Role of hypoxia-inducible factor-1α as a cancer therapy target

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Abstract

Hypoxia occurs in solid tumours due to a mismatch between tumour growth and angiogenesis. Hypoxia in solid tumours is associated with an aggressive phenotype and resistance to radiation therapy and chemotherapy leading to poor patient prognosis. Hypoxia-inducible factor-1 (HIF-1) is a transcription factor, which is activated in response to intratumoural hypoxia and as a result of genetic alterations that activate oncogenes and inactivate tumour suppressor genes. It plays a key role in the adaptation of tumour cells to hypoxia by activating the transcription of genes, which regulate several biological processes including angiogenesis, cell proliferation and survival, glucose metabolism, pH regulation and migration. This makes HIF-1 an attractive target for the development of anticancer agents. The success of these agents depends on reliable methods to identify those patients most likely to benefit from HIF-1-targeted therapy. Several novel small molecule inhibitors of HIF-1 have been identified and are moving towards clinical trials, but none of these are specific for HIF-1. Further work is ongoing to identify more selective HIF-1 inhibitors.

Introduction

Hypoxia is a reduction in the normal level of tissue oxygen tension and occurs in many disease processes including cancer. It results in the death of both cancer cells and normal cells if it is severe or prolonged but cancer cells can adapt to this hostile environment by undergoing genetic changes, which allow them to survive and even proliferate. It is in part this ability to adapt to a hostile environment that predicts the malignant potential and aggressive phenotype of a tumour (Harris 2002).

Hypoxia can be broadly categorised into two types: acute and chronic. ‘Acute’ or ‘transient’ hypoxia occurs due to aberrant blood vessels shutting down and then reopening, thus reperfusing hypoxic tissue with oxygenated blood leading to an increase in free radical concentrations and tissue damage, a process known as ‘reoxygenation injury’. Free radicals are molecules that contain oxygen and present unpaired electrons in their outer orbit. This characteristic makes free radicals a potential source of damage to cells and tissues since they are able to react with and modify the molecular structures of lipids, carbohydrates, proteins and DNA. Cellular metabolism produces free radicals via the mitochondria but the production rate significantly increases if tissues are exposed to hypoxic microenvironments followed by reoxygenation (or to ischaemia followed by reperfusion). ‘Chronic’ or ‘diffusion-limited’ hypoxia results from tumour angiogenesis lagging behind tumour growth. The perinecrotic regions of a tumour are located at a median distance of 130 μm from blood vessels, reflecting the limit in oxygen diffusion (Beasley et al. 2001).

Hypoxia-inducible factor (HIF) is a transcription factor found in mammalian cells cultured under reduced oxygen tension and plays a key role in the cellular response to hypoxia. HIF is a heterodimer consisting of two subunits, the oxygen-sensitive HIF-α and the constitutively expressed HIF-β (also known as aryl hydrocarbon receptor nuclear translocator (ARNT), the heterodimeric partner of aryl hydrocarbon receptor (Ahr); Wang et al. 1995). Both the subunits are members of the basic helix–loop–helix-Drosophila period clock protein (PER)-ARNT-Drosophila single-minded protein family.
its COOH-terminal half: the NH2-terminal transactivation domain of transcription factors (Makino et al. 1997).

Three HIF-α homologues have been identified: HIF-1α, HIF-2α and HIF-3α (Ema et al. 1997, Tian et al. 1997, Gu et al. 1998). HIF-1α and HIF-2α share a high degree of sequence identity, which is highlighted by their common ability to heterodimerise to HIF-β (Ema et al. 1997, Tian et al. 1997). Heterodimers that contain HIF-1α or HIF-2α appear to have overlapping but distinct tissue-specific expression patterns and target genes. Less is known about HIF-3α compared with the other HIF-α homologues. It has been shown that the inhibitory PAS domain protein (IPAS) is an alternatively spliced variant of HIF-3α and functions as a dominant-negative regulator of HIF-α, adding to the complexity in the regulation of hypoxia-inducible genes by the HIF family of transcription factors (Makino et al. 2002).

HIF-1α has two transactivation domains located in its COOH-terminal half: the NH2-terminal transactivation domain or (N-TAD) (amino acids 531-575) and the COOH-terminal transactivation domain or C-TAD (amino acids 786-826; Li et al. 1996, Jiang et al. 1997, Pugh et al. 1997).

**Downregulation of HIF-1α protein levels**

In normoxia, the von Hippel-Lindau tumour suppressor (pVHL), which is the recognition component of an E3 ubiquitin ligase complex, targets HIF-1α (Iwai et al. 1999, Lisztwan et al. 1999), leading to its ubiquitylation and consequent proteasomal degradation (Lisztwan et al. 1999, Cockman et al. 2000, Kamura et al. 2000, Ohh et al. 2000, Tanimoto et al. 2000). The oxygen-dependent degradation domain (ODDD), which overlaps the N-TAD, controls the degradation of HIF-1α by the ubiquitin–proteasome pathway. Deletion of this entire domain renders HIF-1α stable even in normoxic cells (Huang et al. 1998). It has been shown that the interaction of pVHL with HIF-1α is regulated by the hydroxylation of two proline residues, proline-402 and proline-564, located within the ODDD, which are conserved between HIF-1α and HIF-2α (Ivan et al. 2001, Jaakkola et al. 2001, Masson et al. 2001). Further, detailed analysis has revealed two subdomains within the ODDD: an NH2-terminal subdomain or NOODD (amino acids 380-417; Masson et al. 2001) and a COOH-terminal subdomain or CODDD (amino acids 549–582) that overlaps the N-TAD (Pugh et al. 1997).

Epstein et al. (2001) identified egg-laying defect (EGL)-9 as a prolyl hydroxylase capable of hydroxylating HIF-1α in Caenorhabditis elegans. Three prolyl hydroxylase domain (PHD) enzymes, known as PHD1, PHD2 and PHD3, were subsequently identified in mammalian cells and shown to hydroxylate HIF-1α, although at varying levels of activity (Bruick & McKnight 2001, Epstein et al. 2001). This post-translational modification of HIF-1α by the PHDs is oxygen, iron, 2-oxoglutarate and ascorbate dependent, which may be part of the mammalian oxygen-sensing mechanism (Kivirikko & Myllyharju 1998). Interestingly, each of the PHDs hydroxylates human HIF-1α at proline-564, whereas only PHD1 and PHD2 hydroxylate the second site of prolyl hydroxylation, proline-402. Both proline residues subjected to hydroxylation lie within the LXXLAP amino acid motif in the ODDD, conserved between the worm and the mammalian HIF-1α isoforms (Epstein et al. 2001). The relative role of the three mammalian PHDs in terms of their oxygen-sensing capabilities and target proteins for modification remains to be determined. PHD2 has been shown in certain cell lines to be the predominant enzyme that hydroxylates HIF-1α in normoxia, on the basis that inhibition of PHD2 expression with RNA interference (RNAi) stabilised HIF-1α in normoxia, where inhibition of PHD1 and PHD3 had no effect (Berra et al. 2003). PHD genes have been shown to be induced in hypoxia, and that the hypoxic induction is mediated by HIF-1α (Epstein et al. 2001, Berra et al. 2003, Cioffi et al. 2003, Metzen et al. 2003). The increase in PHD expression under hypoxia may reflect the function of PHDs in degrading HIF-1α upon reoxygenation or alternatively may be part of the mechanism to balance HIF-1α in hypoxia (Nakayama & Ronai 2004). Since PHDs are components of the HIF pathway, induction of PHDs by hypoxia suggests a regulatory feedback loop in this signalling pathway. PHD activity is sensitive to changes in oxygen within a physiologically relevant range as the Km values for oxygen for PHD1, 2 and 3 are close to atmospheric oxygen concentrations. Thus, even small decreases in oxygen are likely to influence activity of the PHDs, indicating their effectiveness as oxygen sensors (Hirsila et al. 2003).

Nakayama et al. (2004) demonstrated that PHD1 and PHD3 are targeted for proteasomal degradation by the E3 ubiquitin ligases Siah1a and Siah2 under hypoxic conditions. Siah2 null fibroblasts exhibited increased stability of PHD3, resulting in lower levels of HIF-1α expression during hypoxia. Hypoxia-induced HIF-1α expression was completely inhibited in fibroblasts lacking both Siah1a and Siah2, which was reversed by inhibition of PHD3 using RNAi. Siah2 null mice exposed to hypoxia showed an impaired respiratory response and lower levels of haemoglobin compared with wild-type mice. Thus, the modulation of PHD1 and PHD3 activity by Siah1a and Siah2 adds...
a further layer of complexity in the regulation of HIF-1α in hypoxia (Nakayama et al. 2004).

A recent study by Baek et al. (2005) identified OS-9, a common protein of unassigned function, as a common binding partner for both HIF-1α and HIF-1α PHDs. OS-9 gain of function enhances prolyl hydroxylation and degradation of HIF-1α. OS-9 loss of function increases HIF-1α levels and consequently increases HIF-1-mediated transcription under normoxia.

The interaction of HIF-1α and pVHL was reported to be enhanced by acetylation of lysine-532 through a mouse homologue of an N-acetyltransferase, ADP-ribosylation factor domain protein 1 (ARD1) (Jeong et al. 2002). However, further studies have failed to validate this finding, reporting that human ARD1 (hARD1) does not affect HIF-1α stability (Arnesen et al. 2005, Murray-Rust et al. 2006). Kim et al. (2006) recently demonstrated different effects of mouse and human ARD1 variants on HIF-1α stability and acetylation.

An additional mechanism for negatively regulating HIF-1α protein levels involves interaction of the p53 tumour suppressor gene with HIF-1α, either directly or indirectly via Mdm2, itself a downstream target of p53 (Ravi et al. 2000, Bardos & Ashcroft 2005).

**Transactivation function of HIF-1**

In hypoxia, the proline residues within the ODDD are not hydroxylated and thus HIF-1α is stabilised and the protein levels increase. Stabilised HIF-1α is translocated to the nucleus where it dimerises with HIF-1β (ARNT) and associates with co-activators, such as CREB-binding protein (CBP)/p300 (Yamashita et al. 2001). The HIF-1 heterodimer induces the transcription of several hypoxia-response genes, such as the proangiogenic vascular endothelial growth factor (VEGF), by binding to hypoxia-response elements in their promoters (Forsythe et al. 1996; Fig. 1; Table 1).

The C-TAD of HIF-1α is involved in modulating the transcriptional activation of HIF-1α under hypoxic conditions, in contrast to the N-TAD, which is involved in the stabilisation of HIF-1α. Under hypoxia, the C-TAD is able to interact with transcriptional co-activators, such as CBP/p300. This interaction is unable to occur under normoxia due to the oxygen-dependent hydroxylation of asparagine-803, located within the C-TAD (Lando et al. 2002b). Hydroxylation of asparagine-803 is mediated by an asparaginyl hydroxylase, known as factor inhibiting HIF-1 (FIH-1), which prevents HIF-1α from interacting with the transcriptional co-activators CBP/p300 (Mahon et al. 2001, Lando et al. 2002a). RNA synthesis can also be inhibited by chetomin, a fungal metabolite of the Chaetomium species, which blocks the interaction of the C-TAD with p300, by disrupting the HIF-interacting domain of p300, CHI (Kung et al. 2004).

An additional mechanism of controlling HIF-1 transactivation function is through redox (reduction–oxidation)-dependent processes. Transfection of thioredoxin-1 (Trx-1), belonging to the thioredoxin family of small redox active proteins, increases HIF-1α protein levels under both normoxic and hypoxic conditions. This increase is associated with increased HIF-1 transactivation and the expression of downstream targets including VEGF and nitric oxide synthase-2 (Welsh et al. 2002). Redox effector factor-1 (Ref-1) is a protein which functions not only as a DNA repair endonuclease but also as a redox regulatory factor maintaining transcription factors in an active reduced state. Thiol-redox regulation of C-TAD activity by Trx-1 via the Ref-1 system has been reported to promote interaction of the C-TAD with CBP/p300 resulting in increased HIF-1 transactivation (Ema et al. 1999).

**Regulation of HIF-1 by oncogenic signalling pathways**

In addition to the oxygen-dependent regulation of HIF-1α, multiple other oxygen-independent oncogenic pathways serve to regulate HIF-1α, including growth factor signalling and loss of tumour suppressor genes such as pVHL (Bardos & Ashcroft 2005). Two main differences exist between growth factor and hypoxic regulation of HIF-1α expression. First, growth factor stimulation of HIF-1α is cell-type specific, whereas hypoxia increases HIF-1α expression in all cell types. Secondly, whereas hypoxia stabilises HIF-1α, growth factors and other signalling genes induce HIF-1α synthesis through activation of the phosphatidylinositol3-kinase (PI3K) or mitogen-activated protein kinase (MAPK) pathways. PI3K mediates its effects through its target Akt and the downstream kinase mTOR (mammalian target of rapamycin which is inhibited by rapamycin, a macrolide antibiotic; Semenza 2003).

mTOR regulates protein synthesis through phosphorylation of two targets: the eukaryotic translation initiation factor 4E (eIF-4E) binding protein (4E-BP1) and p70 S6 kinase (S6K). Phosphorylation of 4E-BP1 causes it to dissociate from eIF-4E and consequently increases the rate of translation initiation, as binding of 4E-BP1 to eIF-4E inhibits cap-dependent mRNA translation. Phosphorylation of S6K induces phosphorylation of its substrate, the ribosomal protein S6, leading to enhanced protein translation (Gingras et al. 2001). In contrast to the PI3K pathway, evidence for the
phosphorylation of 4E-BP1 and S6K by the MAPK pathway is contradictory (Fukuda et al. 2002, Treins et al. 2005). However, it has been shown that MAPK-integrating kinase (MNK), a downstream component of the

MAPK pathway, directly phosphorylates eIF-4E leading to increased protein synthesis (Waskiewicz et al. 1999).

Human MCF-7 breast cancer cells stimulated with heregulin, which activates the human epidermal growth

Figure 1 Oxygen regulates the rate of degradation of the HIF-1α protein. In normoxia, the proline (P) residues 402 and 564 in HIF-1α are hydroxylated by the prolyl hydroxylase domain (PHD) enzymes 1–3, allowing it to interact with the von Hippel-Lindau (VHL) tumour suppressor protein, which is the recognition component of an E3 ubiquitin ligase complex. Activity of the PHDs is oxygen, iron, 2-oxoglutarate and ascorbate dependent. The protein OS-9 promotes PHD-mediated hydroxylation of HIF-1α in an oxygen-dependent manner. In contrast, the PHDs are negatively regulated via their targeting for degradation by the Siah E3 ubiquitin ligases, under hypoxic conditions. Interaction with VHL is also promoted by acetylation of lysine (K) residue 532 by the acetyltransferase ARD1. Ubiquitylation of HIF-1α leads to its degradation by the 26S proteasome. Oxygen also regulates the interaction of HIF-1α with transcriptional co-activators. Oxygen-dependent hydroxylation of asparagine residue 803 by the asparaginyl hydroxylase, factor inhibiting HIF-1 (FIH-1), prevents HIF-1 from binding to its transcriptional co-activators CBP/p300 and therefore inhibits HIF-1 transcriptional activity. In hypoxia, the asparagine (N) residue is not hydroxylated allowing interaction between HIF-1α and CBP/p300 and the proline residues are not hydroxylated resulting in stabilisation of HIF-1α. Stabilised HIF-1α is translocated to the nucleus where it dimerises with HIF-1β (ARNT) and associates with co-activators, such as CBP/p300. The HIF-1 heterodimer binds to hypoxia-response elements (HREs), inducing transcription of target genes. Trx-1 via the Ref-1 protein promotes interaction of HIF-1α with CBP/p300 through redox processes in both normoxic and hypoxic conditions. In addition to the oxygen-dependent regulation of HIF-1α, multiple other oxygen-independent growth factor signalling pathways regulate HIF-1α through activation of the phosphatidylinositol3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, resulting in increased HIF-1α protein synthesis. Arrows indicate activating effects, truncated arrows indicate inhibitory effects and dashed lines indicate possible interactions for which only limited evidence is available. BHLH, basic helix–loop–helix; PAS, PER-ARNT-SIM; N-TAD, NH2-terminal transactivation domain; C-TAD, COOH-terminal transactivation domain; ODDD, oxygen-dependent degradation domain; NODDD, NH2-terminal subdomain; CODDD, COOH-terminal subdomain; mTOR, mammalian target of rapamycin; ERK, extracellular signal-regulated kinase; MEK, MAP/ERK kinase; MNK, MAPK-integrating kinase; eIF-4E, eukaryotic translation initiation factor 4E; 4E-BP1, eIF-4E binding protein; ARNT, aryl hydrocarbon receptor nuclear translocator.
factor receptor 2 (HER2)/Neu receptor tyrosine kinase, resulted in increased HIF-1α protein synthesis that was dependent upon activity of PI3K, Akt and mTOR (Laughner et al. 2001). The oncogene H-Ras upregulates HIF-1α protein levels leading to increased promoter activity via the PI3K pathway, known to be downstream of Ras (Chen et al. 2001). Thus, blocking the oncogenes HER2/Neu or Ras may block protein synthesis of HIF-1α or its transcriptional activity.

Conversely, loss of function mutations in tumour suppressor genes, such as pVHL and phosphatase and tensin homologue (PTEN), are associated with an increase in HIF-1α activity. Mutations in pVHL observed in renal cancers and cerebellar haemangiomas result in increased HIF-1α activity due to prevention of VHL-dependent ubiquitylation (Maxwell et al. 1999, Stebbins et al. 1999). In contrast to VHL, mutations in PTEN lead to increased stabilisation of HIF-1α due to loss of the antagonistic effect on the PI3K pathway (Zundel et al. 2000). The effect on HIF-1α activity from mutation of several other oncogenes and tumour suppressor genes was reviewed by Semenza (2003).

Several growth factors are HIF-1 target genes but also activate signal-transduction pathways on binding to their receptors, notably insulin-like growth factor-II (IGF-II) and transforming growth factor-α (TGF-α) binding to their receptors IGF-I receptor (IGF-IR) and epidermal growth factor receptor respectively. This results in a signal-transduction response leading to HIF-1α expression, thus forming an autocrine signalling pathway important for tumour progression (Semenza 2003).

**Pathways regulated by HIF**

HIF regulates the transcription of several genes involved in biological processes, such as angiogenesis, cell proliferation and survival, glucose metabolism, pH regulation and apoptosis (Semenza 2001, Harris 2002; Table 1). Erythropoietin was the first gene discovered to be under HIF regulation (Wang et al. 1995). Besides erythropoietin, another well-characterised HIF-regulated gene is VEGF, which is involved in regulating endothelial cell proliferation and blood vessel formation in both normal cells and cancer cells. Hypoxia also promotes the undifferentiated cell state in stem cells through interaction with the Notch signalling pathway. The Notch intracellular domain interacts with HIF-1α, leading to recruitment of HIF-1α to Notch-responsive promoters and activation of Notch downstream genes (Gustafsson et al. 2005). HIF-1α and VEGF, which is a direct transcriptional target of HIF-1, have also been shown to upregulate Delta 4, a ligand for the Notch receptor, important for physiological and pathological angiogeneses (Patel et al. 2005). Many of the known oncogenic signalling pathways overlap with hypoxia-induced pathways and a recent review placed HIF at the centre of the major oncogene and tumour suppressor gene pathways (Vogelstein & Kinzler 2004).

**HIF and prognosis in cancer – is HIF a good target for therapy?**

Immunohistochemical analysis has shown nuclear expression of HIF-1α in various solid tumours.

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**Table 1** Genes induced by HIF-1

<table>
<thead>
<tr>
<th>Function</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron metabolism</td>
<td>Ceruloplasmin, Transferrin, Transferrin receptor</td>
</tr>
<tr>
<td>Erythropoiesis</td>
<td>Erythropoietin, pH regulation, Carbonic anhydrase-9 and -12, Apoptosis</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>Adrenomedullin, Angiopoietin-2, Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>Cell proliferation and survival</td>
<td>Cyclin G2, Insulin-like growth factor-2, IGF-binding protein-1, -2, -3</td>
</tr>
<tr>
<td>Vascular tone</td>
<td>α1-adrenergic receptor, Endothelin-1, Haem oxygenase-1, Nitric oxide synthase-2</td>
</tr>
<tr>
<td>Collagen metabolism</td>
<td>Prolyl-4-hydroxylase-α (l)</td>
</tr>
<tr>
<td>Glucose metabolism</td>
<td>Aldolase-A, -C, Enolase-1, Hexokinase-1, -2</td>
</tr>
<tr>
<td>Glycolysis and gluconeogenesis</td>
<td>Glucose transporter-1, -3, Glyceraldehyde-3-P-dehydrogenase</td>
</tr>
<tr>
<td>Lactate dehydrogenase-A</td>
<td>Phosphofructokinase-L, Phosphoglycerate kinase-1, 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase-3</td>
</tr>
<tr>
<td>Pyruvate kinase-M</td>
<td>Triosephosphate isomerase</td>
</tr>
<tr>
<td>Regulation of HIF-1 activity</td>
<td>p30srj</td>
</tr>
</tbody>
</table>

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HIF-1α overexpression is a favourable independent prognostic factor in renal cell carcinoma (RCC) (Lidgren et al. 2005), which expresses both HIF-1α and HIF-2α due to loss of VHL function (Turner et al. 2002). In pVHL-defective RCC cells, prooncogenic genes encoding cyclin D1, transforming growth factor-α and VEGF respond specifically to HIF-2α and the proapoptotic gene encoding BNIP3 responds specifically to HIF-1α, indicating the contrasting properties of HIF-1α and HIF-2α in the biology of RCC. Overexpression of HIF-1α suppresses HIF-2α, thus suppressing the transcriptional activity of HIF-2α, which would explain the favourable prognosis associated with HIF-1α overexpression. In keeping with this, RCC tumour xenografts expressing HIF-2α showed enhanced tumour growth, in contrast to HIF-1α expressing tumours which showed retarded growth (Raval et al. 2005). Covello et al. (2005) showed a tumour-promoting role for HIF-2α by knock-in replacement of HIF-1α by HIF-2α, which stimulated teratoma growth through increased angiogenesis and cell proliferation.

HIF-1α has a dual role in that it is important for tumour growth but also apoptosis. HIF-1α may exert its proapoptotic effects through stabilisation of p53 or upregulation of BNIP3 (Greijer & van der Wall 2004). Hypoxia-induced HIF-1-mediated apoptosis might promote the survival of cells that express mutations in tumour suppressor genes and so contribute to their selection. Such a mechanism is supported by evidence that p53-null tumour cells are clonally selected and overgrow similar cells expressing wild-type p53 in hypoxic tumour regions (Graeber et al. 1996).

The question has been raised as to whether HIF-1α is a good target for cancer therapy if HIF-1 inhibition can lead to decreased expression of proapoptotic genes such as BNIP3 which may increase tumour growth. This does not appear to be the case, presumably because the proliferative and antiapoptotic effects of HIF-1 in tumours appear to outweigh any proapoptotic effects (Powis & Kirkpatrick 2004).

**Profiling tumours for hypoxia**

**How to select patients for treatment**

Hypoxia in solid tumours is associated with resistance to radiation therapy and chemotherapy, but is also associated with a poorer outcome regardless of treatment modality (Harris 2002). The evidence showing that hypoxia is important in tumour progression and prognosis has spurred research into developing therapies that target hypoxic cells. Therapeutic strategies include modification of the hypoxic
environment or targeting components of the HIF-1 signalling pathway (Wouters et al. 2002, Semenza 2003, Brown & Wilson 2004). There is therefore a need for reliable methods to identify hypoxic tumours and those patients most likely to benefit from hypoxia-targeted therapy. Presently the level of tumour oxygenation is assessed by direct or indirect methods. The main direct approach is to measure intratumoural pO2 with needle electrodes. Indirect techniques include the immunohistochemical analysis of biopsies after injection of bioreductive drug markers of hypoxia, immunohistochemical analysis of expression of hypoxia-regulated proteins, imaging techniques and more recently gene microarray analysis.

**Oxygen needle electrode**

The Eppendorf polarographic needle electrode is the main technique of direct pO2 measurement. With this technique, a calibrated needle electrode is passed into the tissue of interest along a track and a series of pO2 measurements are recorded at different points along the track. Typically multiple independent tracks are used in both normal and tumour tissue and several pO2 measurements are taken. Tissues are then defined as hypoxic or normoxic based on the median pO2 level or the ‘hypoxic fraction’ (percentage of pO2 values below a set level, typically, 2.5, 5 or 10 mmHg, designated HP2.5, HP5 and HP10 respectively; Adam et al. 1999). The ‘hypoxic subvolume’ is another parameter of tumour oxygenation, defined as the hypoxic fraction multiplied by the total tumour volume, designed to more closely correlate with the absolute number of hypoxic cells in the tumour (Stadler et al. 1999). The major advantage of the Eppendorf electrode is the ability to provide direct tumour pO2 measurements. However, use of the technique is limited by the tumour sites that are accessible to the probe (e.g. HNSCC and cervical cancer), dependency on a technically skilled user, and failure to differentiate between acute and chronic hypoxia. Nevertheless, several studies using the technique, reviewed by Evans & Koch (2003), have shown a correlation between low tumour pO2 and poor radiation treatment outcome, although the concordance between the endpoints used is variable.

**2-Nitroimidazole binding agents**

Pimonidazole hydrochloride is a 2-nitroimidazole that has been used in human clinical trials (Raleigh et al. 2000, Kaanders et al. 2002). When injected intravenously, it undergoes enzymatic reduction and binding to macromolecules at low cellular oxygen tensions. Antibodies raised against the bound products are used to visualise hypoxia. Cells binding to pimonidazole are interpreted as existing at pO2 values < 10 mmHg (Gross et al. 1995). Pimonidazole binding assays provide hypoxia measurements with a high degree of spatial resolution. Another advantage of this technique is that pimonidazole will only bind to hypoxic tumour cells that are metabolically active, thus distinguishing necrosis from viable tissue. This contrasts with oxygen electrode readings, which characterise necrotic areas as hypoxic. In a study of cervical cancer, there was no correlation between pimonidazole binding and oxygen electrode measurements of hypoxia (Nordsmark et al. 2003).

Only one study to date has examined the relationship between pimonidazole binding and radiation treatment outcome, and has shown increased rates of early locoregional recurrence (Kaanders et al. 2002).

**Immunohistochemical staining for endogenous markers of hypoxia**

Several of the proteins induced by hypoxia or the HIF-1 pathway have been evaluated as intrinsic markers of hypoxia in both serum and tissue samples, most notably carbonic anhydrase-9 (CA9), the glucose transporter, Glut-1, and VEGF, as well as HIF-1 itself. Due to the scope of this review only HIF-1α and CA9 will be discussed.

Immunohistochemical detection of HIF-1α demonstrates patterns of expression similar to that of CA9, with typical perinecrotic staining, consistent with the distribution of diffusion-limited hypoxia (Zhong et al. 1999, Talks et al. 2000, Beasley et al. 2001). The relationship between tumour HIF-1α expression and patient outcome has been discussed earlier. The difficulty in using tumour HIF-1α expression as an intrinsic marker of hypoxia is that its activation can occur in the presence of oxygen, for example in RCC due to the loss of pVHL (Lidgren et al. 2005). Since other cytokines, such as CA9, can also be regulated by oxygen-independent pathways, it is imperative that these markers are assessed in the light of comparative studies with specific hypoxia markers.

CA9 is a zinc metalloenzyme that catalyses the reversible hydration of carbon dioxide to bicarbonate and hydrogen ions and is involved in acid–base balance. The intratumoural expression of CA9 has been characterised in a variety of tumours and high CA9 expression was associated with decreased survival in HNSCC, lung, cervix and breast cancer (Chia et al. 2001, Giatromanolaki et al. 2001a, Koukourakis et al. 2001, Loncaster et al. 2001, Swinson et al. 2003). Koukourakis et al. (2001) showed HNSCCs with high CA9 expression had a poorer complete response rate to
chemoradiotherapy, whilst Loncaster et al. (2001) demonstrated that CA9 expression was an independent prognostic factor for disease-specific and metastasis-free survival but not related to local control after radiotherapy in cervical cancer.

Several studies have examined the correlation between the different techniques. In cervical cancer, there was a significant positive correlation between the level of tumour hypoxia (HP5) and the extent of CA9 expression (Loncaster et al. 2001). In another study of cervical cancer, there was a weak negative correlation between HIF-1α expression and median pO2 but no relationship with HP5 (Hutchison et al. 2004). However, Mayer et al. (2004, 2005) showed no correlation between tumour oxygenation and CA9 or HIF-1α expression in tissue samples originating from oxygen electrode tracks of locally advanced cervical cancers. To the authors’ knowledge, no studies to date in HNSCC have compared HIF-1α or CA9 expression with oxygen electrode measurements.

In cervical cancer, CA9 showed a substantial but incomplete overlap with pimonidazole in two studies (Wykoff et al. 2000, Olive et al. 2001), but no correlation in a third study (Airley et al. 2003). There was a weak but significant correlation between pimonidazole and CA9 in HNSCC, with CA9 expression observed at shorter distances from blood vessels, implying that CA9 upregulation occurs at pO2 levels higher than those required for pimonidazole binding (Kaanders et al. 2002). Additional reasons for the difference between pimonidazole and CA9 staining patterns may be temporal fluctuations of hypoxia in certain regions, the time course required for CA9 upregulation, the long half-life of CA9 once induced, and the oxygen-independent regulation of CA9 in some tumours. In a study of HNSCC, no correlation was shown between HIF-1α and pimonidazole (Janssen et al. 2002), whilst in cervical cancer, there was a weak correlation between HIF-1α and pimonidazole (Hutchison et al. 2004).

Endogenous markers have the added advantage that analysis can be performed on a biopsy, which would improve treatment planning. These markers have shown potential to provide prognostic information, but their use as a predictor of response to therapy and thus a selection tool to identify patients who may benefit from hypoxia-targeted therapy has yet to be validated. It is also possible that secreted markers of hypoxia could be monitored by a blood sample. Osteopontin (secreted phosphoprotein 1, SPP1) is a marker that is associated with tumour hypoxia and its plasma concentration is easily obtainable (Le et al. 2003).

Imaging

Positron emission tomography (PET) allows the in vivo measurement and quantification of physiological processes using short-lived positron emitting radiopharmaceuticals. Most radiopharmaceuticals under development for hypoxia detection use 2-nitroimidazole as the targeting moiety and a radioactive element, such as 18F, 67Cu or 64Cu, amenable to nuclear medicine imaging. PET and PET/CT (computed tomography) offer the possibility of in vivo mapping of regional tumour hypoxia as well as monitoring of therapy through follow-up mapping of hypoxia. In addition, PET/CT has the potential to be a single imaging modality for whole body staging. It also allows a more accurate estimation of the hypoxic tumour volume, which is important in planning radiation treatment. Numerous PET studies evaluating hypoxia in different tumour types have been conducted and were recently reviewed by Krause et al. (2006).

Gene expression profiling

DNA microarrays are platforms on which several hundred or thousand oligonucleotides or cDNA of known genes are printed. Microarray technology allows simultaneous visualisation of the expression of potentially all the genes within a cell population or tissue sample, revealing the ‘transcriptome’. The analysis of microarray data is commonly called ‘gene expression profiling’ (GEP). ‘Prognostic signatures’ can be obtained from GEP data, representing a relatively small number of genes that can be valuable in directing appropriate therapy and predicting outcome (Bucca et al. 2004). A ‘gene expression signature’ of the hypoxia response was prognostic in several different tumour types (Chi et al. 2006).

The ability to measure the expression of tens of thousands of genes in a single experiment is an enormous increase over the previously available techniques for measuring gene expression, which included northern blot and reverse transcription-PCR, where only a few genes could be studied at a time; however, these techniques are still required to confirm expression of genes after microarray analysis. The interpretation of microarray data is further complicated by the complexity of the biological systems, such as the relationship between mRNA levels and protein abundance.

The largest drawback of microarray analysis is the lack of well-defined standards for their use, interpretation and validation. In an attempt to standardise the presentation and annotations associated with microarray experiments, the Minimum Information about Microarray Experiments (MIAME) criteria have been established in order
that sufficient information is recorded about each experiment to interpret the results, enable comparisons and permit replication (Brazma et al. 2001).

GEP can distinguish between normal, premalignant and malignant epithelium and may be able to characterise more accurately the malignant or premalignant status of surgical resection margins (Akervall 2005). Evidence for the extent of its use as a prognostic biomarker for response to therapy and survival is limited at present.

### HIF-1α inhibitors

The central role of HIF-1α in the adaptive response to hypoxia and its association with poor prognosis make it a potential target for anticancer drug development. Antisense therapy against HIF-1α has been shown to reduce HIF-1α expression and transcriptional activity; however, with present technology it is only experimentally relevant in cell culture and would be difficult to apply clinically (Yeo et al. 2004). The potential of HIF-1α as a target for cancer therapy, therefore, lies in the development of small molecule inhibitors.

#### Table 2: Anticancer agents that target HIF-1 activity

<table>
<thead>
<tr>
<th>Class</th>
<th>Inhibitor</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small molecule inhibitors of HIF-1 activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP90 inhibitor</td>
<td>Geldanamycin</td>
<td>Destabilisation of HIF-1α protein or inhibition of DNA binding of HIF-1</td>
</tr>
<tr>
<td></td>
<td>17-AAG (geldanamycin analogue)</td>
<td></td>
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<tr>
<td></td>
<td>Radicicol</td>
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<tr>
<td></td>
<td>KF58333 (radicicol analogue)</td>
<td></td>
</tr>
<tr>
<td>Topoisomerase inhibitor</td>
<td>Topotecan (topoisomerase I)</td>
<td>Inhibition of HIF-1α protein expression via undetermined mechanism</td>
</tr>
<tr>
<td></td>
<td>GL331 (topoisomerase II)</td>
<td>Inhibition of HIF-1α protein expression by reduction of HIF-1α mRNA</td>
</tr>
<tr>
<td></td>
<td>Anthracycline (topoisomerase II)</td>
<td></td>
</tr>
<tr>
<td>Microtubule modifier</td>
<td>Taxane: taxol, taxotere</td>
<td>Inhibition of HIF-1α protein expression via undetermined mechanism</td>
</tr>
<tr>
<td></td>
<td>Vinca alkaloid: vincristine, vinblastine</td>
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<tr>
<td></td>
<td>2-Methoxyoestradiol (2ME2)</td>
<td></td>
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<tr>
<td></td>
<td>Epothilone B</td>
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<tr>
<td></td>
<td>Colchicine</td>
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<tr>
<td>sGC stimulator</td>
<td>YC-1</td>
<td>sGC/cGMP-independent inhibition of HIF-1α protein expression via undetermined mechanism</td>
</tr>
<tr>
<td>Trx-1 inhibitor</td>
<td>Pleurotin</td>
<td>Destabilisation of HIF-1α protein or inhibition of HIF-1 transactivating activity by inhibition of redox signalling</td>
</tr>
<tr>
<td></td>
<td>PX-12/1-methylpropyl 2-imidazolyl disulphide</td>
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</tr>
<tr>
<td>Histone deacetylase inhibitor</td>
<td>FK228</td>
<td>Inhibition of HIF-1α protein expression via undetermined mechanism</td>
</tr>
<tr>
<td>P300 CH1 inhibitor</td>
<td>Chetomin</td>
<td>Inhibition HIF-1 transactivating activity</td>
</tr>
<tr>
<td>Proteasome inhibitor</td>
<td>Bortezomib (Velcade)</td>
<td>Inhibition of HIF-1 activity via undetermined mechanism</td>
</tr>
<tr>
<td>Other</td>
<td>PX-478</td>
<td>Destabilisation of HIF-1α protein or inhibition of HIF-1 transactivating activity</td>
</tr>
<tr>
<td></td>
<td>Echinomycin</td>
<td>Inhibition of DNA binding of HIF-1</td>
</tr>
<tr>
<td></td>
<td>Flavopiridol</td>
<td>Inhibition of HIF-1α protein expression via undetermined mechanism</td>
</tr>
<tr>
<td>Inhibitors of signal transduction pathways</td>
<td></td>
<td></td>
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<tr>
<td>PI3K inhibitor</td>
<td>Wortmannin</td>
<td>Inhibition of HIF-1α protein expression by blocking translation of HIF-1α mRNA</td>
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<tr>
<td></td>
<td>LY294002</td>
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<tr>
<td>mTOR inhibitor</td>
<td>Rapamycin</td>
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<td></td>
<td>CCI-779</td>
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<td></td>
<td>Rad-001</td>
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<tr>
<td>MEK inhibitor</td>
<td>PD98059</td>
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<tr>
<td>ErbB2 receptor tyrosine kinase inhibitor</td>
<td>Trastuzumab (Herceptin)</td>
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<tr>
<td>Tyrosine kinase inhibitor</td>
<td>Imatinib (Glivec)</td>
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<tr>
<td>EGFR tyrosine kinase inhibitor</td>
<td>ZD-1839 (Iressa)</td>
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<tr>
<td></td>
<td>Erlotinib (Tarceva)</td>
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<tr>
<td>COX2 inhibitor</td>
<td>Celecoxib</td>
<td></td>
</tr>
<tr>
<td>Tyrosine kinase inhibitor</td>
<td>Genistein</td>
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</tr>
</tbody>
</table>

HSP90, heat-shock protein 90; HIF, hypoxia-inducible factor; sGC, soluble guanylate cyclase; cGMP, cyclic guanosine monophosphate; PI3K, phosphatidylinositol-3-kinase; mTOR, mammalian target of rapamycin; MEK, MAP/ERK kinase; EGFR, epidermal growth factor receptor.
development of small molecule inhibitors of HIF-1. There has been considerable drive to identify and develop compounds that inhibit HIF-1α and to establish their mechanisms of action. A number of anticancer drugs have been shown to inhibit HIF, but none of these drugs have been shown to directly and specifically target HIF-1 (Giacca et al. 2003, Semenza 2003, 2006, Powis & Kirkpatrick 2004, Yeo et al. 2004, Belozerov & Van Meir 2005, Escuin et al. 2005, Wiedmann & Caca 2005, Generali et al. 2006; Table 2). This lack of specificity increases the difficulty in attributing any anti-tumorigenic effects of these drugs specifically to inhibition of HIF-1α. However, this does not disqualify these drugs as potential anticancer agents; indeed, the fact that these drugs target multiple pathways may be beneficial if the other targets are also involved in tumourigenesis. A high-throughput screen of 2000 compounds that represent the ‘Diversity Set’ of the National Cancer Institute chemical repository, identified four specific HIF-1 inhibitors (Rapisarda et al. 2002). Further work is needed to determine their mechanism of action and their efficacy in vivo. It is likely that sensitive high-throughput screening methodologies will be instrumental in identifying specific HIF-1 targets in the future.

Summary

HIF-1 expression is increased in tumours and clinical studies have shown a correlation between HIF-1 overexpression and poor patient outcome. The activity of HIF-1 is increased in response to intratumoural hypoxia and as a result of genetic alterations that activate oncogenes and inactivate tumour suppressor genes. HIF-1 plays a key role in the adaptation of tumour cells to hypoxia by activating the transcription of genes involved in several biological processes, making it an attractive target for anticancer therapy. Several novel small molecule inhibitors of HIF-1 have been identified and are moving towards clinical trials. However, none of the presently available inhibitors exclusively target HIF; many were developed with signal transduction proteins intended as their primary target, and were subsequently shown to inhibit HIF-1, either in addition to or as a result of inhibition of their primary target. Further work is ongoing to identify more selective HIF-1 inhibitors, to determine their mechanism of action and to translate these developments into clinical trials.

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