REVIEW

HIF expression and the role of hypoxic microenvironments within primary tumours as protective sites driving cancer stem cell renewal and metastatic progression

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Hypoxic microenvironments frequently exist in many solid tumours with oxygen levels fluctuating temporally and spatially from normoxia to hypoxia. The response to hypoxia in human cells is mainly regulated by hypoxia-inducible factors (HIFs), a family of transcription factors which orchestrate signalling events leading to angiogenesis and tumorigenesis. Several events conspire together to lead to the stabilization of HIF-α, commonly expressed in many cancer cell types. These events can result from low oxygen tensions occurring within the expanding tumour mass to produce hypoxic microenvironments or from mutations whereby the HIFs cause changes in expression of genes involved in several cellular functions. Hypoxia-mediated HIF-α regulation has gained significant prominence in tumour biology over recent years, and the hypoxic microenvironments have been shown to facilitate and trigger major molecular and immunological processes necessary to drive the progression of tumours to malignancy. More recently, it has been realized that the hypoxic microenvironments also play significant roles in shielding tumour cells from immune attack by promoting immune suppression. In addition, the hypoxic microenvironment promotes many other oncogenic events, such as the metabolic reconfiguration of tumour cells, neovascularization, epithelial to mesenchymal transition (EMT), and cancer stem cell renewal and accumulation. This article reviews the molecular mechanisms underlying tumour hypoxia and their pro-tumour contributions, such as immune suppression, development of nascent and more permeable tumour vasculature, selective cancer stem cell renewal, accumulation, mobilization and promotion of EMT leading to tumour cell metastasis.

Introduction

Hypoxia, a condition of oxygen deprivation that compromises biological function, is a common phenomenon observed in many human pathologies including its growing role as an important factor in cancer (1). Tumour progression is normally sustained by the provision of a constant nutrient supply *via* its associated vasculature, as long as this can remain sufficient to meet the metabolic requirements of the growing tumour (2). As most tumours proliferate rapidly, they commonly undergo periods of aberrant vasculature which then fail to meet the nutritional and oxygen requirements of the growing tumour (3). As a survival mechanism, tissues exposed to oxygen deprivation release signalling

factors leading to neovascularization, often associated with subsequent tumour progression.

Hypoxia-inducible factors (HIFs) are a family of three transcriptional regulators that mediate the effects of key oxygen sensors in the cells. HIFs control the expression of genes that are major contributors to neovascularization, including the signalling factors like vascular endothelial growth factors (VEGFs) (4) and erythropoietin (5). Besides these, HIFs also alter the expression of several genes encoding specific glycolytic protein isoforms (enzymes and transporters) (6,7). The HIF-mediated modification in expression of glycolytic proteins causes a metabolic reprogramming, which helps to address the energy crises inside growing tumours enabling them to survive the state of hypoxia (8) and it also confers resistance to chemotherapy and radiotherapy treatments (9). However, the contribution of the HIFs to drug resistance differs depending on the cancer type and cells of origin (8,10). Thus, tumours showing increased HIF-α protein stabilization often also exhibit higher levels of radioresistance (11), whereas low HIF-α detectable protein levels are not predictive for radiosensitivity. Experiments have shown significant variability in the levels of HIF-1α protein detected in human tumour cell lines, with high levels present even under normoxic conditions (12). These variations directly correlate with the levels of expression of carbonic anhydrase CA-IX, a HIF-1α downstream target gene (13). The variability in HIF-α protein levels may be a result of varying tumour dynamic properties due to cycling hypoxic episodes whereby the cells undergo repeated hypoxia and reoxygenation cycles (14).

HIF-α stabilization shows a distinct correlation with pathophysiological phenomenon like tumour invasiveness and metastasis (15). The present review will focus on factors contributing to HIF-1α stabilization and its multifaceted role in promoting pro-oncogenic events, like epithelial–mesenchymal transition (EMT), metastasis (16), selective accumulation and maintenance of cancer stem cells and tumour survival by immune suppression.

HIFs and tumour hypoxia

The HIFs are a highly conserved family throughout evolution. The HIF transcription factor when formed into its active state comprises a heterodimer of either one of three different α-subunits (HIF-1α, HIF-2α or HIF-3α) together with a β-subunit, HIF-1β. Unlike the HIF-1α subunit, which primarily takes part in the hypoxic response, HIF-1β, also called the aryl hydrocarbon nuclear translocator, participates in cellular responses to environmental toxins including several exogenous ligands, such as natural plant flavonoids, polyphenols and indoles, as well as synthetic polycyclic aromatic hydrocarbons and dioxin-like compounds (17). The human HIF-1α and HIF-1β comprise 826 and 789 amino acids, respectively, and both proteins contain a basic helix–loop–helix domain involved in DNA binding and Per/aryl hydrocarbon nuclear translocator/Sim domain which enables the HIF-1α-β subunit dimerization (18). The oxygen-dependent domain present on the HIF-1α protein makes it vulnerable to degradation under normoxia. Besides the oxygen-dependent domain, HIF-1α also contains two domains required for transcriptional activation: the C-terminal activation domain and the N-terminal activation domain (19). C-terminal activation domain controls the regulation of HIF target genes, whereas N-terminal activation domain contributes to gene-specific targeting (20) (Figure 1).

HIF-2α shares structural similarity to HIF-1α, but it shows tissue-specific expression, limited to the kidneys, small intestine, endothelium, lungs and heart (21). Under hypoxic conditions,

HIF-2α fails to undergo proteasomal degradation but forms dimers with HIF-1β to activate hypoxia response element-mediated gene regulation (22). The function of HIF-3α is not fully understood, but its splice variant is known to inhibit transcriptional activity of HIF-1α (20). HIF-1α stabilization and function inside cancer cells are represented schematically in Figure 2. Recently, bifunctional activity of the two HIF-1α transcriptional domains was demonstrated by Dayan *et al.* (23), when they demonstrated that both C- and N-terminal transcriptional activation domains differentially contributed towards HIF-1α activity. Each of these domains was shown to have their own independent function in that they were hydroxylated by prolyl hydroxylase (PHD) and asparaginyl hydroxylase, respectively (23). Reversal of the hypoxic effect was demonstrated by selectively inactivating HIF-1α using small interfering RNA-mediated repression in cells undergoing hypoxia, resulting in a complete or partial shift in EMT markers back to the prehypoxic state, accompanied by a significant decrease in the hypoxia-induced migration and invasion of tumour cells (24).

Role of PHD, factor-inhibiting hypoxia and Von Hippel Lindau in HIF regulation

The prolyl hydroxylases (previously also known as EgLN) are a family of enzymes that includes three members, termed PHD1, PHD2 and PHD3 (25), each having a highly conserved gene structure (26). Although all isoforms are known to catalyse hydroxylation reactions *in vitro*, each isoform has a different subcellular localization, substrate specificity and tissue expression and contribute differently to the regulation of HIFs (27,28). The PHD enzyme is part of an intrinsic oxygen sensing mechanism that under normoxic conditions prevents the stabilization of the HIF-1α subunits and their ability to contribute to tumorigenesis. The PHDs act by promoting the physical interaction between Von Hippel Lindau (VHL) and HIFα, which targets HIF-αs for ubiquitination and degradation by the proteasomal complex. For example, in human cells, the enzymatic function of PHD is to hydroxylate the proline residues P402 and P564 on HIF-1α to promote the VHL binding (22,29). A 10% decrease in the oxygen concentration has demonstrated a rapid lowering in hydroxylation of
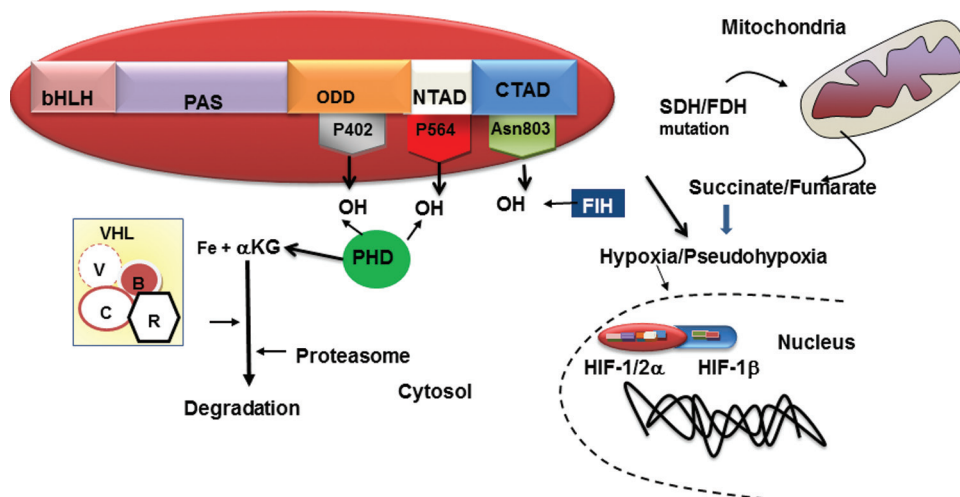

Fig. 1. Schematic representation showing HIF-α domains and their functions in mediating hypoxic regulation and relationship to different hypoxic states.
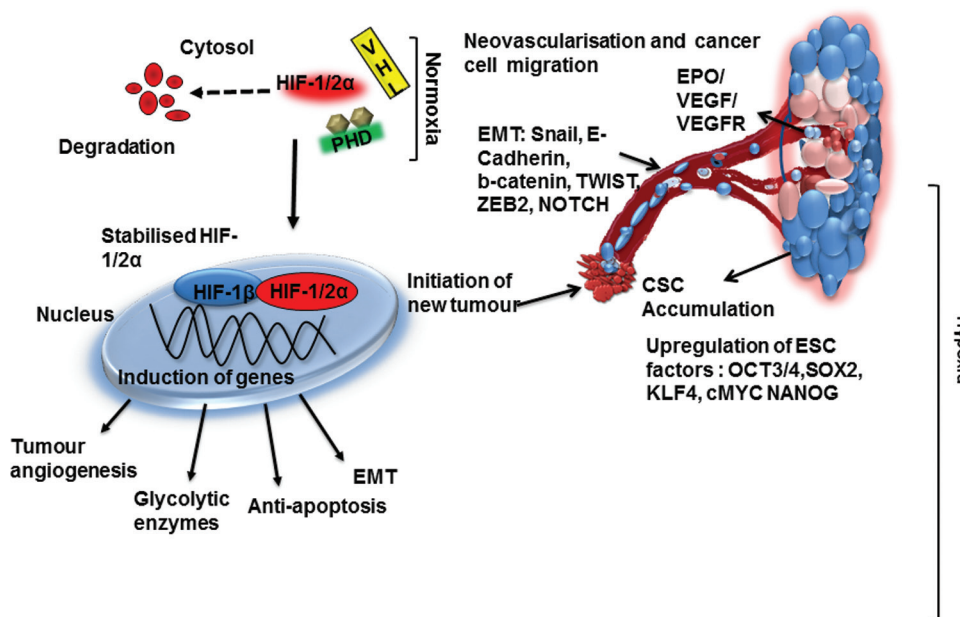

Fig. 2. Schematic representation of factors involved in HIF-α stabilization and the hypoxic response triggering CSC accumulation, leading to the development of new tumours.

proline residues on HIF-1α resulting in its nuclear stabilization (30) (see Figure 3 for regulation of PHD activity as a function of the O_2 concentration).

The PHD1 messenger RNA (mRNA) levels are highest in testes, whereas PHD2/PHD3 mRNA levels are highest in heart tissue (17). Studies have indicated that on reoxygenation, HIF-1α regulates the self-degradation of PHD2 and PHD3 (31). This observation reinforces the importance of the PHD2/PHD3 isoforms in the hypoxic response, and in addition, mammalian PHD2 and PHD3 mRNAs are HIF-1α inducible, probably through the presence of HIF transcription sites in their promoters, whereas PHD1 is not induced by HIF-1α (32,33). It has been speculated that PHD2 and PHD3 are efficient regulators of HIF-α, even under hypoxic conditions. Under prolonged hypoxic conditions, increased PHD2/PHD3 expression was found to be associated with a decrease in HIF-1/2α protein levels, while their mRNA levels showed no significant alteration (31). Silencing experiments conducted in a range of different cell lines targeting the genes encoding the PHD enzymes revealed that PHD1 and PHD3 had no effect on elevation of HIF-1α levels. On the contrary, silencing of PHD2 (EglN1) resulted in an upregulation of HIF-1α expression (17). Small interfering RNA transfection experiments demonstrated that PHD2 plays a pivotal role in hypoxic regulation (34) and showed that the PHD2 isoform is most important for HIF-1α downregulation in normoxia and mild hypoxia, promoting HIF-1α degradation *via* hydroxylation of the P564 residue (34). PHD2, but not PHD1/PHD3, is necessary for normal embryonic development as demonstrated by targeted gene disruption of PHD2 which resulted in embryonic lethality, whereas PHD1/PHD3 double knockout resulted in viable mouse embryos (34). The results summarized here converge to emphasize that PHD2 is the most crucial component of HIF regulation and normal physiology compared with its counterparts. The role of PHD2 in different cell lines, its expression and regulation at the protein level and its correlation with clinicopathological parameters still has yet to be addressed and understood completely.

Factor-inhibiting hypoxia 1 (FIH-1), like PHD, is another oxygen sensor involved in the regulation of HIF-1α activity (23). It indirectly affects HIF-1α levels by preventing the binding of the transcriptional coactivator p300/CBP and inhibits HIF-1α by inactivating the C-terminal transactivation domain rather than affecting its level of expression (35). The FIH1 gene encodes an asparaginyl hydroxylase

Fig. 3. Regulation of the prolyl hydroxylase activity by O_2 and physiological competitive inhibitors of 2-OG. The figure indicates that at saturating levels of fumarate (High Fum), the PHD activity can be severely decreased (6–10 times) when the O_2 concentration is 5–10 mM (the hypoxic range for solid tumours). Under this last condition, the PHD activity cannot be further decreased by elevating the concentration of either succinate (Succ) or the other inhibitors, because they all compete for binding to the same enzyme site. It should also be noted that the PHD activity under low succinate is low, because the O_2 concentration range examined is well below the *Km* value of oxygen.

which hydroxylates the Asn-803 residue in the C-terminal activation domain of the HIF-1α protein (36) (Figure 1). Hypoxic abrogation of asparaginyl hydroxylase activity results in the recruitment of a large transcription activating complex onto hypoxic response target genes, providing a second oxygen sensing mechanism participating in the hypoxia response pathway (37). Recently, a null mutation introduced into the murine FIH gene revealed that it had no significant effects on HIF function. Instead, this FIH mutation caused a hypermetabolic phenotype whose modifications included a lower body weight as well as increased glucose and lipid metabolism (38). Interestingly, this would make FIH a potential target for treatment of diseases based on hypermetabolic symptoms and obesity. However, little investigation has occurred into whether FIH in humans plays the same role in metabolism as that in the mouse model, and this is an area that requires further study.

Once HIF-1α subunits become hydroxylated by PHD, the VHL protein comes into play by recognizing and binding to the hydroxylated HIF-1α, leading to ubiquitination and proteasomal degradation, thereby keeping HIF-1α at low levels under normoxic conditions. Hence, VHL is a HIF-1α regulatory protein (39) and loss of function mutations in VHL results in a variety of tumours, including pheochromocytoma, although precisely how these tumours develop is still under intensive investigation. Many different mutations are responsible for VHL gene inactivation and several missense mutations have been linked to pedigree families with pheochromocytoma. VHL is a multipurpose adaptor involved in interprotein interactions controlling diverse HIF-1α-dependent functions. VHL gene loss of function or inactivating mutation is an early requirement in cancer development and can occur spontaneously (40), initiating pathological events due to the localization of the gene product in the mitochondria, an organelle that contains angiogenic factors and enzymes required for HIF-1α regulation (41).

The Von Hippel Lindau (VHL) gene in humans encodes a 213-amino acid protein that has two domains with as yet unknown enzymatic function (42). VHL protein forms a multimeric complex with elonginB, C, Cul2 and Rbx1 proteins, known as the VBCR complex, that recognizes hydroxylated HIF-1α or HIF-2α and targets them for proteolytic degradation (Figure 1) (43,44). Disruption of the VHL–HIF-α interaction can lead to HIF-α stabilization, followed by nuclear translocation and dimerization with HIF-β, and the complex then switching on the angiogenic and tumorigenic signals (22,45,46) (Figure 2). Knock-in of wild-type VHL genes into VHL−/− renal clear carcinoma cells suppressed their ability to form tumours *in vivo* (47), and interestingly, studies have also shown that VHL is able to target non-hydroxylated HIF-1α for degradation during hypoxia (48).

Mitochondrial metabolites in tumour hypoxia and a key role for succinate as a PHD regulator

Pseudohypoxia is a phenomenon whereby HIF-1α becomes aberrantly stabilized, even under normoxic conditions (49). It is often associated with the mutational inactivation of genes encoding the mitochondrial enzymes succinate dehydrogenase (SDH) and fumarate hydratase (FH) and is proposed to be the reason for the commonly associated mutations of these genes found in tumours such as pheochromocytoma and paraganglioma (50), as well as in clear cell renal carcinomas (51). The mutations in the SDH, FH and isocitrate dehydrogenase genes result in the accumulation of their respective substrates (52) increasing the levels of carboxylic acids, most significantly, succinate, fumarate and citrate, as well as pyruvate and lactate (53). Several of these carboxylic acids can directly inhibit the activity of the PHDs (52,54) (Figure 3). Consequently, in a similar fashion to the VHL mutation, the inhibition of PHDs by these metabolites results in prevention of HIF-α degradation, increasing its cellular levels (54) (Figure 1). The high intracellular lactate level found in cancer cells can induce increased HIF-1α stabilization but works indirectly *via* its conversion to pyruvate in a reaction catalysed by lactate dehydrogenase (55).

A build-up in succinate levels accumulating in the cytosol inhibits PHD and thereby can trigger hypoxic signals, even under normoxia
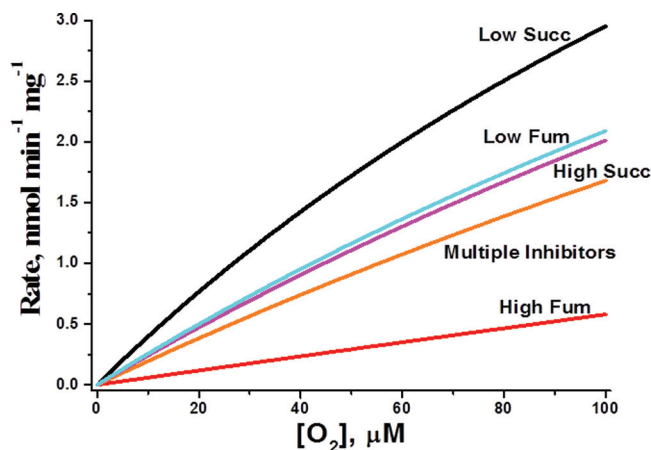
(55). In the mitochondria, succinate couples the tricarboxylic acid (TCA) cycle to mitochondrial respiration where succinate is used as a substrate of complex II. However, it can also be transported out of the mitochondrial matrix to the cytosol, and succinate is the downstream product of the oxidative decarboxylation of 2-oxoglutarate (2-OG), the one 2-oxo acid metabolite required for activating the PHDs. In the cytosol, succinate acts as a stable regulator, high concentrations of which result in inhibition of PHD (Figure 3), leading to HIF-1α stabilization and activation (56). Kinetic analysis of PHD activity based on published studies shows that these enzymes greatly depend on the concentrations of the two limiting substrates 2-OG and O₂ and of the 2-OG derived product, succinate and the substrate analogues citrate, fumarate, oxaloacetate, pyruvate and malate (Figure 3 and see below for further detail). Cellular succinate and fumarate concentrations in tumours with SDH or FH mutations can markedly change basal levels from <0.1 mM found in normal cells to increase up to 1–10 mM in cancer cells (57). The intracellular concentrations of 2-OG (58) and citrate, pyruvate and malate (59,60) determined in tumour cells are, respectively, 2–2.3, 1.7–2.5, 2.1–8.5 and 2.1 mM. The inhibition constant (*Ki*) values of PHD for succinate, fumarate, citrate, pyruvate and malate (61–63) are, respectively, 430 μM, 50 μM, 180 μM and 1.2 mM. Succinate-mediated PHD inhibition is often detected in neuroendocrine tumours with SDH mutations, which show nuclear stabilization of HIF-αs even under normoxic conditions (64). The *Km* values of 2-OG for human PHD are in the range of 55–60 μM (65). A higher concentration of 2-OG is also known to weaken the succinate-mediated inhibition of PHD, and hence, the balance of these two TCA metabolites regulates PHD activity in cancer cells. Thus, *in vitro* studies have demonstrated the reversal of the PHD inactivation caused by succinate when the cells were provided with excess 2-OG (49), indicating that product inhibition is regulated by the ratio of 2-OG to succinate, as these compounds directly compete with each other for PHD binding and regulation (66).

The accumulation of succinate levels is a common mechanism for HIF-1α stabilization most often detected in tumours harbouring mutations in SDH genes like pheochromocytoma and paraganglioma (67), cutaneous leiomyomas, leiomyosarcoma and renal cell carcinoma (68,69) but may also apply commonly to progression of other cancers as well. FH is a tumour suppressor gene whose mutation has been associated with uterine and cutaneous leiomyomas, leiomyosarcoma and renal cell carcinoma (68,69). Mutation in the FH gene causes an increase in the cytosolic concentration of fumarate, which in turn activates hypoxic signals by inhibition of the 2OG-dependent dioxygenases such as the PHD genes, resulting in HIF-1α stabilization (67). Despite its primary role as a mitochondrial gene, FH is also present in the cytosol, as it is dual targeted to both the cytosol and the mitochondria. While the mitochondrial function of FH is to convert fumarate to malate and *vice versa* (70), it has a different cytosolic function as a DNA damage response protein. This additional role is not surprising given that it is also a tumour suppressor gene (70). Mutation in the gene encoding isocitrate dehydrogenase, the enzyme catalysing the formation of 2-OG by decarboxylating isocitrate, affects the ratio of these metabolic intermediates in the same way as mutations in the SDH gene. Hence, decreased 2-OG with increasing succinate (and/or citrate, fumarate, pyruvate, malate) levels will trigger HIF-1α activity, thereby leading to a state of pseudohypoxia (71,72).

Besides the association of pseudohypoxia with defective genes encoding mitochondrial enzymes, mutated VHL can also cause pseudohypoxia (73,74). However, pseudohypoxia in VHL defective renal clear cell carcinomas operates by a slightly different mechanism because these tumours show consistent HIF-2α overproduction and stabilization, but not HIF-1α (75). HIF-2α then likely interacts with the Myc pathway for cell cycle arrest and tumour progression (21). Given the role of mitochondrial metabolites in regulating PHDs and inducing pseudohypoxia, it is still unclear which comes first and whether the mutation leads to the cancer malignancy or whether the cancer becomes established first before the onset of the mutations in TCA cycle genes. Another question is why are these phenomenon restricted to cancers of neuroendocrine origin?

Kinetic analysis of the influence of TCA cycle intermediates on PHD activity

We have undertaken kinetic analysis to explain the influence of the TCA cycle intermediates on PHD activity detected *in vivo*, by using a simulation model assuming a kinetic Bi–Bi ordered mechanism for binding of 2-OG and then O₂ to the enzyme, and further release of first CO₂ followed by succinate (66) (see rate equation below). Assumptions included a maximal velocity (V_{max}) value of 10 nmol/min/mg, under saturating levels of Fe^{2+}, peptide (i.e. HIF-1α) and ascorbate. The *Km* values of PHD for 2-OG (A) and O₂ (B) are 60 and 230 μM, respectively (65), with the *Ki* values for succinate, fumarate, citrate (61) and pyruvate as, respectively, 430, 50, 180 and ~500 μM. The PHD activity was modelled at the physiological concentrations of 2 mM 2-OG existing in cancer cells (58) with different levels of the other metabolites, using the Microcal Origin v. 5 software, as follows:

(i) At low succinate concentration of 0.1 mM determined in heart (76);
(ii) At higher succinate concentration of 10 mM as determined in tumours with FH mutations (57);
(iii) 0.1 mM succinate and a lower fumarate concentration of 1 mM; or
(iv) A high concentration of 10 mM fumarate which are metabolite levels found in tumours with FH mutations (57); and
(v) With multiple inhibitors, using 1 mM succinate plus 1 mM fumarate plus 2 mM citrate plus 2 mM pyruvate. Figure 3 shows the results from this modeling

$$v = \left[\frac{\frac{V_{max}}{Km_{2\text{-}OG} \cdot Km_{O_2}}([2\text{-}OG][O_2])}{1 + \frac{[2\text{-}OG]}{Km_{2\text{-}OG}} + \frac{[2\text{-}OG][O_2]}{Km_{2\text{-}OG} \cdot Km_{O_2}} + \frac{[Fum]}{Ki_{FUM}} + \frac{[Cit]}{Ki_{CIT}} + \frac{[Pyr]}{Ki_{PYR}} + \frac{[CO_2][Succ]}{Km_{CO_2} \cdot Ki_{SUCC}} + \frac{[Succ]}{Ki_{SUCC}}} \right]$$

The relationship of intratumoural hypoxia to angiogenesis and metastasis

Recurrence and metastatic spread from residual disease after initially removing primary solid tumours and draining lymph nodes remains a major clinical problem, with many cancers recurring at high frequency (77,78). One of the major contributing factors to tumour metastasis is the process of neovascularization, which occurs as an outcome of the induction of hypoxia regulated proteins (79). It is well known that intratumoural hypoxia is one of the major factors that drive tumour angiogenesis, and hypoxia-driven angiogenesis is primarily mediated by HIF-1α, often considered as a master regulator of angiogenesis in hypoxia (80). HIF-2α also contributes to hypoxia-driven angiogenesis by regulating the expression of VEGF, VEGF receptors 1 and 2 and angiopoietins needed for the development of blood vessels (81). HIF-2α triggers the chronic hypoxia response and acts as a key inducer of genes involved in tumour invasion such as the matrix metalloproteinases (82) and stem cell factor OCT4 (21). The HIF-regulated genes provide dual protection to the growing tumour by, first, promoting the development of new blood vessels (Figure 1) and, second, helping the tumour to metabolically acclimatize to the decreased levels of O₂ (15). Myc oncogenic activation collaborates with HIF-2α expression and causes the tumour cells to adapt the metabolic shift from oxidative phosphorylation to substrate level phosphorylation occurring within the tumour milieu under prolonged hypoxia (7,21,83)

One key question that arises is what is the driver that makes tumour cells migrate to form metastases? Within the heterogeneous tumour exists a metastatically predisposed subpopulation of tumour cells (84) that are exposed to the hypoxic microenvironments and as a result undergo an EMT (85). These phenotypic changes are brought about by Myc-mediated activation of the Snail transcription factor (86). Myc also contributes to cellular migration and invasion by altering cell–cell matrix interactions as a transactivator of the LGALS1 gene expression as well as cytoskeletal remodelling *via* Myc-activated

RhoA expression (87). Activation of c-Myc and the associated glycolytic shift (88), together with slower electron transfer and O_2 consumption in the mitochondrial respiratory chain, stimulates the production of reactive oxygen species (89). However, HIF-2α induction by Myc helps to shield the cells from the harmful effects of reactive oxygen species by activating those genes responsible for the production of antioxidants (90). HIF-1α counteracts the effects of antioxidants by triggering the calpain-dependent degradation of HIF-2α (90). The resulting decrease in HIF-2α protein levels promotes p53 serine15 phosphorylation, thereby hampering cellular redox homeostasis (91). Reactive oxygen species induction, besides causing DNA damage, also inhibits apoptosis and disables the P53 protein pathway which otherwise regulates homeostasis against cellular stress (92). Deregulated c-Myc-mediated DNA damage occurs prior to the S phase of the cell cycle, enabling unrepaired DNA to remain present during the cell cycle causing genomic instability, one of the hallmarks of cancer leading to tumour invasion and dissemination from its primary site (92). Tumour cell mobilization is also brought about by secretion of matrix metalloproteinases by tumour stromal cells such as adipocytes and carcinoma-associated fibroblasts, which brings about the digestion of the extracellular matrix (93). This dissemination of tumour cells helps to initiate the process of metastasis leading to organ-specific homing of the migratory tumour cells, a process governed by chemotactic interactions (94). In this regard, it is interesting that HIF-2α displays opposing roles in tumour development to HIF-1α and this relationship is an area of current research focus.

Relationship between hypoxia and the induction of EMT

EMT is a crucial phase in embryological development and cancer metastasis during which the epithelial cells lose their polarity switching to adopt migratory mesenchymal cell phenotypes (95). In order to achieve this, the cells change their cellular signalling pathways (96). Loss of the E-cadherin protein content and overexpression of Snail are hallmarks of EMT and increased levels of Snail, in itself, are indicative of EMT, the levels of which correlate with the degree of clinical aggressiveness displayed by tumours (97). HIF-1α induced by hypoxia has been found to promote EMT in many human malignancies in which the hypoxic microenvironment promotes overexpression of Snail, while attenuating the expression of E-cadherin, leading to EMT and increased cancer aggressiveness (98). Cancer cell lines of epithelial origin, including HT-29 (colon carcinoma), MCF-7 (breast carcinoma), HepG2 (human hepatoblastoma) and PANC-1 (pancreatic carcinoma), when subjected to hypoxic conditions develop EMT-associated characteristics such as overexpression of Snail, repression of E-cadherin and the nuclear localization of β-catenin (99) (Figure 2).

HIF-1α stabilization activates Twist, a transcription factor that regulates early embryonic mesoderm formation and gastrulation, which also facilitates tumour development and metastasis. HIF-1α induces Twist gene expression *via* an hypoxia response element present in the proximal promoter region (24). It has been proposed that the EMT pathway in tumours differs depending on whether the level of hypoxia is acute or chronic, thereby affecting the extent of protein expression and recruitment. Acute hypoxia upregulates Twist and has a reversible effect on EMT, whereas more chronic, long-term hypoxia results in an irreversible EMT *via* the activation of the ZEB2 zinc finger binding protein (100). The NOTCH signalling protein is a mediator in the convergence of hypoxic responses to promote the EMT signal (101). The HIF-1α-mediated EMT pathway still remains undefined as to the precise mechanisms operating to regulate this multigene/protein interactive cascade.

The hypoxic tumour microenvironment aids cancer cells to escape immunosurveillance

The immune responses occurring within the tumour microenvironment represent another critical aspect for the important role of hypoxia in tumour biology. Immune cells can recognize and eliminate cancer or precancerous cells based on the types of tumour-associated or tumour-specific antigens that these malignant cells can present. However, tumours arise more readily when the cancer cells overcome the ability of the immune system to eliminate them by processes involving immune-editing and tumour-mediated immune suppression (102). Despite decades of research, the mechanisms for the immune suppression within the tumour microenvironment are still not completely understood. The emerging evidence suggests that intratumoural hypoxia may also be a major contributor to the immunosuppressive microenvironment. Thus, hypoxia can systemically impair the tumour antigen-specific immune responses from being produced within a tumour (103). First, the hypoxic state suppresses maturation of the dendritic cells by inhibiting CD40 and major histocompatibility complex class II expression, which is associated with diminished Th1 responses, including decreased Th1 cytokines, interferon-γ, tumour necrosis factor-1α and interleukin-12 expression (104).

Studies have shown that intratumoural hypoxia promotes negative immunoregulatory cell populations to exist within tumours. Thus, hypoxia-exposed tumour cells produce greater levels of the CC-chemokine ligand 28, which selectively attracts and recruits regulatory T (Treg) cells into the tumour stroma, negatively regulating antitumour immune cell responses (105). Furthermore, CD39 (ecto-apyrase) and CD73 (ecto-5′-nucleotidase), which convert adenosine triphosphate and adenosine diphosphate into adenosine, are upregulated on the intratumoural Treg cells during hypoxia (106). As a result, the increased intratumoural levels of soluble adenosine bind *via* the A_{2A} receptor on effector T-cell surfaces, promoting T-cell growth inhibitory signals (107). In addition, the intratumoural hypoxic conditions recruit monocytes, mainly derived from the bone marrow into the tumour where they can rapidly differentiate into tumour-associated macrophages. The accumulated tumour-associated macrophages in the hypoxic regions of tumours express tumour-promoting cytokines as well as inhibiting cytotoxic T lymphocyte (CTL) function (108), preventing antitumour immune responses.

Hypoxia also directly regulates CTL function, because hypoxia-exposed CTLs are less sensitive to the antigens presented by the tumour cells (109). By using HIF-1α deficient CD4+ and CD8+ T cells, it was shown that these cells had a greater capacity to proliferate and to produce interferon-γ within the intratumoural hypoxic domains (110). An additional strongly immunosuppressive relationship has been found between expression of galectin-1, intratumoural hypoxia and defective antitumour immune responses (111,112). Galectin-1 belongs to the family of animal lectins known to block effector T-cell activation and to promote their apoptosis (reviewed in ref. 91) and galectin-1 is linked to HIF directly in that the galectin-1 gene is HIF-inducible, containing two hypoxia response elements. It has been shown that it is possible to enhance anticancer effector T-cell responses within tumours by inhibiting galectin-1 function using disaccharides (79). Intratumoural hypoxia can bring about metabolic reconfiguration by increased expression of lactate dehydrogenase (113,114), inducible nitric oxide synthase and indolamine-2,3-dioxygenase (115), resulting in the accumulation of lactic acid, nitric oxide (116) and kynurenine (117) within the tumour, which in turn significantly inhibits tumour-specific CTL function (115,118). Thus, hypoxia-mediated immunosuppression has been found to be a novel and significant mechanism for promoting tumour evasion from immune cell attack.

The intratumoural hypoxic microenvironment shields the cancer stem cells

The relationship between tumour hypoxia, post-treatment relapse and distant metastasis has become well established (119). This is probably because intratumoural hypoxia contributes to providing a heterogeneous tumour microenvironment by promoting regions containing increased numbers of cancer stem-like cells (CSCs). Many cancers are now considered to contain small subsets of stem-like cells called tumour-initiating or cancer stem cells, whose numbers are elevated by hypoxia. These cells acquire many phenotypic characteristics with greater capacity for self-renewal, differentiation,

antiapoptotic features, anchorage independence and are highly pluripotent and thus can migrate to distant sites to initiate new tumour formation (120–125). There is increasing evidence for stem cell accumulation associated with post-treatment relapse occurring within tumours. Thus, a recent study by Chen *et al.* (124) on glioblastomas demonstrated that CSCs not only confer chemotherapeutic resistance but can also cause post-treatment relapse and the propagation of new tumours. In addition, the hypoxic microenvironment within tumours has been proposed to provide a conducive CSC niche where they can be protected and maintained by fuelling them with necessary intercellular and intracellular signalling (Figure 2). Stabilized HIF-1α can lead to CSC accumulation and propagation by promoting their proliferation and greater self-renewal (121,126). Cycles of hypoxia and reoxygenation were shown to enhance the proliferation of human breast CSCs that were then highly tumorigenic and metastatic when implanted into immune compromised mice (85). Therefore, it is likely that cycling hypoxia, which normally occurs within the tumour microenvironment, is an important physiological phenomenon enabling CSCs to maintain their stem-like capacity and regeneration within the heterogenic tumour microenvironments, promoting metastasis (127).

Embryonic stem cell (ESC) markers, such as the transcription factors OCT3/4 and SOX2, are instrumental in the maintenance and self-renewal of ESCs and primordial germ cells. The expressions of OCT3/4 and SOX2 are also being increasingly recognized for their roles in cancer cell survival, self-renewal, differentiation and proliferation in solid tumours, including lung, gastric, colorectal, rectal, bladder, breast, prostate and ovarian cancers (128–130). Hypoxia through HIF-αs induces the ESC factors as part of the altered transcriptional programme promoting ESC proliferation, and in many cancer cell types, hypoxia causes expression of the induced pluripotent stem cell factors, OCT4, NANOG, SOX2, KLF4 and c-Myc (in prostate, brain, kidney, cervix, lung, colon, liver and breast tumours) (123) (Figure 2). Recently, a HIF/hypoxic switch has been described in a number of different cancers, which proceeds initially *via* a transient HIF-1α activation, followed by constitutive HIF-2α expression occurring after more prolonged periods of hypoxia, and this hypoxic switch to HIF-2α enhances the tumour-initiating cell population (82,131,132). Human cancers share a common tumorigenic trait with an obligatory growth axis requiring HIF-2α as a major genetic convergence point in cancer, because inhibiting HIF-2α expression using short hairpin RNAs prevents the *in vivo* growth and tumorigenesis of a wide range of cancers, regardless of their mutational status or tissue of origin, including highly aggressive glioblastoma, colorectal and non–small-cell lung carcinomas and the *in vitro* autonomous proliferation of several others (133).

Therapeutic resistance and anticancer therapies targeting hypoxia

Hypoxic regions within tumours develop at a distance of 100–150 μm from the available blood vessels (134). In addition, to having inchoate vasculature, solid tumours also have fewer lymph nodes causing build-up of interstitial fluid pressure inhibiting intratumoural transport of macromolecules which restricts intratumoural drug delivery (135). Lactic acid produced as result of hypoxic episodes generates an acidic pH which in turn decreases the efficacy of drug uptake by tumours (136). HIF also contributes to drug resistance by irreversible cell cycle arrest, increased repair of DNA damage and inhibition of apoptosis (8,137). In more general terms, tumour resistance to chemotherapy can be due to drug pharmacokinetic constraints imposed by the tumour microenvironment and/or tumour cell intrinsic resistance by expression of the multidrug-resistance P-glycoproteins (*aka* ABC drug transporters) (8). One question that remains is whether there is an obligatory target of HIF-α activation that is essential for the subsequent tumour progression to metastasis, because targeting HIF-α is likely to be toxic (138). Several drug manufacturers and pharmaceutical companies have designed drugs whose aim is to selectively target

the chemoresistant and radioresistant hypoxic tumour cells (139). Most drugs have been aimed at inhibiting HIF proteins directly or *via* the downstream HIF-regulated proteins such as CA-IX or lysyl oxidase (140).

The Food and Drug Administration of the USA approved the camptothecin analogue, topotecan, as one of the first HIF-1α inhibiting drugs to be tested on humans, followed by PX-478 and YC-1 (139). Hypoxic tumours can also be targeted with pro-drugs that form cytotoxic adducts under low oxygen concentrations. Thus, tirapazamine, a hypoxia-activated pro-drug, was found to be effective in mouse models but failed further development due to reports of high non-specific toxicity in Phase III clinical trials (140). Other hypoxia-activated pro-drugs such as 3,5-dinitrobenzamide-2-mustard (PR-104) have shown antitumour activity, especially by targeting the hypoxic regions of tumours, as they form reactive cytotoxic hydroxylamines at low oxygen concentrations (141). Proacta's proprietary hypoxia-activated irreversible multikinase inhibitor, PR610, was recently approved by the Food and Drug Administration for Phase I and II clinical trials being conducted in the USA and New Zealand (142). Given that Wilson *et al.* (143) have recently reviewed targeting hypoxia in cancer therapy and have listed all the HIF-activated cytotoxic pro-drugs and their clinical status, these approaches will not be further discussed here.

Another interesting strategy for drug delivery targeting hypoxia is by using anaerobic facultative bacteria which possess oncolytic properties and can specifically colonize only within the hypoxic regions of solid tumours. This procedure was proposed to be particularly effective as the bacteria would fail to grow under aerobic conditions and thereby offered no threat to the well-oxygenated normal tissues. One such example is the YB1 strain of *Salmonella typhimurium*, engineered to grow under hypoxic conditions (144). Recombinant bacteria were able to produce tumour necrosis factor, HIF-1α antibody or other oncolytic biomolecules and are being developed into a potential tool for drug delivery within solid tumours (145).

Conclusion

The intratumoural hypoxic microenvironment favours tumour adaptability during states of low oxygen tension. An analogy would be like the renewed phoenix in Greek mythology arising from the embers of the nest. Stabilization of HIF-αs is identified as one of the major contributors to the hypoxic microenvironment, which in turn promotes a battery of events favouring cancer cell survival and progression. It is now clear that the hypoxic microenvironment favours primary tumours by helping them to overcome multiple hurdles on the pathway to malignancy. It shields some of the tumour cells, enabling them to escape attack and predation by the immune system. The mechanisms involved in these escape processes are being elucidated, although one essential feature appears to be the decreased activity of the PHD regulatory enzymes, thereby enhancing HIF-α protein stability, triggering production of factors like VEGF and erythropoietin to increase nourishment for the expanding cell mass *via* neovascularization. These tumour-promoting effects of HIF-α also help to maintain an expanding/renewing population of CSCs ready to be distributed much like seeds or pollen blowing in the wind. The analogy extends to these cells waiting for the right favourable opportunity or signals before planting themselves in more fertile soil elsewhere and then proceed to proliferate through the entire development and differentiation stages of cancer as distant metastases. It is evident from the review that tumour hypoxia plays a pivotal role in the tumour development, metastasis, drug resistance and post-treatment relapse and hence should be included as one of the hallmarks of cancer (146). Further research is aimed at understanding the complex relationships of the HIF family to cancer biology and their multifaceted roles within the hypoxic tumour microenvironment. These studies will help to drive the development of novel drugs that are capable of more precisely targeting tumours as well as the initiating cells, regardless of their staging to destroy the roots of origin, thereby eliminating the cause of cancer as a disease.

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