO_2s (38, 42). This process is very efficient in normoxic cells, such that under normal circumstances, HIF-1α is not measureable (43, 44).

An asparaginyl hydroxylase, named Factor Inhibiting HIF-1 (FIH-1) provides an additional point of regulation of this promoter (45). It also results in HIF-1α hydroxylation; however, the hydroxylation occurs at the C-terminal transactivation domain. This alters the affinity between HIF-1α and its coactivator proteins p300/CBP, barring the transactivation of target genes under normoxia (46, 47).

A recent study has indicated that HIF-1 is regulated by transcription-dependent degradation. Demidenko et al showed that under hypoxic conditions, tumor cells treated with a transcription inhibitor showed a dramatic increase in HIF-1α levels. This increase in HIF-1α was partially inhibited by the use of deacetylases, offering a potential therapeutic target (48).

Under hypoxic conditions (*in vitro* K_m for HIF signaling was determined to be 15-20 μM (12)), HIF-1α almost instantaneously accumulates and is translocated to the nucleus (49, 50), along with HIF-1β. In the nucleus, HIF-1α and HIF-1β dimerize and bind to target gene motifs called hypoxia responsive elements (HREs) to alter gene expression (51).

HIF-1 can also be regulated in tumor cells through O_2-independent genetic alterations. Although this type of HIF regulation is not strictly within the scope of this paper (see reviews (52, 53)), uncoupling the O_2-dependent and -independent HIF-1 levels is difficult. Perhaps the key difference for O_2-independent regulation of HIF-1 is that the genetic alterations usually result in an increase in HIF-1α production through loss-of-function or gain-of-function mutations (38, 54-58). These genetic alterations affecting HIF-1 expression in specific tumor cell lines have been crucial to determining possible targets for therapeutics, which are discussed below.

3.1.2. HIF-1 targeting

High HIF-1 levels in human tumor biopsies are associated with increased mortality in a variety of solid tumors (52, 59-62). Decreased HIF-1 activity has been shown to decrease tumor growth in preclinical models (63-65). Combined with the knowledge of the many downstream effects of HIF-1 activation, HIF-1 is an excellent target for therapy.

Due to the well-characterized mechanisms of HIF-1 regulation, many approaches for HIF-1 targeting have been developed. Perhaps one obvious technique has been to target the co-activator proteins of HIF-1α (66, 67), preventing its translocation to the nucleus. This approach has been shown to decrease tumor growth in xenograft models, as well as mitigate some of the downstream effects of hypoxia related to angiogenesis and metabolism (68, 69). Similarly, HSP90, which is known to be involved in the folding of HIF-1α, has also been targeted in an effort to reduced HIF-1 activity. The HSP90 inhibitor geldanamycin has been shown to destabilize HIF-1α under both normoxia and hypoxia, resulting in transcriptional inhibition of VEGF in several cancer cell lines (70, 71). Other targets for therapy might target dimerization between HIF-1α and HIF-1β , inhibit its synthesis or prevent accumulation of HIF-1α under hypoxia (72-75).

Genetic approaches for HIF-1 targeting have also shown promising results. Antisense HIF-1α plasmids have been shown to suppress HIF-1α expression in tumors, reducing both VEGF levels and vascular density (76). In one preclinical study done in two human tumor lines, cells transfected with antisense HIF-1α significantly inhibited tumor cell growth *in vivo* (77) (Figure 3). Adenoviral delivery of antisense HIF-1α to established tumors also showed an anti-tumor effect when combined with radiation (77). This result is consistent with previous studies, which have shown that suppression of HIF-1 gene expression inhibited tumor growth in a xenograft model (60, 78). Silencing HIF-1 in preclinical models has also been shown to increase drug penetration.

An enormous effort is on-going within the scientific community to develop effective, non-toxic methods of targeting HIF in human patients; meanwhile, there are also on-going efforts to develop methods of

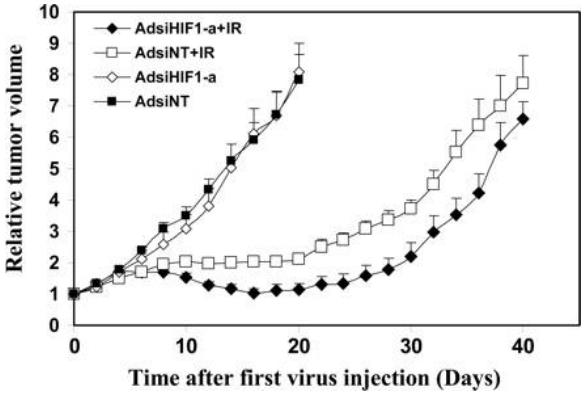

Figure 3. Results of tumor growth delay using adenoviral delivery of antisense HIF-1α with and without radiation. Established HCT116 tumors 6-8 mm in diameter were given three doses of an adenovirus containing antisense HIF-1α or a scrambled sequence every other day. If appropriate, tumors were irradiated 24 hrs after adenoviral injections. While antisense HIF-1α alone has no anti-tumor effect, antisense HIF-1α combined with radiation results in a tumor growth delay greater than that of radiation alone. From (77) with permission of the author and publisher.

targeting some of the larger down-stream molecular effects of HIF.

3.1.2. Angiogenesis

Pro-angiogenic pathways are an integral part of the HIF-1 response to hypoxia. Since the seminal paper by Folkman on tumor angiogenesis in 1971 proposing that the size of a tumor was limited by its ability to grow new vasculature (79), the factors regulating blood vessel growth have been explored in great detail. An attractive aspect of targeting angiogenesis is the fact that while tumor cells themselves are inherently genetically unstable, endothelial cells, which are part of the host cellular component of tumors, are not genetically unstable. A number of pharmacological means to target angiogenic pathways have been identified.

3.1.2.1. Targeting VEGF

The VEGF family is composed of seven homodimeric glycoproteins: VEGF A-F, and placental growth factor (PlGF) (80-82). VEGFA is believed to be the main regulator of angiogenesis in tumors, and is known to have at least six different isoforms, of which $VEGFA_{145}$ and $VEGFA_{165}$ are the most commonly found (81-84).

The angiogenic effects of VEGF are potentiated by their binding to VEGF receptors, two of which are receptors for VEGFA: VEGFR-1 (or Flt-1) and VEGFR-2 (or KDR/Flk-1) (80, 81). VEGFRs include an extracellular domain, transmembrane region, and a split tyrosine-kinase domain interrupted by a kinase-insert sequence (85, 86). The signal transduction of VEGF is mediated by the intracellular tyrosine-kinase domain (87), whose autophosphorylation at different sites is believed to activate different signaling pathways (i.e. result in different cellular responses) (80, 81).

VEGFA, VEGFR-1, and VEGFR-2 have all been shown to be up-regulated by hypoxia at least partially through the HIF pathway (82, 88). VEGFR-1 is believed to primarily act as a negative regulator of VEGFR-2 due to its competitive binding with VEGFA (88-90); in any case, VEGFR-2 is considered to be the dominant mediator of the angiogenic response from VEGFA. VEGF is upregulated in many human tumors, and has been shown to correlate with poor prognosis in several tumor types.

VEGF pathway has long been considered an excellent target for therapy; numerous pre-clinical and clinical studies have reinforced that belief (91-94). VEGF-targeting agents which are now in clinical trials include a humanized monoclonal antibody to VEGF, and anti-VEGFR-2 antibody, a soluble VEGFR, and small molecule inhibitors of VEGFR-2 signal transduction (95, 96). A monoclonal antibody targeting VEGF, to prevent binding to its receptor has been approved for human use in metastatic colorectal cancer.

3.1.2.2. Targeting angiopoietins

The angiopoietin-Tie2 pathway is another important angiogenic pathway in endothelial cells and some tumor cells (97). Tie-2 is a tyrosine kinase receptor with four known ligands, Angiopoietins 1-4 (Ang1-4) (98). Most studies have focused on the roles of Ang1 and Ang2 with Tie-2, perhaps due to their competitive inhibition of each other. Ang1 is the primary agonist for Tie-2; in normal tissues Ang1 is constitutively expressed and maintain vessels in an adult state by promoting interactions between endothelial cells and pericytes or other endothelial support cells (99, 100). However, overexpression of Ang1 in adult animals has been shown to produce neovascularization, and angiogenic effects *in vitro* such as endothelial cell tube formation, sprouting, migration, and survival (101). Recent studies have linked the Tie-2 signaling mechanisms to some of these endothelial cell behaviors through the PI3-K, PAK-1, MAPK, and/or Erk1/2 pathways.

Ang2 has been shown to be upregulated by HIF-1. Although Ang2 has a similar affinity for Tie-2 binding, it does not induce autophosphorylation of the Tie-2 receptor. In fact, studies have shown conflicting results concerning the role of Ang2 in angiogenesis. Initial studies suggested that Ang2 inhibited Ang1 binding to Tie-2 (102), resulting in little neovascularization. More recent studies indicated that Ang2 activates Tie-2 (103, 104), advancing endothelial cell survival and differentiation and angiogenesis independently of Ang1. This dual role for Ang2 can be attributed to VEGF, whose presence in conjunction with Ang2 has been shown to promote an angiogenetic response pathways (105).

High microvascular density in human breast cancer has been found to be an independent prognostic factor, and to significantly correlate with Ang2 expression (106). Strong expression of Ang2 and VEGF, but not Ang2 alone, was also shown to correlate with disease free survival in breast tumors (106). Similar results were found in patients with epithelial ovarian cancer, Ang1/Ang2 gene

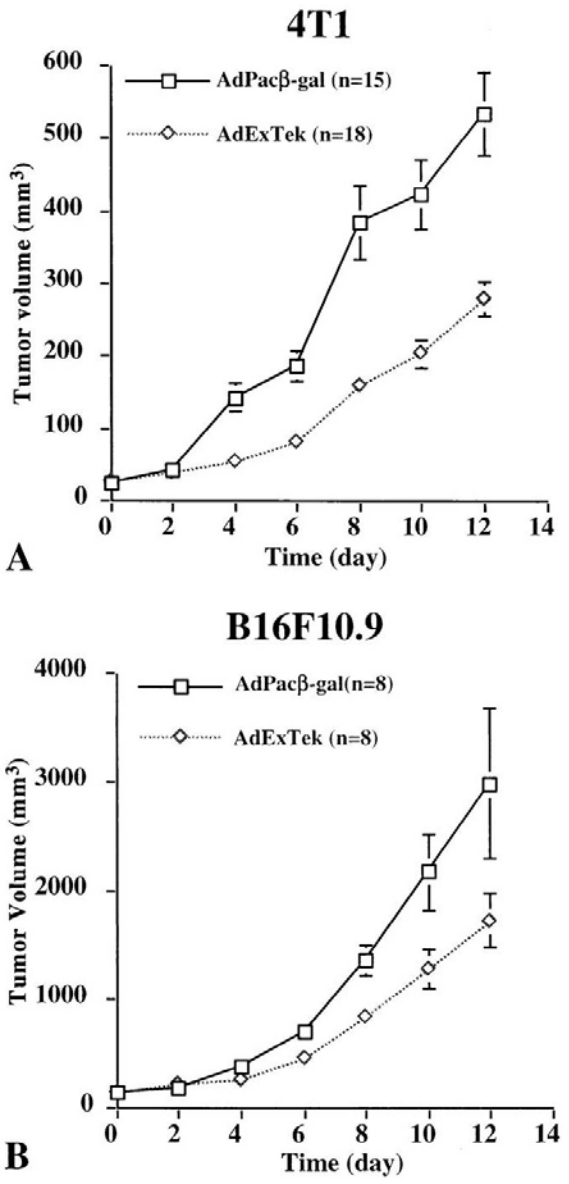

Figure 4. Well established 4T1 (A) and B16F10.9 (B) treated with recombinant adenoviruses containing either soluble Tie2 receptor (AdExTek), which blocks Tie2 activation, or a control virus (AdPacβ-gal). Twelve days after treatment, ExTek showed a significant anti-tumor effect in both tumor lines. From (110) with permission of the author and publisher.

expression was found to significantly correlate with poor prognosis (107).

Preclinically, blocking Ang1 in human gastric cancer cells using an antisense vector resulted in a decreased tumor growth rate and decrease in microvascular density (108). In another preclinical study, blocking Ang2 function has been shown to reduce endothelial cell proliferation, prevent VEGF-stimulated angiogenesis, and resulted in tumor growth arrest (109). Combined, this data suggests that angiopoietins are potential candidates for therapeutic targeting. However, targeting all angiopoietins is of some concern, since Ang1 binding to Tie2 is required to maintain vascular maturity. Therapeutic approaches, therefore, may have to target Ang2 specifically, or target Tie-2.

This approach was taken by Lin et al., who developed a soluble specific inhibitor of the Tie-2 extracellular domain, ExTek (110, 111) (Figure 4). ExTek was shown to inhibit binding of both Ang1 and Ang2 to Tie2, but did not alter tumor cell proliferation rate in treated cells (110, 111). Systemic administration of ExTek using an adenovirus resulted in decreased growth and inhibition of metastases in murine mammary carcinoma and melanoma tumors (112). Even the effects of VEGF stimulated angiogenesis in the corneal pocket were decreased with ExTek administration, suggesting possible interdependence of these two pathways (112). The results of these studies have been confirmed by other investigators (113, 114). Overall, the results from these studies suggest that targeting Tie-2 may have important therapeutic implications.

3.1.3. Invasion/metastasis

In the most basic sense, this section is merely an extension of the previous one on angiogenesis: tumor metastases occur through the vasculature, thus targeting angiogenesis also targets tumor metastases. However, hypoxia also alters tumor cell and matrix phenotypes, as discussed below.

3.1.3.1. Targeting MMPS

Matrix metalloproteinases (MMPs) play an important role in the maintenance and degradation of the tumor extracellular matrix (ECM). MMPs are grouped into eight classes: minimal domain MMPs, simple hemopexin-like domain-containing MMPs, gelatinases, furin-activated secreted MMPs, transmembrane MMPs, GPI-linked MMPs, vitronectin-like insert, linker-less MMPs, and cysteine/proline-rich IL-1 receptor-like domain MMPs (115). All MMPs have an N-terminal predomain, followed by a prodomain. This prodomain interacts with the catalytic zinc ion, and is followed by the catalytic domain which contains the distinct configurations for zinc-dependent metalloenzymes (115). While the zinc-binding motif within this catalytic domain is highly conserved between MMPs, C-terminal domains vary within different groups for specific functionalities (115). MMPs are involved in regulation of cell growth, apoptosis, angiogenesis, invascion, metastases, and adhesion.

MMPs are regulated by endogenous inhibitors, including tissue inhibitors of MMPs (TIMPs) (116, 117), α2-macroglobulin (117), and the glycoprotein reversion-inducing cysteine-rich protein with kazal motifs (RECK) (118). TIMPs are the principle inhibitors of MMPs (119). They are small, cysteine rich proteins which form high-affinity complexes with MMPs (115). TIMPs 1, 2, and 4 are secreted proteins, and are believed to have distinct physiologic roles (119). TIMPs have been shown to prevent endothelial cell tube formation, migration, and invasion *in vitro* (120-123).

MMP-2 and MMP-9 have been extensively studied in cancer. Both of these gelatinases are expressed in cancer cells and endothelial cells, and have been shown to be altered by hypoxia (124-128). Preclinical studies have shown MMPs to be good targets as potential anti-angiogenic or anti-metastatic therapies: MMP-2 deficient mice have greatly reduced tumor angiogenesis and growth (129), and MMP-9 deficiencies in premetastatic lung endothelial cells resulted in decreased metastases (130). However, clinical studies for MMP inhibitors (MMPIs) have not shown significant improvements in patient outcome (131-134). This disconnect between preclinical work and clinical studies is not understood, and continued work on this promising target is needed.

3.1.4. Metabolism

3.1.4.1. Targeting GLUT1

GLUT1 is a member of the GLUT family of glucose transporters which transports glucose into cells through facilitated diffusion along its concentration gradient (135, 136). GLUT1 consists of several amphiphatic helices clustered together in the cell membrane creating a barrel-like structure containing an aqueous pore (135). The "altering confirmation" model for glucose transport suggests that substrate binding sites are alternately exposed at different sides of the membrane through conformational changesin the transporter. The glucose molecule is proposed to hydrogen-bond to polar amino acid side-chains comprising the wall of the aqueous pore (135).

GLUT1 is the most widely conserved isoform across species and is the most widely expressed GLUT transporter among different tissues, including tumors (135, 136). GLUT1 levels have been shown to be increased in multiple different tumor types (137-145), consistent with an increased energy requirement in tumors cells, which often proliferate rapidly. Increased expression of GLUT1 in tumors has been shown to correlate with decreased patient survival in several different human tumors (137, 143, 146, 147).

GLUT1 expression has been shown to be increased by hypoxia (135, 148) through a HIF-1 dependent pathway. This increased expression of GLUT1 in hypoxic tumor cells (137) may provide a specific target for cancer therapy; blocking glucose transport could kill hypoxic tumor cells which are more dependent on glucose for their energy production (149, 150).

One approach to targeting GLUT1 could be to block the GLUT1 transporter One phytoestogen, genistein, directly interacts with GLUT1, inhibiting glucose uptake in cells in a dose-dependent manner (151). Other Tyrosine kinase inhibitors can also compete with the ATP binding site of tyrosine kinase toinhibit GLUT1 (152). These inhibitors work in a competitive, dose-dependent manner (152).

Although inhibiting glucose transport into tumor cells by blocking GLUT1 could enhance tumor cell kill, it could also have adverse effects in normal cells such as endothelial cells and red blood cells, which showhigh expression of GLUT1 (135). Blocking GLUT1 could have harmful systemic effects, as neurons and red blood cells are dependent on glucose for energy production under normal conditions. Despite these potential problems, blocking GLUT1 remains a promising method to specifically kill hypoxic tumor cells.

3.2. Non-HIF regulated hypoxic targets

While the HIF family of proteins dominates the cellular response to hypoxia, there are other pathways which also response to low pO_2 in the cell.

3.2.1. Targeting AP-1

Activator protein 1 (AP-1) transcription factor is involved in a diverse variety of cellular functions including apoptosis, proliferation, differentiation, invasion, and metastases (153). Hypoxia has been shown to activate AP-1, and, although the mechanism of oxygen-sensing is not well-known, it is generally believed to be indirect (154-159). ROS may also alter AP-1 activation as it is known to be a redox sensitive transcription factor (160, 161).

The AP-1 transcription factor is a dimer composed of members of the JUN, FOS, activating transcription factor (ATF), and musculoaponeurotic fibrosarcoma (MAF) protein families. Ap-1 proteins dimerize through a leucine-zipper motif which recognizes different promoters of target genes. The main DNA response element is the 12-O-tetradecanoylphorbol-13-acetate (TPA) responsive element (TRE) (162), but different dimers also bind to other response elements (163, 164).

Elevated AP-1 activity has been detected in several tumors (165, 166) and activation of AP-1 is required for tumor promotion (167). Additionally, dominant negative inhibitors of AP-1 blocked tumor promotion in a pre-clinical model (168). While no small molecule inhibitors of AP-1 have been identified, AP-1 presents an excellent target for the development of these inhibitors.

3.2.2. Targeting NF-κB

Proteins in the NF-κB mammalian family share a highly conserved homology domain which plays an important role in dimerization and DNA binding. This domain is also responsible for the interactions of NF-κB with its inhibitor IκBα (169, 170). In resting cells NF-κB is bound to IκBα, concealing its nuclear localization sequence and forcing NF-κB to remain in the cytoplasm (169, 170). Cellular stimulation results in phosphorylation and subsequent ubiquitination and degredation of IκBα; NF-κB enters the nucleus and is further regulated by phosphorylation of its p65 subunit under certain cellular stimuli (169, 170). All proteins in the NF-κB family have been shown to bind to a common κB binding motif of specific genes, and some dimers also recognize slightly altered κB motifs (169, 170).

Hypoxia has been shown to activate nuclear factor kappa-B (NF-κB) through a variety of mechanisms.

Although the exact signaling pathways are still ambiguous, they are believed to include phosphorylation of IκBα through the Ras/Raf pathways (171, 172), signaling through the p42/p44 MAPK and PIK3 pathways (173), or reactive oxygen species (ROS) generation (174). NF-κB activation has been linked to cell adhesion, apoptosis, and survival.

The inhibition of NF-κB by IκBα offers a clear target for therapy. Several approaches toward this goal have been attempted: upstream blocking of signaling resulting in IκBα phosphorylation (175), inhibition of IκBα degredation (176, 177), and enhanced synthesis of IκBα (178-180). While some of these efforts have been successful, none of these mechanisms of IκBα potentiation are specific for NF-κB and could result in disregulation of other important cellular functions.

Another promising approach for targeting NF-κB has been to competitively inhibit binding of NF-κB to its κB motif with specially designed oligonucleotides or transcription factor decoys. While this strategy has not yet been examined in tumor cells, the use of a transcription factor decoy significantly decreased downstream effects of NF-κB for 72 hours in murine B cells (181), suggesting this method could have significant anti-tumor effects in cancer.

3.3. Other targets

3.3.1. p53

An essential physiological mechanism which cells use to guard themselves against the consequences of cellular stress is apoptosis, or programmed cell death. An important regulator of apoptosis is the p53 gene, which plays a key role in mediating apoptosis in normal and damaged cells.

p53 may be the most complex target in cancer therapy due to the many roles it plays in the tumor cell. Depending on its location within a cell, p53 is known to affect transcriptional activity, DNA repair, mitochondrial membrane permeability, and exonuclease activity possibly leading to apoptosis, senescence, cell-cycle arrest, or differentiation (182-187). Combined, these potential functions in tumor cells have ensured that p53 is a well-studied, hotly debated protein. For the purposes of this review, p53 will be examined as a transcriptional activator or suppressor.

The p53 protein consists of four identical subunits, each consisting of five well-characterized domains. Three of these domains, the N-terminal transactivation (TA) domain, the highly conserved core DNA-binding domain (DBD), and the C-terminal domain (CTD), are known to be integral to the efficacy of p53 (188). Further complexity is added to the function of this protein by the many post-translational modifications it can undergo, which include phosphorylation, acetylation, ubiquitination, neddylation, and sumoylation (184).

In the nucleus, p53 binds to its recognition elements (RE) near its target genes. The DNA-binding domain has high affinity for a consensus RE, which is attributed to the interactions of the pentamers in the consensus RE with the p53 tetramer (189, 190). Alternatively, the C-terminal domain is known to bind to nonspecific DNA sequences, although its binding behavior is heavily influenced by post-translational modifications (189, 190). Less is known about the TA domain binding, although its secondary structure has been shown to have conserved regions which are believed to act as recognition sites for p53 interacting proteins (189). Despite their differences in binding domains, several studies have shown that mutations in either the DNA-binding domain or C-terminal domain can cause significant alterations in p53 transcriptional activity (191).

p53 is known to be negatively regulated in the nucleus by direct interaction with the MDM2 protein through two different mechanisms. In a mechanism of regulation reminiscent of HIF-1α and VHL, a ring finger in MDM2 contains an E3 ubiquitin ligase for p53, leading to its degradation and maintaining low levels of p53 in unstressed cells (192). In response to various types of cell stress, p53 is stabilized, and a second level of regulation is triggered. High levels of nuclear p53 activate transcription of the *mdm2* gene. MDM2 interacts with p53 through a binding pocket which attaches to the N-terminal TA domain of p53, inhibiting the ability of p53 to activate transcription (193). A recent study has also shown a potential third binding site between MDM2 and p53 within the DBD, suggesting that two different p53 molvecules within its tetramer may bind to MDM2, further strengthening the binding between these two proteins (194).

3.3.1.1. Targeting p53

More than half of human tumors are believed to have mutations which significantly alter the function of p53 (195), making it a therapeutic target with tremendous potential. Mutant p53 is also known to upregulate or downregulate several genes which promote treatment resistance. Almost all tumor-derived p53 mutations contain a mutation which alters the DNA binding sites, often inactivating the transcriptional ability of p53 and leading to a decrease in apoptotic function. Hypoxia has been shown to select for cells with mutant p53 (196, 197), showing decreased levels of apoptosis in response to traditional therapies (198-200). Thus two main approaches to targeting p53 have emerged: increasing wild-type p53 levels to stimulate apoptosis and returning normal p53 activity to cells which have lost this phenotype.

A strategy for potentiation of p53 which has recently been successfully implemented preclinically is blocking the interaction between p53 and MDM2 to stop the degradation of p53. Nutlins, specific small molecule agonists of MDM2, have been shown increase expression of p53 upregulated genes and to greatly inhibit tumor growth in a murine model (201). This strategy has the potential for additional efficacy as agonists of MDM2 have also been shown to enhance the effects of chemotherapy (202).

The use of gene therapy is one strategy for the reintroduction of wild-type p53 into cells. Preclinical

studies have successfully shown the ability to introduce the p53 gene into tumor cells. Phase I and Phase II trials have been conducted using a replication incompetent adenovirus delivering p53 expression in combination with chemotherapy or radiation in non-small cell lung cancer, with more than half of the patients achieving a complete or partial response (203-205). Biopsies taken after treatment showed a significant increase in p53 regulated gene expression compared to pretreatment biopsies, suggesting some gain of function of p53 from the gene therapy treatments (203-205).

The creation and use of synthetic peptides derived from the p53 CTD has also been shown to induce p53-dependent apoptosis in tumor cells. In one pre-clinical study, a small molecule, PRIMA-1 (206), was shown to restore this function and had a significant anti-tumor effect in a human osteosarcoma (207). Further studies on PRIMA-1 showed a synergistic effect on tumor cell colony formation when used in combination with Cisplatin, and an increased effect on tumor volume in lung adenocarcinoma xenografts (207).

However, the many pathways in which p53 is involved also make it a tremendously difficult therapeutic target. Although targeting the MDM2/p53 pathway would seem to be a highly desired therapeutic target, MDM2 is also involves in nuclear export of p53, which is integral for apoptotic p53 activity in the mitochondria. This suggests that complete inhibition of MDM2 binding to p53 could decrease the apoptotic potential in some cancer cells. Recalling that only the role of p53 as a transcription factor has been examined here, careful deliberation of the impact on all of the activities of p53 should be conducted before attempting to target this multifunctional protein.

4. OPTIMIZING THE BENEFITS OF TARGETED THERAPIES

Although scientists and clinicians have embraced the concept of targeting downstream effects of hypoxia as a method to improving tumor therapy, clinical trials using angiogenesis inhibitors have shown these inhibitors to be ineffective as monotherapies, with a less than 4% overall response rate (95).

In combination with traditional therapies, however, inhibitors of the downstream targets presented above have been shown to have greater anti-tumor effects than either single mono-therapy. Additionally, preclinical studies with multiple downstream targets have had greater success than those with only one. This strongly suggests that multi-targeting of molecular determinants of the tumor microenvironment in combination with traditional therapies is the best approach for increased anti-tumor effect.

The potential benefits of multi-targeting are excellently highlighted in a paper by Cao et al. (208). In this paper human colon carcinoma and murine mammary carcinoma cells were grown in dorsal window chambers. Incipient angiogenesis preceded HIF-1 mediated angiogenesis, suggesting that strategies targeting HIF-1 alone would not prevent tumor angiogenesis. This strongly suggests the use of other anti-angiogenic targeting in combination with HIF-1 targeting.

Preclinical studies have suggested that the scheduling of the therapies may have a significant effect on the efficacy of the combined therapies. One traditional therapy which has been examined in combination with numerous molecular inhibitors is ionizing radiation. In experimental models, anti-VEGF compounds have consistently had a greater anti-tumor effect than radiation alone, independent of scheduling of the therapies.

The scheduling of radiation in combination with inhibiting any one molecular target is an unresolved topic, despite numerous studies in the area. Studies have produced conflicting reports on the importance of scheduling, with some studies showing no effect due to different dosing regimens and some studies showing a significant effect due to different dosing regimens. These results are further complicated by different doses of radiation, different drugs targeting a single molecular agent, different tumor lines, and different study end-points (209).

At least two groups have made thoughtful, well-supported arguments vis-à-vis scheduling of targeting the molecular effects of hypoxia and radiation (Figure 5).

Using a VEGFR2 inhibitor given three times over one week, Winkler et al. have shown that by day 4 the phenotype of tumor vessels is more like the phenotype of normal cells, a process named “vascular normalization” (210). Vascular normalization is a process in response to the VEGFR2 inhibitor used which causes a temporary improvement in solute transport, leading to an increase in oxygenation, radiosensitivity and drug penetration In this scenario blocking VEGFR2 resulted in less tortuous, more uniform tumor vasculature for a limited time period occurring several days after beginning administration of the inhibitor. This normalized vasculature could be taken advantage of by scheduling oxygen-dependent therapies such as radiation during this normalization window.

Moeller et al. have shown that the HIF-1 pathway has significant effects on vascular and tumor cell radiosensitivity. In two tumor cell lines, radiation was shown to upregulate HIF-1 and its downstream targets (211). Irradiated tumor cells also produced cytokines, such as VEGF, through a HIF-1 dependent pathway that were radioprotective for endothelial cells. Blocking HIF-1 in combination with radiation was shown to have a significantly larger effect than radiation or anti-HIF-1 therapy alone. This data suggested that blocking HIF-1 in tumor cells before they can produce cytokines that decrease endothelial cell radioresponsiveness would be an optimal scheduling regimen.

A follow-up study by Moeller et al. further examined the relationship between HIF-1 and radiation. In this study, the tumor microenvironment was shown to influence the effects of HIF-1 and tumor cell radiosensivity (212). Low glucose combined with hypoxia showed HIF-

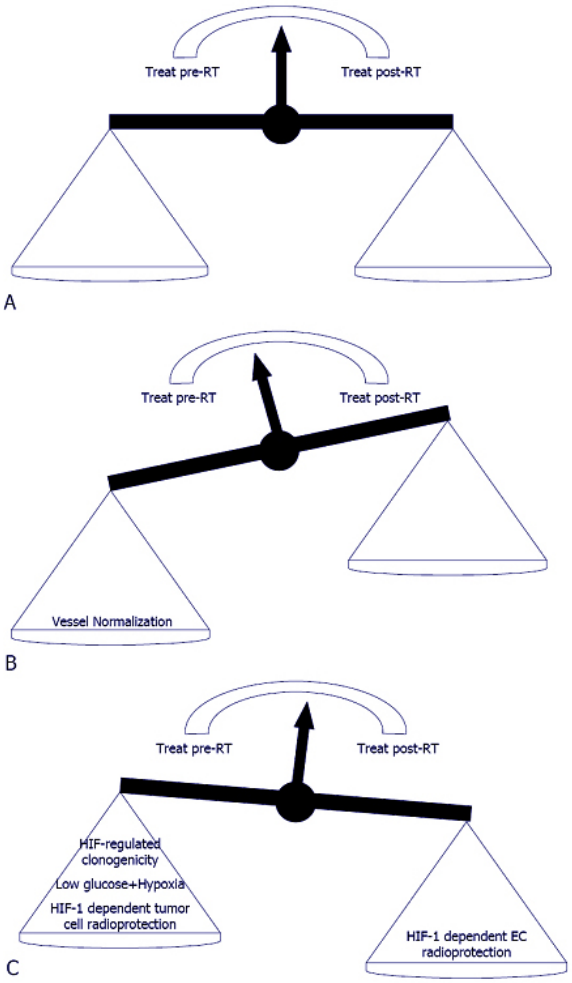

Figure 5. Optimizing RT with modification of the downstream effects of hypoxia. In A) no treatment other than RT is given so no protocol optimization is necessary. In B) a VEGF/VEGFR blocking agent is given, leading to a window of vessel "normalization;" to take advantage of this normalization, RT is scheduled several days after administration of the VEGF/VEGFR blocking agent. In C) blocking the effects of HIF on clonogenicity, metabolism, and endothelial cells are balanced; although there are both pro- and anti-tumor effects due to blocking HIF in combination with RT, the optimal protocol suggests blocking HIF immediately after RT.

1-dependent ATP production and increased HIF-1-dependent proliferation; blocking HIF-1 under hypoxia resulted in increased tumor cell clonogenicity. This data presents conflicting potential pro- and anti-tumor effects of a HIF-1 blockade, and muddies the role for HIF-1 inhibitors and inhibitors of downstream targets in combination with radiation. In the two tumor lines examined, the balance for targeting the pro- and anti-tumor effects of HIF-1 due to hypoxia and radiation seems to be found by blocking HIF-1 immediately after irradiating the tumors. This sequence of therapy led to a significant tumor growth delay in both cell lines. Interestingly, the two tumor lines showed differences in their relative tumor growth delay from scheduling the HIF-1 blocking pre- or post-irradiation; this is consistent with the theory that the tumor lines will have different tumor microenvironments and thus a different balance of pro- and anti-tumor effects due to HIF-1 (213).

While Winkler et al. finds an optimal therapy to maximize the effects of an anti-VEGFR2 therapy, the studies only examine the effects of vessel normalization and its subsequent effects on the tumor microenvironment. However, since only some of the environmental effects of hypoxia are caused by tortuous vasculature, this strategy can only, at best, mitigate those effects. A more comprehensive, although not all-inclusive, strategy to optimizing anti-HIF-1 treatments with radiation is the one developed by Moeller et al.; in this strategy the balance of the contradictory effects of HIF-1 on apoptosis, glucose metabolism, proliferation, and radioresistance are balanced. However, this tactic of broadly approaching the effects of modifying the downstream effects of hypoxia does not provide the scientific community with a definitive solution; it merely provides an excellent framework for future studies to examine the pleiotropic effects of altering downstream effects of hypoxia and their interaction with traditional therapies.

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