Review Article

Tumor hypoxia: A target for selective cancer therapy

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Tumor hypoxia has been considered to be a potential therapeutic problem because it renders solid tumors more resistant to sparsely ionizing radiation (IR) and chemotherapeutic drugs. Moreover, recent laboratory and clinical data have shown that tumor hypoxia is also associated with a more malignant phenotype and poor survival in patients suffering from various solid tumors. Therefore, selective targeting of hypoxic tumor cells has been explored, and since severe hypoxia (pO_2<0.33%, 2.5 mmHg) does not occur in normal tissue, tumor hypoxia could be exploited for therapeutic advantage. However, the following three characteristics of hypoxic tumor regions present obstacles in targeting hypoxic cells. First, it is difficult to deliver a sufficient amount of drug to a region that is remote from blood vessels. Second, one must specifically target hypoxic tumor cells while sparing normal well-oxygenated tissue from damage. Finally, the severely hypoxic tumor cells to be attacked have often stopped dividing. Therefore, high delivery efficiency, high specificity and selective cytotoxicity are all necessary to target and combat hypoxic tumor cells. The current review describes progress on the biological aspects of tumor hypoxia and provides a compilation of the recent molecular approaches used to target hypoxic tumors. These approaches include our work with a unique hypoxia-targeting protein drug, TOP3, with which we have sought to address the above three difficulties. (Cancer Sci 2003; 94: 1021–1028)

In solid tumors, oxygen delivery to the respiring neoplastic and stromal cells is frequently reduced or even abolished by deteriorating diffusion geometry, severe structural abnormalities of tumor microvessels, and damaged microcirculation.[1)] It has been known for some time that tumor cells can survive and even grow in such a deteriorated microenvironment. Moreover, as described in detail below, tumor hypoxia has been shown to be a cause of malignant transformation and a source of resistance to current cancer therapies. For these reasons, approaches to targeting hypoxic tumor cells have been sought over a period of years. Recent progress in understanding the molecular mechanisms of hypoxic response, and especially the discovery of a key molecule, Hypoxia Inducible Factor (HIF), as well as improvements in diagnostic techniques through which the tumor microenvironment can be assessed, has opened the door to a solution of this longstanding issue.

1. Biological characteristics of hypoxic tumor cells

Unlike in normal tissues, the vasculature is often inadequate in a solid tumor mass. Thus, solid tumors are highly heterogeneous, and often exhibit low oxygen tension, low pH, and low glucose concentration. Such a microenvironment induces a variety of biological changes in tumor cells, including metabolic changes and changes in gene expression (Fig. 1).

Two types of hypoxia have been recognized: chronic or diffusion-limited hypoxia and acute or perfusion-limited hypoxia (reviewed in ref. 2). Chronically hypoxic cells are usually situated remotely from capillaries (Fig. 1), whereas acutely hypoxic cells are found adjacent to capillaries. Chronically hypoxic cells in a growing tumor have been observed to survive and to continue to proliferate, in contrast to those in perfusion-limited areas, and this may cause cellular changes that can result in a more clinically aggressive phenotype. Acute hypoxia occurs when aberrant blood vessels are shut down. The closed vessels can be subsequently reopened, leading to reperfusion of hypoxic tissue with oxygenated blood. This leads to an increase in free-radical concentrations, tissue damage and activation of stress-response genes.

1.1 Oxygen levels in tissues and tumors

Oxygen levels are typically very heterogeneous, both among patients and within individual tumors. The oxygenation status has primarily been measured using either polarographic oxygen electrodes or biochemical techniques that rely upon antibody detection of nitroimidazole-based adducts in hypoxic tissue (pimonidazole, EF5, EF1). By using oxygen electrodes, Vaupel and his colleagues have convincingly demonstrated that human solid tumors contain regions with significantly lower oxygen tension than that found in normal tissue.[3)] Unlike in normal organs, tissue oxygenation in human primary tumors is not regulated only by metabolic demand, and the pO_2 of the tissue is often markedly low and heterogeneous. It is also linked to the tumor size, and a tumor with a volume of more than 2 cm^3 typically has an extremely hypoxic region. Direct measurements of oxygen tensions in human tumors show a range of median oxygen tensions from 1.3–3.9% (10–30 mmHg), with readings recorded as low as 0.01% (0.08 mmHg), whereas concentrations in normal tissues are significantly higher; 3.1–8.7% O_2 (24–66 mmHg). More importantly, 82% of all readings taken in solid tumors were less than 0.33% O_2 (2.5 mmHg).[3)]

1.2 Metabolic parameters

Low oxygen tension in tumors is frequently associated with low extracellular pH, low glucose concentration, and high lactate concentration. Although limited data exist, electrode-measured pH values of human tumors and adjacent normal tissues consistently show that the pH is substantially and consistently lower in the tumor than in normal tissue. The reader is referred to other review articles for detailed discussions of this issue.[1, 2)]

1.3 Glycolysis and glucose transport

Under hypoxic conditions, cells switch their glucose metabolism pathway from the oxygen-dependent tricarboxylic acid (TCA) cycle to glycolysis, which is an oxygen-independent metabolic pathway. Hypoxic cancer cells use glycolysis as a primary mechanism of ATP production, and cellular transformation has been associated with induction of glycolysis. The

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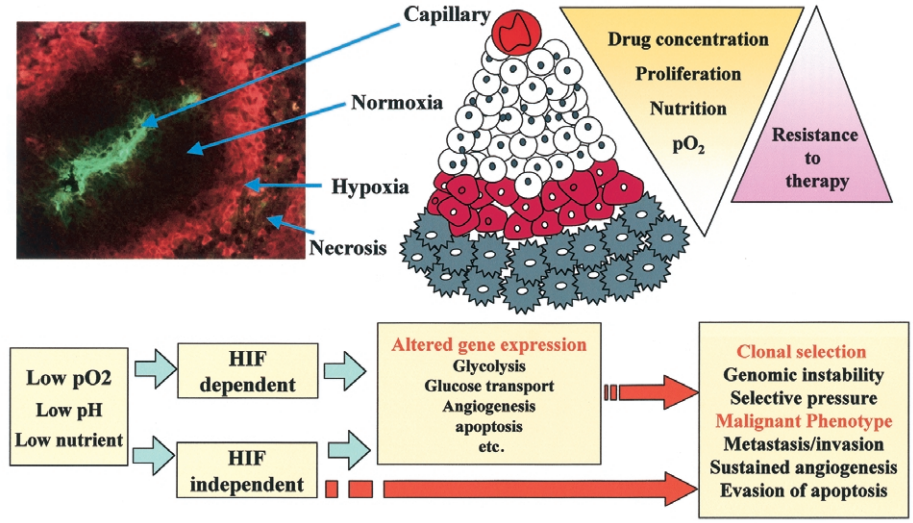

Fig. 1. Hypoxia in solid tumors. The photograph shows a section of experimental human colon tumor (HCT116) treated with an antibody against CD31 (green), an endothelial cell marker, and with pimonidazole (red), a hypoxic marker, and detected with secondary antibodies conjugated to fluorescent proteins giving the corresponding color. The cartoon on the right shows part of a tumor cord surrounding a capillary. With increasing distance from the capillary, cell proliferation, the oxygen and drug concentrations, and nutrition all decrease, while resistance to radiotherapy and chemotherapy increases. The lower figure shows a schematic representation of the paramount importance of hypoxia in the malignant progression of solid tumors. See text for details.

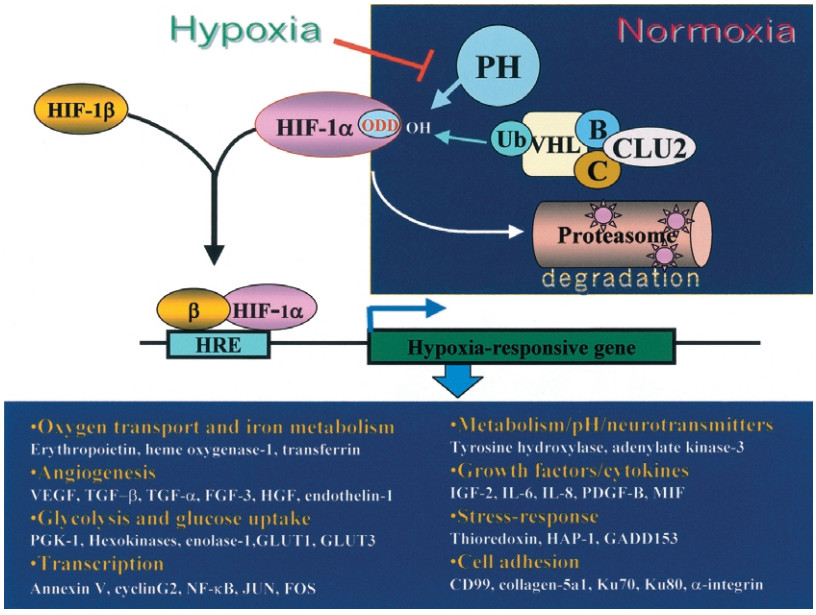

Fig. 2. Oxygen-dependent regulation of HIF-1α protein stability. In the presence of oxygen (normoxia), prolyl hydroxylase (PH) hydroxylates proline residues on HIF-1α, allowing it to interact with an ubiquitin-protein ligase complex (VHL, CLU2, elongin-B and -C) through VHL. Ubiquitination of HIF-1α targets it for degradation by the 26S proteasome. Under hypoxic conditions, binding of VHL to HIF-1α is inhibited, resulting in the accumulation of HIF-1α and its dimerization with HIF-1β. The heterodimer then translocates to the nucleus and binds to HRE elements in the promoter region of genes, inducing the expression of various hypoxia-responsive genes.

perceived glycolytic character of cancer cells is being increasingly exploited by the use of glucose-based positron emission tomography (PET) in detection and diagnosis. This approach is based upon the assumption that tumors take up glucose more rapidly than surrounding normal tissue. The expression levels of genes associated with glycolysis and glucose transport are significantly elevated by hypoxia, as will be discussed below.

Recently, Lu *et al.*[4] reported a novel role for pyruvate in metabolic signaling and suggested a mechanism through which high rates of aerobic glycolysis can promote malignant transformation and survival of cancer cells. Hence, lactate and pyruvate regulate hypoxia-inducible gene expression independently of hypoxia by stimulating the accumulation of HIF-1α, a key regulator of hypoxia-responsive gene expression.

1.4 Apoptosis

Recent evidence indicates that hypoxia induces cell death through apoptosis. Activation of caspase-3, an executioner caspase, under hypoxic conditions has been reported in several cell types, including ventricular myocytes, endothelial cells and squamous cell lung carcinomas (reviewed in ref. 5). Apoptotic signaling under hypoxic conditions has been shown to occur through the release of cytochrome *c* and Apaf-1-mediated caspase-9 activation.[6] The upstream regulators of cytochrome *c* release are members of the Bcl-2 family, and pro-apoptotic Bcl-2 family members are clearly required to initiate cytochrome *c*/Apaf-1/caspase-9-mediated cell death during oxygen deprivation. How oxygen deprivation is coupled to activation of pro-apoptotic Bcl-2 family members remains unknown. Potential regulators of the Bcl-2 family members, such as Akt, p53 or HIF-1, might influence oxygen deprivation-induced apoptosis in a particularly cell-specific manner.

A number of studies have suggested that oxygen deprivation-induced apoptosis is transcriptionally regulated (reviewed in ref. 5), and the transcription factor p53 has been implicated in regulating hypoxia-induced apoptosis through induction of apoptosis-related expression of genes such as *Bax*, *NOXA*, *PUMA* and *PERP*.[7]

2. Hypoxia-induced changes in gene expression

The biological parameters of hypoxic tumor cells are closely related to altered gene expression under conditions of oxygen deficiency. Hypoxia-inducible transcription factor 1 (HIF-1) is

a master transcription factor that plays a central role in hypoxic expression of a variety of genes.[8] Therefore, the mechanisms that regulate HIF-1 are mostly responsible for the altered gene expression.

2.1 HIF-1

HIF-1 is a heterodimeric basic HLH-PAS protein that consists of α and β subunits.[8] Although HIF-1β (ARNT) is constitutively expressed, HIF-1α is precisely regulated by cellular oxygen levels. Under hypoxic conditions, HIF-1α is induced, dimerizes with a β subunit, translocates to the nucleus, and initiates gene transcription (Fig. 2). More than 40 genes have been reported to be induced by HIF-1 under hypoxic conditions (reviewed in ref. 3). For example, HIF-1 activates the transcription of glycolytic enzymes such as aldolases A and C, enolase 1, hexokinases 1 and 3, lactate dehydrogenase A, phosphofructokinase L and phosphoglycerate kinase (PGK) 1; glucose transporters such as glucose transporters 1 and 3 (GLUT1 and GLUT3); angiogenic molecules such as vascular endothelial growth factor (VEGF) and angiogenin; survival and growth factors such as platelet-derived growth factor-B (PDGF-B), transforming growth factor-β (TGF-β) and insulin-like growth factor-II (IGF-II); and enzymes and proteins involved in tumor invasiveness and metastasis, such as urokinase-type plasminogen activator, chaperones and other apoptosis resistance-related proteins.

2.2 Regulation of HIF-1α expression

The oxygen-dependent regulation of HIF-1α occurs at the level of protein degradation (Fig. 2). Under hypoxic conditions, stabilization of HIF-1α is negatively regulated by a series of oxygen-dependent posttranslational modifications, which are mediated by three prolyl hydroxylases[9, 10] and one or more asparaginyl hydroxylases.[11] A conserved proline residue, proline 564, is hydroxylated under normoxic conditions, allowing the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex to bind to HIF-1α.[9, 10] The VHL complex adds ubiquitin to HIF-1α, which is then degraded by proteasomes. Under hypoxic conditions, oxygen becomes rate-limiting for prolyl hydroxylation, resulting in decreased ubiquitination of HIF-1α (Fig. 2).

In addition, oxygen-dependent hydroxylation of Asn803 has also been shown to inhibit the interaction of HIF-1α with the co-activator p300.[11] The ability of HIF-1α to activate transcription is also oxygen-regulated, as a result of the cooperative binding of VHL and the co-repressor FIH-1 (factor inhibiting HIF-1), which recruits histone deacetylase to HIF-1α under normoxic conditions.[12]

HIF-1α expression is also regulated at the translational level and is maintained at low levels in most cells under normoxic conditions. Growth factor stimulation induces HIF-1α protein synthesis via a signal transduction pathway leading from receptor tyrosine kinases (RTKs) such as HER2 to phosphatidylinositol-3-kinase (PI3K) to the serine/threonine kinase Akt (protein kinase B) and FRAP (FKBP-rapamycin associated protein).[13]

2.3 p53

The transcription factor p53 lies at the center of a protein network that controls cell cycle progression and commitment to apoptosis. p53 is inactive in proliferating cells, largely because of negative regulation by the Mdm2 oncoprotein, with which it physically associates. Release from this negative regulation is sufficient to activate p53 and can be triggered in cells by multiple stimuli through diverse pathways. This diversity is achieved in part because Mdm2 uses multiple mechanisms to inactivate p53: it targets p53 for ubiquitination and degradation by proteosomes, shuttles it out of the nucleus and into the cytoplasm, prevents its interaction with transcriptional coactivators, and possesses an intrinsic transcriptional repressor activity.[14]

Homozygous deletion of p53 promotes neovascularization and growth of tumor xenografts in nude mice. Because p53 promotes Mdm2-mediated ubiquitination and proteasomal degradation of HIF-1α, loss of p53 in tumor cells enhances HIF-1α levels and augments HIF-1-dependent transcriptional activation of the *VEGF* gene, which is a master gene for malignant progression in response to hypoxia.[15]

Mirnezami *et al*.[16] have shown that Mdm2 can repress p53 activity through the recruitment of a transcriptional co-repressor, hCtBP2. This interaction, and the consequent repression of p53-dependent transcription, is relieved under hypoxic or hypoxia-mimicking conditions that are known to increase levels of intracellular NADH. CtBP proteins can undergo an NADH-induced conformational change, which results in a loss of their binding affinity for Mdm2.

2.4 Other transcription factors

Although most hypoxia-responsive genes are induced by HIF-1, several genes have been reported to be controlled by other transcription factors. Induction of inhibitor of apoptosis protein-2 (IAP-2) by severe hypoxia is regulated at the transcriptional level through cAMP-response-element-binding protein (CREB) sites in the promoter/enhancer sequences.[17] Cyclooxygenase-2 is induced by hypoxia via the NF-κB p65 transcription factor,[18] while placental growth factor (PlGF), a ligand for VEGFR1, and metallothionein[19] are induced by another hypoxia-activated transcription factor, metal-transcription factor-1. The *early growth response-1* gene is upregulated by hypoxia and induces cell-surface protein tissue factor gene expression, leading to vascular fibrin deposition and blood clot formation.[20]

3. Rationale for hypoxia targeting

3.1 Treatment resistance associated with tumor hypoxia

For many years, the importance of hypoxia in solid tumors was linked solely to the fact that hypoxic cells are intrinsically more resistant to treatment. A markedly lower intratumoral oxygen level is a primary problem because ionizing radiation (IR) and certain drugs, such as bleomycin, require oxygen-derived free radicals to destroy target cells. In addition, chemotherapeutic drug resistance can be caused by hypoxia-induced inhibition of cell cycle progression and proliferation, since a number of drugs specifically target highly proliferating cells. Proliferation decreases as a result of decreasing oxygen levels,[21] and it has been shown that drug toxicity falls off as a function of distance from blood vessels (Fig. 1). Furthermore, inefficient drug delivery to hypoxic regions is a fundamental problem. Because the blood flow to hypoxic tumor cells is very low, drugs are inefficiently delivered and the drug concentration never reaches an effective level.[22] Putting all the above problems together, it is apparent that high delivery efficiency, high specificity and selective cytotoxity are necessary for hypoxia-targeted tumor therapy.

3.2 Hypoxia-mediated malignant progression

Chronically hypoxic tumor cells undergo genetic and adaptive changes that allow them to survive and even proliferate in a hypoxic environment. The microenvironment described above exerts a strong selection pressure and promotes genomic instability, which may increase the number of genetic variants. These processes contribute to the malignant phenotype and to aggressive tumor behavior, including metastasis, sustained angiogenesis, and evasion of apoptosis.

3.2.1 Metastasis and invasion: Vaupel and his colleagues have shown that low oxygen tension in tumors is associated with increased metastasis and poor survival in patients suffering from squamous tumors of the head and neck, and from cervical or breast cancers.[3, 23] The initiation of metastasis is a multi-step pathway that involves three major processes: degradation of the basement membrane and extracellular matrix (ECM), modulation of cell adhesion molecules, and cell migration.[23] Hypoxia induces expression of gene products involved in the matrix degradation step, such as metalloproteinases and uPAR.[24] Cad-

herins involved in cell-cell adhesion are downregulated by hypoxia,[25] while autocrine motility factor (AMF)[26] is induced, thus enabling the tumor cells to move about. Taken together, these factors suggest that hypoxia could play a key role in facilitating tumor invasion and metastasis in a comprehensive manner.

Recently, Pennacchietti *et al.*[27] demonstrated that Met tyrosine kinase (Met) is induced under hypoxic conditions both *in vitro* and *in vivo*. Met, a high affinity receptor for hepatocyte growth factor (HGF), plays a crucial role in controlling invasive growth and is often overexpressed in cancer. Pennacchietti *et al.*[27] also showed that Met upregulation by hypoxia results in increased sensitivity to HGF stimulation, and that HGF and hypoxia have a synergistic effect in inducing invasive growth. Based on these results, they suggested that hypoxia may promote tumor invasion by sensitizing cells to HGF stimulation.

3.2.2 Sustained angiogenesis: The switch to an angiogenic phenotype is a fundamental determinant of neoplastic growth and tumor progression. The potent angiogenic factor, VEGF, is promptly induced in both tumor and stromal cells under hypoxic conditions.[15] Since the VEGF promoter has a typical hypoxia response element (HRE),[28] which is a binding site for HIF-1, amplification of HIF-1-dependent responses to hypoxia with or without loss of p53 function contributes to the angiogenic switch during tumorigenesis. HIF-1 also positively regulates other angiogenic factors, such as adrenomedullin and IL-8.[26] Tumor-associated macrophages (TAM) that accumulate in the tumor hypoxic region may also be involved in tumor angiogenesis through the secretion of angiogenic factors.[29] Thus, hypoxia plays a major role in promoting tumor angiogenesis. Conversely, it should be noted that the angiogenesis inhibitors that are currently being tested in clinical trials could expand the hypoxic region by blocking newly formed blood vessels. A tumor in which the cells are already resistant to hypoxia would not respond to angiogenesis inhibitors, and moreover, prolonged administration of such inhibitors might promote malignant conversion through hypoxic stress, as described above.

3.2.3 Evasion of apoptosis: The increased malignancy of hypoxic tumors has been attributed to the ability of hypoxia to select for cells that are more resistant to apoptosis. The importance of hypoxia-mediated clonal expansion of tumor cells with diminished apoptotic potential has been demonstrated both experimentally and clinically. Graeber *et al.*[30] used embryonic fibroblasts derived from wt and p53-deficient mice to investigate the role of p53 in hypoxia-induced apoptosis and showed that oncogenic transformation predisposed cells to hypoxia-induced killing through an apoptotic pathway modulated by p53. Based on the observation that in a mixture of transformed $p53^{-/-}$ and $p53^{+/+}$ cells in a 1 to 1000 ratio, more $p53^{-/-}$ cells than $p53^{+/+}$ cells were present after multiple rounds of hypoxia and aerobic recovery, they concluded that hypoxia could also select for apoptosis-resistant cells. Recent clinical results showing that hypoxic cervical cancers with a low apoptotic index are highly aggressive strongly support this basic experimental concept.[23] Dong *et al.*[17] have also recently reported that IAP-2 induction plays an important role in apoptosis resistance of hypoxic cells at the level of caspase activation.

4. Hypoxia-targeted therapy

There are two classical approaches to hypoxia-targeted tumor therapy. One involves improving oxygenation of the hypoxic region of solid tumors and combining this with radiotherapy or chemotherapy. Examples of such an approach include combination therapy using erythropoietin (EPO) and chemotherapy, and accelerated radiotherapy in combination with carbogen and nicotinamide (ARCON), radiosensitizers such as KU-2285, nimorazole, sanazole, TX-1845, TX-1846 and so on.[31, 32] The other approach to hypoxia-targeted tumor therapy involves exploiting the microenvironment of hypoxic tumor cells and has been extensively studied and reviewed in detail.[33, 34] We describe representative examples of this approach in the following sections.

4.1 HIF-1 targeting

The importance of HIF-1 as a transcription factor suggests that it could be a novel, tumor-specific target for anticancer therapy. Chen *et al.*[35] have demonstrated that dominant-negative HIF-1α rendered pancreatic cancer cells sensitive to apoptosis and growth inhibition induced by hypoxia and glucose deprivation, and reduced their tumorigenicity. The regulators of HIF-1α expression described above could also be tumor-specific targets (Fig. 3). For example, tyrosine kinase inhibitors (Herceptin, Iressa, herbimycin), a protein kinase C (PKC) inhibitor (calphostin C), PI3K inhibitors (wortmannin, LY294002), a MAP kinase inhibitor (PD98059), a FRAP/mTOR inhibitor (rapamycin), a redox signaling blocker (diphenylene iodonium) and a glucokinase inhibitor (mannoheptulose) are all able to block HIF-1α function (reviewed in ref. 2).

Recently, Mabjeesh *et al.*[36] identified 2-methoxyestradiol (2ME2) as a novel antitumor and antiangiogenic agent that inhibits tumor growth and angiogenesis at concentrations that efficiently disrupt tumor microtubules (MTs) *in vivo*. 2ME2 downregulates HIF-1 at the posttranscriptional level and inhibits HIF-1-induced transcriptional activation of VEGF expression. Inhibition of HIF-1 occurs downstream of the 2ME2/tubulin interaction, as disruption of interphase MTs is required for HIF-1α downregulation. These data establish 2ME2 as a small molecule inhibitor of HIF-1 and provide a mechanistic link between the disruption of the MT cytoskeleton and inhibition of angiogenesis.

4.2 Bioreductive drugs

Bioreductive drugs are compounds that are reduced by biological enzymes to toxic, active metabolites. They are designed

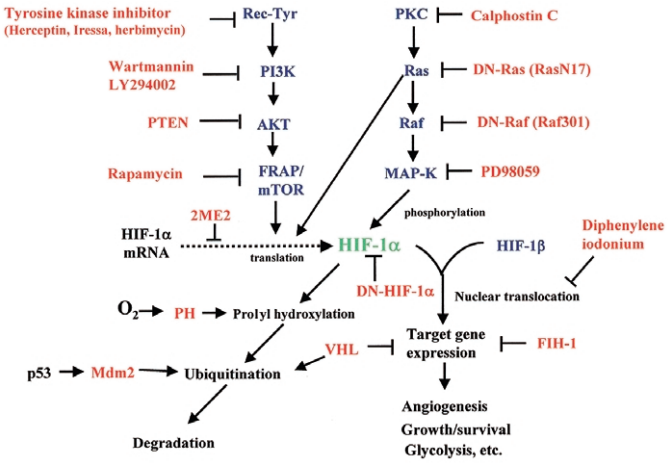

Fig. 3. Inhibitors of HIF-1α and its activation pathways. Activation of receptor tyrosine kinases (Rec-Tyr), such as HER2, IGF and EGF receptors, stimulates the PI3K-Akt-FRAP signal transduction pathway, which leads to increased translation of HIF-1α mRNA into protein. The PKC-RAS-RAF-MAPK signal transduction pathway also induces translation and activation of HIF-1α. Each component of these pathways (blue) can be inhibited by component-specific inhibitors (red). 2ME2 inhibits HIF-1α expression at the posttranscriptional level. Diphenylene iodonium abrogates IL-1β-dependent nuclear translocation and activation of HIF-1α. VHL and FIH-1 also recruit histone deacetylases, which repress the HIF-1α transactivation domain function. Binding of p53 to HIF-1α under hypoxic conditions causes recruitment of Mdm2, which targets HIF-1α for ubiquitination and degradation. Abbreviations: PTEN, phosphatase and tensin homolog deleted on chromosome 10; DN, dominant negative; FRAP, FKBP-rapamycin-associated protein; FKBP, FK-506-binding protein.

such that this metabolism occurs only or preferentially in the absence of oxygen. Tirapazamine (TPZ) is the leading compound in this class of agents. Under hypoxic conditions, TPZ is reduced to a radical that leads to DNA double-strand breaks (DSBs), single-strand breaks, and base damage. Recently, TPZ has been shown to be a hypoxia-selective topoisomerase II poison.[37] Another promising bioreductive drug is AQ4N, a prodrug that is activated by reduction in hypoxic cells, producing a stable product (AQ4) that intercalates with DNA and blocks topoisomerase II action.[38] AQ4N is not effective when given alone, but shows substantial antitumor activity when combined with methods to increase the hypoxic fraction, with radiation or with other anticancer drugs.

4.3 Gene therapy

To achieve high specificity to hypoxic tumor cells, many hypoxia-response promoters have been constructed to express therapeutic genes. Most of the known hypoxically-inducible genes involve a *cis*-acting HRE, which can be present in either the 5′ or 3′ flanking DNA regions. HREs contain one or more binding sites for either HIF-1α or other related basic helix-loop-helix PAS (bHLH-PAS) proteins.[28] Several hypoxia-inducible promoters have been constructed using HREs from hypoxia-responsive genes, such as *phosphoglycerate kinase 1* (*PGK-1*), *enolase*, *LDH-A*, *EPO* and *VEGF* (reviewed in ref. 34). Recently, newly-devised approaches have been developed and have been shown to have good outcomes. Some of these recent studies, using hypoxia-responsive promoters, unique delivery systems and hypoxia-specific strategies, are summarized in Table 1.

4.3.1 Adenovirus: Gene-Directed Enzyme Prodrug Therapy (GDEPT) involves the delivery to the target cells of a foreign gene encoding a non-toxic enzyme, which activates specific prodrugs to toxic agents at the site of conversion. Binley *et al*.[39] constructed an optimized hypoxia response promoter (OBHRE) and put the *herpes simplex virus thymidine kinase* (*HSV-TK*) gene under the control of the OBHRE. They then investigated hypoxia-targeted gene expression *in vivo* in the context of an adenovirus vector. Systemic administration of adenovirus followed by treatment with the anti-herpes viral agent ganciclovir (GCV), a HSV-TK substrate, resulted in tumor regression. Post and van Meir[40] have developed a hypoxia/HIF-dependent replicative adenovirus (HYPR-Ad) that displayed hypoxia-dependent E1A expression and conditional cytolysis of hypoxic cells. Hernandez-Alcoceba *et al*.[41] generated AdEHT2 and AdEHE2F, two conditionally replicative adenoviruses for the treatment of breast cancer. Minimal dual-specificity promoter that responds to estrogens and hypoxia controls the expression of the *E1A* gene in both oncolytic adenoviruses. The tumor selectivity of these adenoviruses is increased by introducing the telomerase reverse transcriptase promoter and the E2F-1 pro-

Table 1. Recent molecular approaches to targeting of tumor hypoxia

Delivery system	Reference	Model	Hypoxia-specific strategy	Result
PTD from HIV-tat	Harada *et al*., 2002 (ref. 47)	CF-PACI, human pancreatic tumor cell	Cytotoxic protein fused to a part of HIF-1-ODD was specifically activated.	Tumor growth delay and regression
Adenovirus	Binley *et al*., 2003 (ref. 39)	MDA-MB468, breast tumor	GDEPT: optimized hypoxia response promoter-driven *HSV-TK* gene expression and GCV treatment	Delay in tumor growth
Adenovirus HYPR-Ad	Post and van Meir, 2003 (ref. 40)	brain tumor cell lines (*in vitro*)	HIF-1-dependent adenovirus replication. Virus replication causes cell lysis.	Hypoxia-dependent cell killing
Adenovirus CRADs (AdEHT2, AdEHE2F)	Hernandez-Alcoceba *et al*., 2002 (ref. 41)	MCF-7and BT-474, ER^+ human breast cancer cell lines	3XHRE/5XERE-regulated E1A expression and hTERT (AdEHT2) or E2F-1 (AdEHE2F) promoter-regulated E14 expression. Both E1A and E14 are required for virus replication.	Tumor growth suppression and regression
Anaerobic bacteria *Clostridium novyi*	Dang *et al.,* 2001 (ref. 45)	HCT116 (colon cancer), B16 (melanoma cells)	Non-pathogenic bacteria spore infected and treated with CTX, MMC.	Complete cure in 50% of mice
Anaerobic bacteria *Bifidobacterium*	Yazawa *et al*., 2001 (ref. 44)	DMBA-induced mammary carcinoma	Test delivery to spontaneous tumors	Engineered bacteria located in tumors
Anaerobic bacteria *Clostridium sporogenes*	Liu *et al*., 2002 (ref. 43)	SCCVII	*Clostridium* delivery of enzyme to convert 5-FC to 5-FU	Significant delay in tumor growth
Macrophage	Griffiths *et al*., 2000 (ref. 46)	Established spheroid from T47D, breast cancer cell line (*in vitro*)	GDEPT; hypoxia-regulated adenoviral vector encoding human cytochrome p4502B6 and cyclophosphamide treatment	Significant tumor cell killing under hypoxic conditions *in vitro*
Adenovirus	Kaliberov *et al*., 2002	Human lung carcinoma cells (*in vitro*)	VEGF promoter regulated-BAX	Significant tumor cell killing *in vitro*
ND	Greco *et al*., 2002	T24 bladder carcinoma cells MCF-7 mammary carcinoma cells (*in vitro*)	GDEPT; hypoxia-inducible promoter (Epo HRE/CarG)-regulated HRP and the prodrug indole-3-acetic acid	Significant tumor cell killing and radiosensitization of tumor cells *in vitro*
NA (transfected cancer cell line)	Shibata *et al*., 2002	HT1080 fibrosarcoma cells	GDEPT; hypoxia-inducible promoter (5XVEGF HRE)-regulated NTR transfected HT1080 and i. v. injected anticancer prodrug CB1954	Significant delay in tumor growth
NA (transfected cancer cell line)	Koshikawa *et al*., 2000	Lewis lung carcinoma A11	GDEPT; HRE/TK-transfected A11 was s. c. injected and then i. p. injected with GCV	Regression of tumor

HIV, human immunodeficiency virus; NA, not applicable; ND, not done; GCV, ganciclovir; GDEPT, Gene-Directed Enzyme Prodrug Therapy; HYPR-Ad, hypoxia/HIF-dependent replicative adenovirus; HRP, horseradish peroxidase; NTR, nitroreductase; CRAD, conditionally replicative adenoviruses; ER, estrogen receptor; ERE, estrogen response element; hTERT, human telomerase reverse transcriptase.

moter, which are preferentially activated in cancer cells, into the E4 region of AdEHT2 and AdEHE2F, respectively.

4.3.2 Anaerobic bacteria: *combination bacteriolytic therapy (COBALT)*: Several groups have been studying nonpathogenic and anaerobic bacteria as highly specific gene delivery vectors for cancer gene therapy (reviewed in ref. 42). The use of nonpathogenic anaerobic bacteria spores to deliver toxic agents to tumor cells takes advantage of the unique physiology of hypoxic tumor cells. These strictly anaerobic, Gram-positive, spore-forming bacteria give, after systemic administration, selective colonization of hypoxic/necrotic areas within the tumor. Moreover, they can be genetically modified to secrete therapeutic proteins such as *Escherichia coli* cytosine deaminase (CD) or tumor necrosis factor α. CD can convert the nontoxic prodrug 5-fluorocytosine (5-FC) into the anticancer drug 5-fluorouracil (5-FU).[43] The specificity of this protein delivery system can be further increased when expression is controlled by the use of a radio-inducible promoter, leading to increased spatial and temporal regulation of protein expression.

A strain of domestic bacteria, *Bifidobacterium longum*, which is nonpathogenic and anaerobic, has been shown to selectively localize and proliferate in several types of mouse solid tumors after systemic application.[44] Similarly to wild-type *B. longum*, genetically engineered *B. longum* could be detected in tumor tissue only, and was not found in a survey of a large number of normal mouse tissues after intravenous injection.

Recently, Dang *et al.*[45] have created a new strain of the anaerobic bacteria *Clostridium novyi* that is devoid of its lethal toxin (*C. novyi*-NT). Systemic administration of *C. novyi*-NT spores together with conventional chemotherapeutic drugs led to dramatic and prolonged regression of subcutaneous tumors.

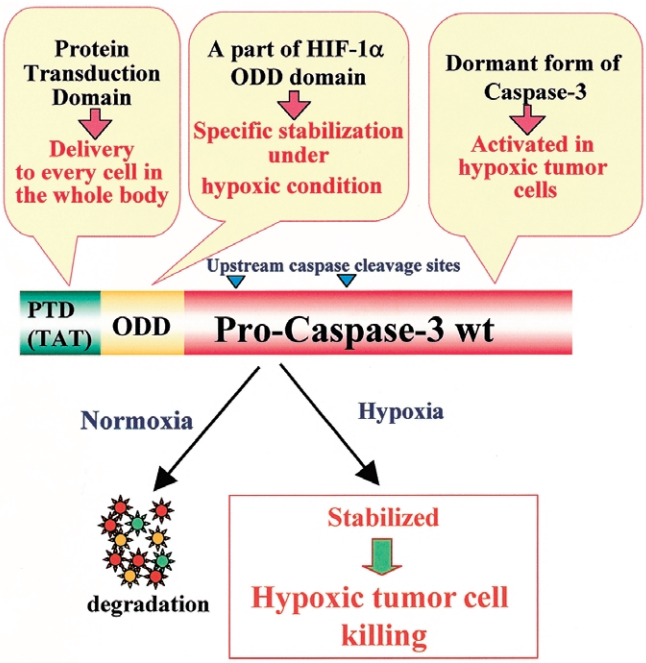

Fig. 4. Structure of TOP3. TOP3 consists of three domains: a protein transduction domain for delivery, an ODD domain for oxygen-dependent regulation, and a functional domain for tumor cell killing. The stability of TOP3 is regulated by the same mechanism as that of HIF-1 through the ODD domain (see Fig. 2). The procaspase-3 can be activated under hypoxic conditions because apoptotic pathways remain active in hypoxic tumor cells, even after they acquire resistance to apoptosis. The accumulation of active caspase-3 induces cell death *in vitro*, but the precise *in vivo* mechanism of cell death is currently unknown. It may occur through the same mechanism as that shown *in vitro* and/or may involve caspase-3-mediated phagocytosis.

4.3.3 Macrophages: The availability of macrophages as vehicles for hypoxia-selective gene therapy has been demonstrated recently.[46] It is known that macrophages infiltrate solid malignancies to form a significant proportion of the tumor solid mass, and that they predominate in areas of hypoxia and necrosis. For example, differentiated macrophages transduced with an adenoviral vector containing the human *cytochrome p450 2B6* (*CYP 2B6*) gene were found to infiltrate human tumor spheroids and to induce tumor cell death when the spheroids were incubated with the prodrug cyclophosphamide, which was converted by CYP 2B6 into the alkylating agent phosphoramide mustard.

4.4 A novel approach using a hypoxia-targeting fusion protein

To address the requirements for hypoxia-targeted therapy, namely high delivery efficiency, high specificity and selective cytotoxity, we have constructed a unique fusion protein, TOP3 (Fig. 4)[47]. We discuss its properties in the following sections in terms of these requirements.

4.4.1 High delivery efficiency: A severe limitation of protein therapeutics is the problem of transporting large proteins into cells. Overcoming this problem of bioavailability would not only enhance the effectiveness of existing therapeutics, but would also broaden the scope of viable cancer therapeutic strategies. The protein transduction domain embedded in the human immunodeficiency virus TAT protein (TAT-PTD; amino acids 47–57) has been shown to successfully mediate the introduction of given peptides and proteins with a molecular weight in excess of 100,000 into mammalian cells *in vitro* and *in vivo* (reviewed in ref. 48). TAT-PTD fusion proteins can be delivered to the entire body, including to the brain.[49] Furthermore, delivery does not occur only via the bloodstream. TAT-PTD fusion proteins are also able to freely diffuse through cell membranes, and therefore through layers of tumor cells. Hence, they can be delivered to tissues where blood vessels are shut down, such as in the ischemic brain.[50]

4.4.2 High specificity: As described above, HIF-1α protein stability is tightly regulated by oxygen-dependent prolyl hydroxylation and ubiquitination (Fig. 2) through its oxygen-dependent degradation domain (ODD), which contains proline 564. *In vitro* experiments have shown that endogenous HIF-1α is degraded within 5 min after exposure of cultured cells to 21% O_2. We have identified the minimum region of ODD (18–50 amino acids) that provides stability to a fusion protein under the oxygen-dependent conditions that degrade HIF-1α.[47] The reporter protein TAT-PTD-ODD-β-galactosidase was examined for delivery efficiency and specificity. Systemic administration of TAT-PTD-ODD-β-galactosidase showed that it was successfully delivered and stabilized in hypoxic tumor cells, but not in normal liver cells.[47] Therefore, if a cytotoxic protein is fused to the ODD polypeptide, its cytotoxicity will be controlled by the oxygen concentration.

4.4.3 Selective cytotoxity: To obtain selective cytotoxity, we chose an endogenous cytotoxic protein, procaspase-3, which is a major executioner protease that is located at the most downstream position in several apoptotic pathways and remains dormant until initiator caspases activate it by direct proteolysis. It is activated specifically in hypoxic tumor cells because, as described above, the apoptotic pathway and, hence, initiator caspases are activated in hypoxic tumor cells, generating active caspase-3.

The final fusion protein product TAT-PTD-ODD-Procaspase-3 (TOP3) (Fig. 4) was examined for its efficacy in tumor-bearing mice. Systemic administration of TOP3 significantly suppressed tumor growth and even reduced the tumor size without any apparent side effects in mice bearing human pancreatic tumor xenografts.[47]

The hypoxic tumor cell-specific effect of TOP3 was further confirmed in a rat ascites model (Inoue *et al.*, in preparation).

The fluid in ascites generated by rat ascites hepatoma cells has an exceedingly low oxygen tension (less than 1% pO_2), low pH, and low glucose and high lactate concentrations. Intraperitoneal administration of TOP3 increased the life span of rats that bore a significant amount of malignant ascites. The effect was so drastic that 60% of the treated animals were cured without recurrence of ascites.

Our ultimate goal is improvement of radiotherapy and chemotherapy for solid tumors by removal of hypoxic tumor cells and, indeed, we have recently shown that the combination of TOP3 with radiotherapy and chemotherapy has additional cytotoxic effects on solid tumors (Harada *et al*., in preparation).

5. Conclusions

As the mechanisms underlying response to hypoxic stress are dissected at the molecular level in solid tumors, the existence of such a microenvironment in tumor cells is becoming apparent as a flaw of current cancer therapies. The difference between hypoxic tumor cells and well-oxygenated normal cells is so distinct that it is possible to design efficient and selective therapies against the sequestered regions. Whether targeting hypoxic tumor cells has a real benefit in clinical cancer therapy still remains an open question, however, since complete obliteration of hypoxic tumor cells has still not been achieved genetically or pharmacologically, even in *in vivo* experimental settings. Accumulation of data showing the impact of emerging hypoxia-targeting therapies, such as TOP3, on solid tumors should confirm tumor hypoxia as a major cause of malignant and refractory conversion. Such evidence would validate the idea of attacking tumor hypoxia as a therapeutic approach, and would provide momentum for further exploitation of this approach in clinical applications.

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