Forum Minireview

Pathophysiological Response to Hypoxia — From the Molecular Mechanisms of Malady to Drug Discovery: Drug Discovery for Targeting the Tumor Microenvironment

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Abstract. The tumor microenvironment, characterized by regions of hypoxia, low nutrition, and acidosis due to incomplete blood vessel networks, has been recognized as a major factor that influences not only the response to conventional anti-cancer therapies but also malignant progression and metastasis. However, exploiting such a cumbersome tumor microenvironment for cancer treatment could provide tumor-specific therapeutic approaches. In particular, hypoxia is now considered a fundamentally important characteristic of the tumor microenvironment in which hypoxia inducible factor (HIF)-1–mediated gene regulation is considered essential for angiogenesis and tumor development. Additional oxygen sensitive signaling pathways including mammalian target of rapamycin (mTOR) signaling and signaling through activation of the unfolded protein response (UPR) also contribute to the adaptation in the tumor microenvironment. This in turn has led to the current extensive interest in the signal molecules related to adaptive responses in the tumor microenvironment as potential molecular targets for cancer therapy against refractory cancer and recurrence in preparation for the aging society. Therefore, we should focus on the drug discovery for targeting the tumor microenvironment to develop tumor-specific cytostatic agents including angiogenesis inhibitors. In this paper, the development of hypoxia-selective prodrugs, HIF-1 inhibitors, and modulators of the tumor microenvironment will be discussed.

Keywords: hypoxia, tumor microenvironment, hypoxia inducible factor (HIF)-1, bioreductive activation

1. Introduction

Recently, it has become imperative to develop a clinical strategy for improving prognosis and preventing recurrences in cancer treatment since cancer morbidity has increased especially in older adults, which indicates that there is a much higher risk of recurrent or refractory cancer. Hence, the tumor microenvironment has attracted much attention as a target for the development of novel cancer treatment strategies because it is a major factor influencing treatment resistance to conventional anticancer therapies, malignant progression, and metastatic potential. Furthermore, the tumor microenvironment might be a general target of solid tumors regardless of cancer types.

The tumor microenvironment is often characterized by hypoxia, nutrient deprivation, acidosis, and aberrant stroma. Hypoxia occurs in the majority of tumors and plays a critical role in various cellular and physiological events characteristic of malignant alteration of the entire tumor. The hypoxia inducible factor (HIF)-1 is a primary transcriptional factor that regulates oxygen homeostasis and the cellular response to hypoxia (1). Recently, it has been revealed that cellular responses for hypoxia are mediated by both HIF-dependent and HIF-independent pathways (2). These oxygen sensitive signaling pathways including mammalian target of rapamycin (mTOR) signaling and signaling through activation of the unfolded protein response (UPR) in addition to HIF-1 signaling pathway that influence each other and contribute to the
adaptation in the tumor microenvironment.

Thus, further research of adaptive mechanisms for stress tolerance in an austere environment might provide new tumor therapeutic and diagnostic strategies (3). Consequently, we should focus on drug discoveries that target the tumor microenvironment to create tumor-specific cytostatic therapy in preparation for the aging society. To show the role of the tumor microenvironment as a potential target for cancer therapeutics, I will introduce and discuss in this paper the development of hypoxia targeting drugs, HIF-1 inhibitors, and modulators of the tumor microenvironment.

2. Hypoxia-targeting drugs based on bioreductive activated mechanisms

Since Thomlinson and Gray proposed the existence of hypoxic cells in solid tumors half a century ago, these hypoxic tumor cells have been recognized as potential problems of resistance to chemo- and radiotherapy and predictors of poor prognosis and recurrence. At first, strategies of cancer therapy that target hypoxia focused on sensitizing the tumor response to cancer therapy by developing oxygen-mimic chemical modifiers and hypoxia-selective cytotoxic prodrugs (4, 5). Key mechanisms of hypoxia selectivity are bioreductions to generate active species in hypoxic tissue, which have long been studied (Fig. 1). By using bioreductive activation mechanisms, not only various hypoxia-selective prodrugs but also hypoxia-imaging agents have been developed (6). Especially, nitro aromatic compounds (e.g., nitroimidazoles) and electron-deficient aromatic heterocycles (e.g., quinones and benzotriazine di-N-oxides) are typical scaffolds for hypoxia-selective compounds due to their appropriate reduction potentials (5). Although no new agents have been approved for clinical use, several hypoxia-selective prodrugs, such as EO9, CB1954, RP104, tirapazamine (TPZ), and AQ4N, are in various stages of clinical trials (7).

We previously reported hypoxic cytotoxins that inhibited angiogenesis and the expression of transcriptional activity of HIF-1α under hypoxia (Fig. 2) (4, 8). TX-402 suppressed induction of the mRNA of Glut-1, Glut-3, and VEGF even at noncytotoxic doses and inhibited angiogenesis significantly at a dose of 5 μg/CAM in the chick embryo chorioallantoic membrane (CAM) assay. Their potent antiangiogenic effects can be attributed to the suppression of VEGF through blocking the HIF-1α pathway. These results suggested that hypoxia-selective cytotoxins should affect adaptive responses to hypoxia including neoangiogenesis, glycolysis, and metastasis, which are associated with their cytostatic effect mediated by the inhibition of the HIF-1 pathway during moderate hypoxia.

As the tumor-specific microenvironment is characterized by hypoxia, application of the principle of hypoxia-specific bioreductive activation has expanded to cancer imaging (6). Nitroaryl compounds are representative exogenous hypoxia markers that recognize severe hypoxia (0.1% – 0.6% O2), while on the other hand, endogenous markers recognize moderate hypoxia (e.g., HIF-1α: <1% O2). The 2-nitroimidazole derivative pimonidazole is the most common hypoxia marker for immunohistochemical detection. For the noninvasive multimodality imaging of tumor hypoxia, fluorinated derivatives of nitroimidazole, such as fluoromisonidazole ([18F]FMISO), [18F]EF5, and fluoroazomycin arabinoside ([18F]FAZA) are available for PET; and hexafluoromisonidazole ([19F]CCI-103F) and [19F]EF5 are used for 19F MRS or MRI (9, 10). All of these nitroaryl probes undergo a hypoxia-dependent bioreductive metabolism by cellular nitroreductase to produce hydroxylamine derivatives via 4-electron reduction of the nitro group. Hydroxylamine is highly reactive and can bind to cellular macromolecules such as proteins, nucleic acids, and non-protein sulfhydryl compounds. These adducts can be detected by multimodality imaging techniques. A pilot study of PET imaging in cancer patients using radioactive [64Cu]Cu-diacetyl-bis (N4-methylthiosemicarbazone) ([64Cu]Cu-ATSM) is currently being conducted. With a high membrane permeability and redox potential, it can easily permeate the cell membranes and reside selectively in hypoxic cells by biological reduction under low cellular oxygen tension (11). Recently, optical techniques, such as bioluminescence and fluorescence, are emerging as future modalities for noninvasive molecular imaging in disease and therapy. Though these optical techniques are still in use for research in small-animal models, we are developing novel

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**Fig. 1.** A scheme for the activation of a bioreductive prodrug (P) by enzymatic reduction. The reduced intermediate of the bioreductive prodrug (P−) can be back-oxidized by the presence of oxygen (O2) with the formation of superoxide anion (O2−). P− may itself be cytotoxic or be further reduced to produce cytotoxic/active species (D1, D2, Dn).
near-infrared fluorescence (NIR) probes for in vivo optical imaging of tumor hypoxia, based on selective bioreductive activating mechanisms (Fig. 3). GPU-167, consisting of a hypoxia-responsive device, 2-nitroimidazole and cyanine dye, was retained in the hypoxic region of the tumor tissue in a mouse xenograft model for over one week after an intravenous injection at a dose of 10 nmol/mouse.
3. Development of HIF-1 inhibitors for cancer therapy

As mentioned above, oxygen-sensing and adaptation to hypoxia are critical for survival of cancer cells in the tumor microenvironment. Since HIF-1α has been identified by Semenza et al. as a master regulator of the cellular response to hypoxia, it has emerged as an attractive molecular target for cancer therapy (12, 13). Accordingly, the broad high throughput screenings (HTS) of chemical libraries and natural product libraries for HIF-1α inhibitors were carried out all over the world, and a wide variety of compounds have been identified over the last decade (13 – 15). Most of them were known therapeutics and biological active agents that possessed multifunctions other than HIF-1 inhibition. A growing number of novel anticancer agents have been shown to inhibit HIF-1 through a variety of molecular mechanisms (15, 16). It is extremely difficult to evaluate how many contributions were made by their inhibitory action on HIF-1 toward their anticancer effects, which indicates that most, if not all, HIF-1 inhibitors identified so far exhibit no specificity for the HIF-1 molecule. Cell-based screenings have the advantage of being able to identify a wide range of compounds targeting known and unknown signal transduction involved in HIF-1 transcriptional activity, but they provide little information about mechanisms of action. A novel and more specific screening system focusing on DNA-binding or protein–protein interaction between subunits of HIF-1 heterodimer were constructed for identifying HIF-1–specific inhibitors and assigning the targeting domain of HIF-1α (17, 18). Bortezomib (PS-341), a proteasome inhibitor, repressed HIF-1α activity by reinforcing the factor inhibiting HIF-1 (FIH)–mediated inhibition of p300 recruitment at much lower concentrations than those required to impair proteasome function, suggesting that the mechanism of HIF inhibition by PS-341 may be independent from proteasome inhibition (19). HIF-1 inhibitors previously discovered could be divided into some groups based on putative target signaling molecules or pathways, which were the oncogenic signaling pathway inducing HIF-1α, mTOR pathway, HIF-1α mRNA transcription, protein translation, proteasome degradation, dimerization, DNA binding, and transcriptional activity (Fig. 4) (20).

There are two main HIF-α isoforms (HIF-1α and HIF-2α), both of which bind hypoxia-response element (HRE) and activate HRE-dependent gene transcription. Despite considerable similarities in structure, function, and regulation, knock-out studies suggest that they play nonredundant roles (21, 22). On clear-cell renal cell carcinoma (CCRCC) 786-0 xenografts, HIF-1α inhibited the c-Myc oncoprotein and suppressed tumor growth, whilst HIF-2α potentiates c-Myc transcriptional activity and promotes tumor growth by an adaptive change to a more oxidative phenotype (23). On the other hand, both HIF-1α and

Fig. 4. Representative HIF-1 inhibitors and their proposed mechanisms of action and putative targets.
HIF-2α showed overlapping roles that promoted angiogenesis and growth in oral squamous cell carcinoma (OSCC), which suggest that combined inhibition of HIF-1α and HIF-2α may be beneficial for the treatment of OSCC (24). From these results, the development of isoform-specific inhibitors is of great interest. However, it is significantly difficult to obtain small molecule inhibitors due to their high structural similarity, except for siRNAs and antisense RNAs.

As a consequence of the validation of antitumor effects of HIF-1α inhibitors in vivo, it has been suggested that HIF-1 inhibitors, even if they were specific to HIF-1α, seem to be tricky to use for cytotoxic cancer treatment as a single agent. However, HIF-1 definitely plays a primary role in the oxygen homeostasis of the tumor microenvironment that mediates the maintenance of tumor cells and stem cell state. Therefore, in my opinion, the non-cytotoxic HIF-1 inhibitors should be more important for restoring the tumor microenvironment to the normal state in conjunction with cytotoxic therapies, resulting in improvement of prognosis and the prevention of recurrences. As mentioned by Gregg Semenza, we require new methods for establishing the proper context for clinical application of HIF-1 inhibitors in order to appreciate basic research achievements and translate them into cancer treatment (15).

4. Targeting the adaptive responses to tumor microenvironment

The heterogeneity in the tumor microenvironment leads to gradients in the rate of cell proliferation and regions of hypoxia and low nutrient availability, which can produce a more resistant and malignant tumor phenotype (25). Such cells with low proliferative capacity become tolerant to hypoxia and nutrient deprivation and adapt to the microenvironment. The adaptive responses to the tumor microenvironment are orchestrated through activation of multi signaling pathways implicated in oxygen and nutrients sensing. Oxygen homeostasis regulated by the HIF-1 pathway is best understood. HIF-1 also functions as a key regulator of cancer bioenergetics in conjunction with c-MYC and p53 to mediate aerobic glycolysis implicated in Warburg’s effect (26, 27). Since aerobic glycolysis has been recognized as the seventh hallmark of cancer, a better understanding of its biological mechanisms may lead to new strategy of anticancer treatment (28). More recently, two other pathways, mTOR and UPR signaling pathways, have been proven to be oxygen sensitive and responsible for hypoxia tolerance and adverse phenotype, as mentioned in the introduction (Fig. 5) (2). Each signaling pathway is activated independently by hypoxia and/or nutrition deprivation to

![Fig. 5. The adaptive responses to oxygen concentration and nutrient deprivation in the tumor microenvironment. Three main O2-sensing pathways, hypoxia-inducible factor (HIF)-1α pathway, unfolded protein response (UPR) pathway by activation through endoplasmic reticulum (ER) stress, and the mammalian target of rapamycin kinase (mTOR) signaling pathway, promote hypoxia tolerance by regulating transcription and mRNA translation. Together these three pathways influence the phenotype of hypoxic cells by altering metabolism, angiogenesis, autophagy, and ER homeostasis.](image-url)
influence tumor metabolism, autophagy, and endoplasmic reticulum (ER) homeostasis. UPR is a program controlling protein production and degradation, cell metabolism, and cell death. It occurs as a consequence of ER stress activated by glucose deprivation and hypoxia through ER-stress sensors including PERK, IRE1, and AFT6. The mTOR pathway is regulated by a wide variety of cellular signals, including growth factors, hormones such as insulin, nutrients (amino acids, glucose), cellular energy levels, and stress conditions. The current data shows moderate hypoxia (~1% O2) inhibits mTOR complex activity, protein synthesis, and proliferation through an AMP-activated protein kinase (AMPK) pathway (29). On the other hand, under severe hypoxic conditions (<0.1% O2), often accompanied by drastic glucose and amino-acid restriction, the autophagic response is induced via the AMPK-mTOR pathway or UPR pathway.

Recently, the UPR has received considerable attention as a potential target of the tumor microenvironment for cancer therapy (30, 31). Saito et al. reported that anti-diabetic biguanides, such as metformin, buformin, and phenformin, could inhibit activation of the GRP78 promoter during glucose deprivation (32). So, we recently evaluated the structure–activity relationship (SAR) of new biguanide analogs as UPR modulators using the luciferase reporter assay of GRP78 promoter. The new analog GPU-231 suppressed GRP78 promoter activity induced by 2-deoxyglucose (2-DG) dose-dependently and showed selective cytotoxicity under 2-DG stress conditions. GPU-231 also reduced HIF-1α–responsive promoter activity under the hypoxic condition. It inhibited the protein accumulation of GRP78 in glucose-free medium and HIF-1α protein induced under hypoxia (unpublished data). Phenformin and GPU-231 not only reduced the UPR signal but also the HIF-1 signal, suggesting that they may be promising lead compounds in developing novel cytostatic cancer agents targeting adaptive responses to the tumor microenvironment.

5. Conclusion

This review highlights the drug discovery target for the adapting responses of tumor cells exposed to the stressful microenvironment. In recent years, molecular mechanisms of hypoxia tolerance in tumor microenvironment have been elucidated intensively. And then, the microenvironment including possible targeting pathways has received much attention for the development of a novel cancer therapeutic agent for intractable malignant tumors. Notably, HIF-1–independent signal transductions such as the mTOR and UPR pathways may also significantly contribute to adaptation and survival of tumor cells in poor living conditions, which should be taken into consideration to formulate future strategies for cancer treatment.

References


