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Hypoxia as a target for combined modality treatments

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Abstract

There is overwhelming evidence that solid human tumours grow within a unique micro-environment. This environment is characterised by an abnormal vasculature, which leads to an insufficient supply of oxygen and nutrients to the tumour cells. These characteristics of the environment limit the effectiveness of both radiotherapy and chemotherapy. Measurement of the oxygenation status of human tumours has unequivocally demonstrated the importance of this parameter on patient prognosis. Tumour hypoxia has been shown to be an independent prognostic indicator of poor outcome in prostate, head and neck and cervical cancers. Recent laboratory and clinical data have shown that hypoxia is also associated with a more malignant phenotype, affecting genomic stability, apoptosis, angiogenesis and metastasis. Several years ago, scientists realised that the unique properties within the tumour micro-environment could provide the basis for tumour-specific therapies. Efforts that are underway to develop therapies that exploit the tumour micro-environment can be categorised into three groups. The first includes agents that exploit the environmental changes that occur within the micro-environment such as hypoxia and reduced pH. This includes bioreductive drugs that are specifically toxic to hypoxic cells, as well as hypoxia-specific gene delivery systems. The second category includes therapies designed to exploit the unique properties of the tumour vasculature and include both angiogenesis inhibitors and vascular targeting agents. The final category includes agents that exploit the molecular and cellular responses to hypoxia. For example, many genes are induced by hypoxia and promoter elements from these genes can be used for the selective expression of therapeutic proteins in hypoxic tumour cells. An overview of the various properties ascribed to tumour hypoxia and the current efforts underway to exploit hypoxia for improving cancer treatment will be discussed. © 2002 Published by Elsevier Science Ltd.

Keywords: Tumour hypoxia; Radiation resistance; Gene-expression; Translational research

1. Introduction

1.1. Hypoxia is present in solid human tumours

During the past 10 years, it has become evident that solid human tumours very often contain regions that are deficient in oxygen. The presence of hypoxia has been demonstrated in cervical cancer [1,2], squamous cell carcinoma (SSC) of the head and neck [3,4], melanoma [5,6], breast [7,8] and more recently in prostate cancer [9]. The oxygen levels are typically very heterogeneous both among patients and within individual tumours. Oxygenation status has primarily been measured using either polarographic oxygen electrodes (Eppendorf) or biochemical techniques that rely upon the antibody detection of nitroimidazole-based adducts within hypoxic tissue (pimonidazole, EF5, EF1). Electrode pO_2 data have been used extensively in clinical studies and are often referred to as the 'gold standard' for determining tumour oxygenation status. However, these electrodes show no discrimination of cell type or viability and thus will record readings from less significant (radiobiologically speaking) tissue. Since pimonidazole and EF5 are selectively reduced only in viable hypoxic cells, they have a theoretical advantage for determination of relevant hypoxia. This may also explain why Eppendorf pO_2 values do not always correlate with the nitroimidazole-based hypoxia marker studies [10–12]. Reliable methods of identifying patients with hypoxic

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tumours will be increasingly important in the coming years as therapies targeting this aspect of the micro-environment approach use in the clinic.

1.2. Hypoxia is associated with poor prognosis

The presence of hypoxic cells in human tumours is considered as one of the multifactorial causes of tumour treatment resistance. Experimental and clinical evidence suggest that the hypoxic fraction in solid tumours reduces sensitivity to conventional treatment modalities, influences growth, and may increase malignant progression. Importantly, tumour hypoxia has been clinically demonstrated to predict an adverse treatment outcome in the radiotherapeutic management of cancer of the head and neck, uterine cervix and soft-tissue sarcomas [2,4,13–16]. In head and neck cancer in particular, there is strong evidence that hypoxia is associated with poor outcome of radiotherapy in terms of locoregional control, disease-free survival and overall survival [13]. This poor prognosis due to hypoxia is independent of known prognostic parameters such as clinical stage. In some cases, the prognostic value of hypoxia was shown to be independent of the treatment modality. Patients with hypoxic tumours in one series had a worse prognosis when treated with surgery alone [2]. This result implies that hypoxia may be associated with more advanced or aggressive tumours.

2. Mechanisms for worse prognosis

2.1. Treatment resistance

For many years, the importance of hypoxia in solid tumours was linked solely to the fact that hypoxic cells are intrinsically more resistant to treatment. For ionising radiation, the dose required to produce the same amount of cell killing is up to 3 times higher for hypoxic cells compared with well-oxygenated cells [17]. Chemotherapeutic drug resistance in hypoxic cells is also partially caused by reduced toxicity in the absence of molecular oxygen. Some agents, such as bleomycin, require free radicals in their mechanism of cell killing. Chemotherapeutic drug resistance can also be caused by the hypoxia-induced inhibition of cell cycle progression and proliferation, since a number of drugs specifically target highly proliferating cells. Proliferation decreases as a result of decreasing oxygen levels [18], and it has been shown that the drug toxicity falls off as a function of distance from blood vessels [19]. Furthermore, chemotherapeutic drug delivery to hypoxic areas is challenged since tumour hypoxia itself arises from insufficient and distorted vasculature. Thus the effective dose to hypoxic regions may be much less than to other parts of the tumour [19,20].

2.2. Increased malignancy

Recently, data have suggested that conditions within the tumour micro-environment, most notably hypoxia, can influence patient prognosis by means other than treatment resistance. These data have come from both the laboratory and the clinic.

2.2.1. Laboratory data

There is a wealth of data from the laboratory that implicates hypoxia as a contributor to the malignant phenotype. Hypoxia has been implicated in promoting metastasis, angiogenesis, and selection of cells with a more malignant phenotype.

2.2.1.1. Metastasis. Several experimental models have shown that tumour hypoxia is associated with an increased ability to form metastases. Young and co-workers demonstrated many years ago that murine tumour cells exposed to severe hypoxia increased their metastatic potential [21]. Similarly, in the murine KHT-C fibrosarcoma model, hypoxic primary tumours exhibit a significant increase in pulmonary metastases [22]. Other *in vitro* experiments utilising the vasculosa area of early chick embryos to grow human glioblastoma cells demonstrated that microvessel density was significantly increased under hypoxia, and that migration of tumour cells outside of the main tumour mass occurred only under hypoxic conditions [23].

Hypoxia is able to promote tumour metastasis in two ways: (1) by inducing the expression of gene products involved in the metastatic cascade and (2) by providing selection pressure for a more aggressive phenotype (see next section). The initiation of metastasis is a multistep pathway that involves three major processes: degradation of the basement membrane and extracellular matrix (ECM), modulation of cell adhesion molecules, and cell migration. Hypoxia plays a role in influencing several of these areas, thereby making it an attractive target to control tumour progression.

The importance of matrix metalloproteinases (MMPs) in tumour invasion and metastasis is widely accepted. This family of enzymes is capable of degrading constituents of the basement membrane and ECM, including fibrillar collagen, but may also contribute to metastasis through interactions with cell adhesion molecules and migration through the ECM [24]. Several studies have shown that MMP expression is associated with poor prognosis and decreased overall survival [25–27]. Canning and co-workers have shown that MDA-MB-231, a highly metastatic breast carcinoma cell line, displays reduced secretion of tissue inhibitor of metalloproteinase-1 (TIMP-1) and increased expression of MMP-9 under hypoxic conditions *in vitro* [28]. In addition, the increased invasion of MDA-MB-231 cells through matrigel filters under hypoxia can be markedly

reduced by addition of a MMP inhibitor. Similarly, in a rabbit model of myocardial infarction, cardiac myocytes show induced MMP-3 and MMP-9 expression, but downregulate TIMP-1 expression following infarction [29]. This pattern of MMP expression could be duplicated *in vitro* by culturing myocytes under hypoxic conditions, thus it seems that hypoxia is responsible for modulating MMP expression in several pathological conditions.

Activation of MMPs under hypoxia may be mediated by increased expression of urokinase-type plasminogen activator receptor (uPAR). uPAR is a cell surface receptor responsible for the binding and activation of urokinase-type plasminogen activator (uPA). Activated uPA is able to convert plasminogen into plasmin, which can then act directly in ECM degradation, and initiate the MMP activation cascade [24]. Cell surface associated uPAR is upregulated under hypoxia *in vitro*, and also contributes to invasiveness [30]. Hypoxia mediates this increased expression by increasing both transcription and stability of *uPAR* RNA [31]. There is also evidence that the association of uPAR with its ligand is directly involved in migration, independent of uPA-mediated proteolysis, which in combination with ECM degradation can markedly enhance invasion [32].

Most research regarding the regulation of cell adhesion molecules by hypoxia has focused on endothelial cells with respect to angiogenesis, with relatively few studies having been conducted using tumour cells themselves. One such study revealed that cell surface integrins and other adhesion molecules, such as CD44 and N-CAM, were transiently downregulated upon exposure to hypoxia, leading to an associated decrease in adhesion to ECM components that returned to normal levels after reoxygenation [33]. If similar changes should occur *in vivo*, this could have a significant effect on the migration of malignant cells from a hypoxic environment to a new site of tumour growth.

In addition to its pro-inflammatory properties, interleukin-8 (IL-8) has been associated with the tumourigenicity, angiogenesis, and metastasis of numerous tumours including melanoma, prostate, bladder, pancreas and ovarian cancer. *In vitro* exposure of several different cell types to hypoxia leads to elevated levels of both *IL-8* mRNA and protein [34,35]. The hypoxic regulation of *IL-8* mRNA involves increases in both the stability and transcription of the message and is dependent upon the cooperation of the AP-1 and NF-B transcription factors. *In vivo* analysis by immunohistochemistry and *in situ* hybridisation of tumour sections has localised IL-8 expression adjacent to necrotic zones, lending even further evidence to the argument that IL-8 expression is regulated by hypoxia within the tumour micro-environment [34,36]. IL-8 expression is often correlated with an aggressive phenotype and has the ability to cause non-metastatic cell lines transfected with *IL-8* cDNA to become highly tumorigenic and invasive [37,38]. IL-8 transfected cells show upregulation of *MMP-2* and *MMP-9* mRNA, collagenase activity, and increased invasiveness through Matrigel-coated filters.

2.2.1.2. Selection. Hypoxia-mediated selection of tumour cells with a diminished apoptotic potential under hypoxic conditions has been suggested as an important biological mechanism for tumour progression [39]. Graeber and colleagues used embryonic fibroblasts derived from wt and p53-deficient mice to investigate the role of p53 in hypoxia-induced apoptosis and showed that oncogenic transformation predisposed cells to hypoxia-induced killing through an apoptotic pathway modulated by p53. They also demonstrated that apoptotic regions were more prevalent in $p53^{+/+}$ tumours than in $p53^{-/-}$ tumours and that apoptotic areas colocalised with hypoxic regions, distal to adjacent blood vessels. Based on the observation that in a mixture of transformed $p53^{-/-}$ and $p53^{+/+}$ cells in a 1 to 1000 ratio, $p53^{-/-}$ cells had overtaken $p53^{+/+}$ cells after multiple rounds of hypoxia and aerobic recovery, they concluded that hypoxia could also select for apoptosis-resistant cells. Drawn primarily from these experimental results, a mathematical model has recently been developed that describes the effects of alternating periods of hypoxia and normoxia on tumours that contain wild-type and mutant p53 cells [40]. Based on independent experimental results, the model can predict the time it takes for a subpopulation of mutant p53 tumour cells to become the dominant population within defined tumour regions, both *in vitro* and *in vivo*, and provides a qualitative insight into the behaviour of mixed populations of wild-type and mutant cells growing under normoxic and hypoxic conditions. By studying the role of the human papilloma virus (HPV) *E6* and *E7* genes in sensitising human cervical epithelial cells to hypoxia, Kim and colleagues [41] consolidated the results of Graeber and colleagues and extended the relevance of these observations made in genetically manipulated rodent cells to human neoplasia. Furthermore, studies using three-dimensional cultures of human multicell spheroids have also shown that tumour cells bearing mutant p53 are able to sustain longer periods of cellular proliferation in hypoxic conditions than those with the wild-type gene [42].

The selective pressure resulting from hypoxia is not limited to the selection of cells with reduced apoptotic potential. It has also been shown to provide a possible selection force for cells that have altered oncogenic pathways that result in a switch to a more angiogenic phenotype [43].

By promoting the clonal expansion of cells with reduced apoptosis and increased angiogenesis, hypoxia can contribute to the development and malignancy of tumours. Recent clinical results showing that hypoxic

cervical cancers with a low apoptotic index are highly aggressive, strongly support this basic experimental concept [44].

2.2.1.3. Angiogenesis. Tumour progression requires the formation of new blood vessels—the process of angiogenesis—in order to provide nutrients and remove catabolites from the expanding tumour mass. Angiogenesis is also essential for the efficient dissemination of primary tumour cells during metastasis. The early steps of angiogenesis and tumour metastasis are nearly identical, as both processes involve degradation of the ECM and directed migration of either vascular or neoplastic cells. In addition, angiogenesis requires proliferation of the migrating endothelial cells. Therefore, it is not surprising to find that many of the molecules that facilitate tumour cell invasion during metastasis are also involved in angiogenesis (i.e. MMPs, the uPA system and cell adhesion molecules), and may also be regulated by hypoxia in this function.

Initiation of angiogenesis begins when cells within the tumour micro-environment respond to hypoxia by the production of the vascular endothelial growth factor (VEGF) [45]. *In vitro* studies by Rofstad's group have shown that D-12 melanoma cells expressing low VEGF levels under aerobic conditions, significantly increase VEGF secretion under hypoxia, and demonstrate increased angiogenesis and metastatic efficiency in mice [46]. In addition to VEGF, hypoxia is also responsible for inducing the expression of the VEGF receptors (VEGFR1 and VEGFR2) through HIF-1 mediated transcription [47]. Thus, it would seem that hypoxia efficiently promotes an angiogenic signal by regulating both the VEGF ligand and its receptors.

Basic fibroblast growth factor (bFGF), like VEGF, is a potent angiogenic factor, but its expression in endothelium does not appear to be directly regulated by hypoxia. bFGF binds with high affinity to heparan sulphate proteoglycans in the ECM where it remains sequestered in an inactive form until released by the FGF binding protein (FGF-BP). Upon mobilisation by FGF-BP, bFGF can exert its biological effects by signalling through one of its four receptor tyrosine kinases [48]. Hypoxia may play an indirect role in upregulating bFGF activity by inducing FGF-BP through the p38 signal transduction pathway [49,50]. Hypoxia can also regulate the amount of extracellular bFGF available to stimulate endothelial cells by inducing its secretion, along with that of platelet-derived growth factor, from macrophages that infiltrate the tumour micro-environment [51].

Integrins αvβ3 and αvβ5 are expressed on the angiogenic endothelium where they mediate adhesion with ECM components such as vitronectin. Human umbilical endothelial cells (HUVECs) exposed to 1% oxygen show increased expression of αv and β3 subunits, while β5 expression remained constant compared with aerobic controls [52]. A concomitant increase in the attachment to fibrinogen, a αvβ3-mediated process, was also observed under hypoxia. There is evidence that this integrin regulates matrix degradation through the binding of proteolytically active MMP-2, which facilitates collagen degradation *in vitro* [53]. Cell–matrix interactions can augment VEGF signal transduction through complexes of αvβ3 and VEGFR-2, whereby binding of vitronectin to its receptor results in increased VEGFR-2 kinase activity [54].

2.2.2. Clinical data

Several clinical studies support the association between hypoxia and malignancy. Data in primary uterine cervical carcinoma [1,2,15,55], soft-tissue sarcoma [4,56] and SSC of the head and neck [3,13,14,57–61] showed that tumour hypoxia was prognostic for poorer outcome, irrespective of the treatment modality. Different end-points were evaluated, locoregional control, disease-free survival, disease-specific survival or overall survival. In the study of Brizel and colleagues [13], 63 patients with head and neck cancer receiving primary radiotherapy underwent pre-treatment polarographic tumour oxygen measurement of the primary tumour or a metastatic neck lymph node. The median pO_2 for the primary lesions was 4.8 mm Hg, and it was 4.3 mm Hg for the cervical nodes. Hypoxia adversely affected 2-year local control (30 versus 73%, $P=0.01$), disease-free survival (26 versus 73%, $P=0.005$), and survival (35 versus 83%, $P=0.02$).

In general, tumour hypoxia does not depend on clinical tumour size, clinical stage, histological type, grade, extent of necrosis, or patient haemoglobin levels, and is therefore an independent predictor of outcome. Based on these results, it has been proposed that tumour hypoxia may directly influence malignancy and that the poor prognosis of hypoxic tumours is not simply a result of resistance to therapy [2,14]. Indeed, tumour hypoxia has been shown to promote lymph-vascular space involvement and parametrial infiltration in SCC of the uterine cervix [2]. Moreover, positive correlations between the lactate concentration of the primary tumour and the incidence of lymph node metastases have been demonstrated in cervical carcinoma [62] and in carcinoma of the head and neck [63]. High lactate level is indicative of extensive anaerobic metabolism and, hence, poor oxygenation in the tumour tissue [64].

There is substantial evidence that hypoxia is associated with clinical metastases and several mechanisms have been suggested. Nordsmark and colleagues demonstrated an inverse relationship between the tumour cell potential doubling time (T_{pot}) and the median tumour pO_2 in human soft tissue sarcomas [56]. The authors suggested that a high proliferation rate was confined to more hypoxic tumours. In human cervical

carcinoma, a low apoptotic index was associated with highly aggressive tumours [44]. Although experimental studies suggest that apoptotic cell kill is compromised in hypoxic tumours due to *TP53* mutations [39], no association between mutant *TP53* and hypoxia could be found in human soft-tissue sarcomas [16] or in cervical cancers [65]. In cervical cancers, a high incidence of metastases in squamous cell carcinoma of the uterine cervix is associated with poor oxygenation of the primary tumour and not with vascular density [66].

The exact mechanisms by which tumour hypoxia leads to distant metastases are still to be elucidated. Some suggestions for improving treatment strategies come from the study of Rofstad and colleagues in SCC of the uterine cervix treated with radiotherapy. The authors argue that treatment failure was primarily a result of hypoxia-induced radiation resistance rather than hypoxia-induced lymph-node metastasis, suggesting that novel treatment strategies aiming at improving tumour oxygenation or enhancing the radiation sensitivity of hypoxic tumour cells may prove beneficial to improve radiation therapy of advanced cervical carcinoma [67].

2.3. Gene expression

The multiple roles assigned to hypoxia, including the induction of angiogenesis, apoptosis and metastasis, likely result in large part from changes in gene expression that accompany hypoxia. A significant number and wide variety of hypoxia-induced genes have been described. Changes in the expression of many of these genes serve to counteract hypoxia and increase oxygenation, while others affect the cellular adaptation to decreased oxygen levels or mediate death signal pathways.

Upregulation of growth factors and hormones such as vascular endothelial growth factor (VEGF) [68], platelet-derived endothelial cell growth factor/thymidine phosphorylase (PDECGF/TP) [69] and erythropoietin (EPO) [70] results in endothelial cell proliferation and increased red blood cell production and serves to restore oxygen availability. Expression of the VEGF receptor Flt-1 is also induced in endothelial cells under hypoxic conditions [71]. Induction of the messenger molecule nitric oxide synthase (NOS) under hypoxia has been postulated as a mechanism to stimulate vasodilation resulting in increased blood flow [72].

As an adaptation to oxygen deprivation, cells need to shift their adenosine triphosphate (ATP) production from oxidative phosphorylation to anaerobic glycolysis. Thus, the activity of glycolytic enzymes such as phosphoglycerate kinase-1 (PGK-1) [73] and pyruvate kinase M (PK-M) [74] is increased during hypoxia, and the expression of glucose importer proteins (GLUTs) [75,76] are also induced.

Several genes involved in regulating cell survival, metabolism and proliferation have been reported to be induced by hypoxia, including *c-jun* [77], insulin-like growth factor-2 (*IGF-2*), IGF-binding protein 1 and 3 (*IGFBP-1* and *IGFBP-3*), transforming growth factor β (*TGF-β*) [78], placental growth factor (*P1GF*) [79], *urokinase receptor* [80], tyrosine hydroxylase (*TH*) [81], $p27^{Kip1}$ [82] and $p21^{Waf1}$ [83].

The regulation of gene expression under hypoxia has been shown to occur through many different mechanisms, including transcription, mRNA stability, translation and post-translational modifications. VEGF expression in particular is controlled at several levels by hypoxia, including increased transcription initiated by the transcription factor HIF-1 [84], enhancement of message stability by association with an RNA-binding protein HuR [85], and by increased production of a required chaperone protein ORP150 [86]. The 5′UTR of *VEGF* mRNA has also been shown to contain a functional internal ribosomal entry site (IRES), which facilitates cap-independent translation. This may serve as an advantage under hypoxic conditions where translation is low and competition for cap-dependent translation factors is high [87–89].

Cells exposed to hypoxia upregulate the expression of several transcription factors, including hypoxia-inducible factor (HIF-1) [90], p53 [91], AP-1 [92], C/EBPβ [93], early growth response 1 (Egr-1) [94] and nuclear factor κB (NFκB) [95]. Perhaps the most important within this group is HIF-1, which induces the expression of more than 30 known genes (for a review see Ref. [96]), including *EPO* [90,97], *VEGF* [98], *NOS2* [99], *Flt-1* [100], *GLUT-1* and *GLUT-3*, *PK-M* [101] and *IGF-2*. The transcription of the HIF-1-responsive genes is stimulated through the binding of HIF-1 and other transcriptional activators to a hypoxia responsive element (HRE) in the gene promoter [102–105].

The HIF-1 transcription factor itself, is regulated by a post-translational mechanism. HIF-1 is a heterodimer consisting of the two subunits, HIF-1α and HIF-1β (identical to the aryl hydrocarbon receptor nuclear translocator (ARNT)) which are both ubiquitously expressed [71,106]. HIF-1β protein is stable, while HIF-1α is targeted for ubiquitination by the von Hippel-Lindau tumour suppressor protein (VHL) and rapidly degraded by the proteasome under well-oxygenated conditions [107–109]. VHL recognises a hydroxylated prolyl residue (P564) in the HIF-1α protein, which remains unhydroxylated under hypoxic conditions [110,111]. Thus, HIF-1α is stabilised during hypoxia and can dimerise with its partner HIF-1β to induce the transcription of HRE-responsive genes.

3. How do we combat hypoxia

The realisation that hypoxia is a common characteristic of human tumours that adversely effects patient

prognosis suggests that targeting hypoxia will be an effective means of improving treatment. Scientists and clinicians alike are using two fundamentally different approaches to tackle the problems of hypoxia. The first approach is to improve or restore normal tumour oxygenation, and the second approach is to exploit the unique property of tumour hypoxia for targeting treatment to the tumour. The success of these two approaches will ultimately depend upon the relative importance of hypoxia in treatment resistance and malignancy.

3.1. Improve oxygenation

Attempts to increase the oxygen supply to the hypoxic yet potentially viable tumour cells has been a major goal of experimental and clinical research for over 40 years. Various strategies have been considered including hyperbaric or increased oxygen breathing, the administration of hypoxic cell sensitisers, and, more recently, erythropoietin to improve the haemoglobin level and to avoid repeated transfusions. Although most of the early attempts to overcome hypoxia have led to mixed results, in head and neck cancer a large meta-analysis of these trials has shown that oxygen modification results in a significant improvement in local control and disease-specific survival [112,113].

3.1.1. Erythropoietin (EPO)

EPO is a glycoprotein hormone produced by the kidney in response to tissue hypoxia that stimulates red blood cell production in the bone marrow. Currently, there is active interest in using recombinant human EPO in patients with low haemoglobin (Hb) levels in order to improve tumour oxygenation. The hypothesis is that some hypoxic tumours may result from low Hb levels in anaemic patients. Hb concentration has been shown to be an important prognostic factor for the outcome of various cancer types treated by radiotherapy. Most of the clinical studies published have shown better tumour control in patients with higher Hb levels than in patients with Hb in the lower part of—or below—the normal range. There seems to be a good documentation for the effect of Hb on radiation response in carcinoma of the uterine cervix [114–117], in head and neck cancer [118–122], in bronchogenic carcinoma [123–125], in bladder carcinoma [126–129] and prostate carcinoma [130]. Overall, patients with low haemoglobin levels have lower local control and survival. The only prospective study on the effect of transfusion on tumour control is a small study in carcinoma of the cervix [131]. Patients who were transfused to maintain their Hb level above 135 g/l showed significantly improved local control rates.

Recombinant human EPO (r-HuEPO) has been evaluated in normal subjects, as well as in subjects with various anaemic conditions. In oncology, EPO is known to increase the Hb level in cancer patients without interfering with their course of radiation therapy. In a study by Lavey and colleagues [132], the 40 participating patients had a Hb value <135 g/l and a malignant tumour located above the diaphragm without evidence of distant metastasis for which they were scheduled to undergo a 5–8 week course of daily radiation therapy. Half the patients also received 150–300 mg/kg of EPO subcutaneously (s.c.) three times per week starting 0–10 days prior to the first dose of radiation. The EPO and control groups did not differ significantly in patient age, gender, tumour type, initial Hb, erythropoietin or iron bioavailability. The Hb level increased more than 6% during radiation therapy in all 20 of the EPO patients, but in only 2/20 of the control patients ($P<0.001$). The Hb rose from a mean $\pm$ standard deviation (S.D.) of 119 ± 13 g/l to >140 g/l during radiation therapy in 80% of the EPO group compared with 5% of the control group ($P<0.001$). The mean change in Hb concentration during radiation (an average rise of 5% per week) in the EPO group significantly higher than in the control group ($P<0.001$).

Abels and colleagues also showed that approximately 50–60% of anaemic cancer patients receiving chemotherapy responded with a Hb rise of at least 20 g/l to EPO therapy given three times weekly at a dose of 150 I.U./kg over a period of 12 weeks [133]. In a subsequent open-label dose titration study, doses up to 300 IU/kg, were sometimes required, demonstrating the relative resistance to the effect of EPO in these patients. In another study, 60 anaemic patients treated with neo-adjuvant radio-chemotherapy and EPO experienced more pathological responses compared with that of a historical control group (67% versus 27%) [134]. At the moment, several phase III trials are running to test the hypothesis that an increase of Hb with EPO during radio- or chemo-therapy has the ability to improve outcome.

3.1.2. ARCON

The ARCON protocol (accelerated radiotherapy combined with carbogen and nicotinamide) is currently being evaluated in the clinic. Carbogen (95% $O_2+5\%$ CO_2) is used to reduce diffusion limited or chronic hypoxia, and nicotinamide is added to reduce acute hypoxia resulting from temporary vasculature shutdown [135–140]. The use of these agents simultaneously has indeed been shown to increase the radiation damaging effect in a variety of rodent tumour models [141–145].

Increased oxygenation of tumours treated with carbogen and nicotinamide has been demonstrated in patients [140]. Promising results have been obtained in several non-randomised clinical studies using this combination in conjunction with accelerated irradiation.

The Nijmegen radiotherapy group reported a significant beneficial effect for the treatment of stage T_3–T_4 SCC laryngeal tumours compared with historical conventional radiation therapy data, both in terms of locoregional control and survival [146,147]. Phase II clinical results obtained for bladder carcinoma also showed a significantly increased local control and overall survival from the triple combination treatment, when compared with previous experiences using standard radiotherapy [148].

However, these positive findings were not confirmed by a phase I/II study of the European Organization for Research and Treatment of Cancer (EORTC) that involved head and neck SCC tumours of various localisations [149]. EORTC studies involving non-small cell lung cancer [150], and glioblastoma were also negative [151]. A randomised phase III clinical trial will be started shortly to ultimately determine the success of this protocol.

3.1.3. Radiosensitisers

Many years have been dedicated to the search and development of compounds that could substitute for oxygen at the time of radiotherapy. This approach was based on the concept that these compounds could mimic the effects of oxygen at the time of radiation delivery, thereby increasing DNA damage and restoring radiosensivity. However, most of the compounds developed could not be administered to patients at effective concentrations with acceptable toxicity. None the less, hypoxic sensitisers continue to be developed and used in some instances. Nimorazole, a 5-nitroimidazole derivative, has been widely used as an antimicrobial agent against *Trichomonas vaginalis* and other protozoa including *Entamoeba histolytica* and *Giardia intestinalis* with little reported toxicity. Similarly, significant or chronic toxicity has been absent from the phase I and II studies involving the use of nimorazole [152,153]. In a large double-blind randomised phase III trial in Denmark, nimorazole was reported to significantly improve the effect of radiotherapy of supraglottic and pharyngeal tumours, while the toxicity of the drug was mild [154]. This result was highly significant, and nimorazole has now been incorporated into the standard treatment of most head and neck cancer patients in Denmark.

3.2. Exploit the microenvironment

The second approach in combating hypoxia is fundamentally different from attempts to restore or replace oxygen. In this scenario, the unique property of tumour hypoxia is used as an advantage for targeting cancer treatment. There are three primary means by which this targeting is currently being attempted. The first is to target the lack of oxygen *per se*, for example by using bioreductive drugs that are only toxic in the absence of oxygen. The second is to exploit the unique features of the tumour vasculature that are both responsible for and a consequence of tumour hypoxia. Finally, one can target the known molecular and cellular biological responses to hypoxia.

3.2.1. Exploit hypoxia per se

3.2.1.1. Bioreductive drugs. Bioreductive drugs are compounds that are reduced by biological enzymes to their toxic, active metabolites. They are designed such that this metabolism occurs only or preferentially in the absence of oxygen. The use of these drugs in combination with traditional therapies has the potential to greatly improve treatment outcome by increasing cytotoxicity to the hypoxic fraction. Tirapazamine (TPZ) is the leading compound in this class of agents and has shown promising results in a number of clinical trials when used in combination with cisplatin and/or radiotherapy [155–157]. A wide number of cell lines are sensitive to TPZ, regardless of their p53 status, and require 50–150 times higher dose for the same toxicity under aerobic conditions [158]. The mechanism of this preferential toxicity is mediated by an enzymatically catalysed one-electron reduction of TPZ, which yields a highly reactive radical capable of causing cell death by producing various types of DNA damage [159]. In the presence of oxygen, the TPZ radical is rapidly oxidised back to the non-toxic parental compound, thus minimising toxicity to well-oxygenated tissues. Preclinical *in vitro* testing has shown TPZ to have a synergistic effect on cell kill when given prior to cisplatin [160]. This synergism reflects the findings in animal studies [158,161] and clinical trials [162,163] showing that this combined chemotherapy potentiates the antitumour efficacy of cisplatin without increasing systemic toxicity. The mechanism of this synergism has yet to be elucidated, but has been postulated to involve the inhibition of cisplatin-induced DNA cross-link repair [160,164].

Another promising bioreductive drug nearing clinical trial is AQ4N, a prodrug that is activated by reduction in hypoxic cells producing a stable product (AQ4) that intercalates within DNA and blocks topoisomerase II action. A key advantage to this drug is that the active AQ4 is stable, thus allowing diffusion to aerobic regions where it can act to produce a 'bystander' effect, or be effective in areas of transient/acute hypoxia [165]. In murine tumour models, AQ4N is not effective as a single agent, but shows substantial antitumour activity when combined with methods to increase the hypoxic fraction (physical clamping or hydralazine), radiation, or anticancer drugs [166,167].

3.2.1.2. Gene therapy. Poor prognosis for many cancer patients prescribed conventional drug or radiation treatments has increased interest in clinical protocols based on gene therapy. The aim is to transfer genetic

material to the tumour cell or its micro-environment in quantities sufficient to obtain a therapeutic level of expression. However, strategies devised to date have limited efficiency, most notably due to deficiencies in the delivery systems employed. A recent approach to this problem employs the concept of targeting anaerobic bacteria to the hypoxic/necrotic areas of solid tumours. An association between bacteria and tumours dates back more than 100 years ago when William Coley found that certain patients who contracted bacterial infections recovered remarkably well from certain cancers. Currently, *Clostridium* spp. [168,169] and attenuated *Salmonella typhimurium* auxotrophs [170,171] are being investigated at several research centres as systems to deliver anti-tumour compounds specifically to the tumour site. The latter strain grows under aerobic and anaerobic conditions, with selectivity for tumours reported as a consequence of its auxotrophic nature. The specificity of clostridia for tumours resides in its obligate requirement for anaerobic conditions, giving *Clostridium* an advantage over *Salmonella*. Intravenously (i.v.) injected spores of a non-pathogenic clostridial species have been shown to localise to, and germinate in, the hypoxic/necrotic regions of solid tumours. Although growth alone in the tumour is not sufficient for therapeutic efficacy, the possibility now exists to engineer *Clostridium* spp. to produce a variety of therapeutic proteins with anticancer properties. Clostridia can thus be used as highly selective *in-situ* cell factories able to produce and secrete antitumour therapeutics specifically at the tumour site. Moreover, it has been shown that the immune response does not hinder repeated administration of clostridial spores, that colonisation can be improved using vascular targeting treatment using Combretastatin A4-phosphate (CA-4P) (see next section) and that gene expression can be stopped at any time using suitable antibiotics [172]. We [173] and others [174,175] demonstrated that it is possible to express therapeutic proteins, not only *in vitro*, but also *in vivo* after administration of the recombinant clostridia to tumour-bearing animals [176]. Moreover, the specificity of this gene delivery system can be further increased, by placing the therapeutic gene under the regulation of a radio-induced promoter, leading to spatial and temporal control of gene expression [177]. Taken together, these experiments demonstrate that the principle of using the *Clostridium* vector system, or other anaerobic bacteria such as *Bifidobacterium* [178], is feasible and holds considerable promise for tumour-specific therapy.

3.2.2. Exploit tumour vasculature

Abundant evidence has demonstrated that solid tumours require an expansion of the blood supply to provide their oxygen and nutritional requirements. Yet in tumours, this process of angiogenesis results in disproportional and inadequate vascular architecture, with vessels that are structurally and functionally different from those in normal tissues [179–181]. Consequently, this abnormal intra-tumoral vessel network, which elicits a high rate of endothelial cell proliferation [182], offers an ideal target for novel therapeutic strategies, such as anti-angiogenesis and vascular targeting.

3.2.2.1. Anti-angiogenesis. Angiogenesis is a complex biological process that offers potential therapeutic targets at many points [183]. The target population most often consists of actively dividing and migrating vascular endothelium from established normal host and tumour vessels. Many of the current strategies for therapeutic anti-angiogenesis involve the blockade of angiogenic growth factors and the suppression of endothelial cell recruitment through small molecule receptor blockers, specific antibodies or the use of endogenous inhibitors. The five classes of angiogenesis antagonists in current clinical trials include molecules that block matrix breakdown, inhibit endothelial cells directly, block activators of angiogenesis, inhibit endothelial-specific integrin/survival signalling and distinct mechanisms of action. Due to the large number of currently investigational anti-angiogenic approaches, we limit our discussion to a select number of drugs currently subject to clinical investigation.

The initial step in the angiogenic process is the degradation of the basement membrane surrounding the endothelial cells [184]. MMPs play a critical role in the degradative process [185]. Thus, inhibitors of MMPs are an obvious choice for anti-angiogenic strategies. Synthetic molecules such as marimastat, prinomastat, and BAY 12-9566 have been investigated as such agents. Unfortunately, phase III clinical trials using these inhibitors alone or in combination with chemotherapy have demonstrated no clinical efficacy [186]. The apparent explanation for this observation is that MMPs may be more important in the early stages of cancer and may not be required once the metastases have been established. Another method to target the enzymatic breakdown of the basement membrane and surrounding tissue is to disrupt the uPA system [187]. The urokinase inhibitor penicillamine is currently being tested in a phase II clinical trial for glioblastoma.

Molecules that inhibit endothelial cell migration and proliferation include the endogenous molecules angiostatin and endostatin [188], as well as the potent teratogen thalidomide. Angiostatin, a fragment of the precursor plasminogen was the first isolated tumour-derived angiogenesis inhibitor [189]. Treatment of experimental animals with angiostatin causes regression of the primary tumour, prevents angiogenesis and metastatic growth [189,190]. Endostatin is a C-terminal fragment of collagen type XVIII [191]. Interestingly, the activity of endostatin and angiostatin are synergistic

Table 1
Vascular targeting strategies with demonstrated preclinical antitumour activity

Hyperthermia	Damage to endothelial cells with subsequent alteration of micro-haemodynamics and vascular stasis	e.g. Refs. [246–248]
Photodynamic therapy	Aims to target directly the tumour cells, but also induces tumour cell loss through the destruction of intratumoral microvasculature	e.g. Refs. [249,250];
Tumour necrosis factor α (TNFα)	Vascular damage and subsequent blood flow failure with acute haemorrhagic intratumoral necrosis; also true for drugs that mediate their action through TNFα induction, such as flavone acetic acid (FAA) and its analogue DMXAA	e.g. Refs. [218,225,251]
Antibody-directed targeting	Targeting tissue factor to initiate thrombosis within the tumour, with the formation of central necrosis	e.g. Refs. [252]
Tubulin-interfering agents	Acute endothelial cell collapse, vessel damage and blood flow reduction, with rapid major haemorrhagic necrosis	e.g. Refs. [219,229,232,253]

DMXAA, 5,6-dimethylxanthenone 4-acetic acid.

when combined suggesting different molecular targets [192]. Both of these molecules are currently the subject of phase I clinical trials. Thalidomide has also been shown to have anti-angiogenic properties and *in vitro* data suggest that it also inhibits endothelial cell and tumour cell proliferation [193,194]. Recent reports from phase II clinical trials have shown encouraging results [195,196].

VEGF, its receptor and its signalling pathway are attractive targets for anti-angiogenic strategies. A series of compounds that target this pathway including small molecule inhibitors of the VEGF-R, such as SU5416 [197], SU6668 [198], a ribozyme that degrades *VEGF* mRNA (angiozyme) [199] and antibodies directed against VEGF [200,201] or VEGF-R (PTK-787/ZK22584) [202] have been developed and are under clinical investigation.

Interactions between tumour cells and the ECM are vitally important for invasion and migration. In particular, αvβ3 and αvβ5 integrins, serve as major receptors for ECM-mediated cell adhesion and migration [203]. These integrin molecules have been demonstrated to be upregulated during repair, retinal neovascularisation and tumour neo-angiogenesis [204–206]. This adhesion event is mediated by an arg-gly-asp (RGD) peptide motif and small peptides containing such a motif have been demonstrated to inhibit integrin function [207]. Angiogenesis is inhibited both by antibodies directed against these integrins and by peptide antagonists that block integrin–extracellular matrix interactions. A humanised monoclonal antibody directed against αvβ3, designated Vitaxin [208,209] and a small molecule blocker of αvβ3, EMD121974 are currently the subject of clinical investigation.

A number of anti-angiogenic strategies work through mechanisms distinct from those described above. CAI is an inhibitor of calcium influx [210] currently in phase I studies in combination with paclitaxel against solid tumours. Interleukin-12 (IL-12) is a multifunctional cytokine determined to be anti-angiogenic [211–213] by inducing interferon gamma and interferon-γ-inducible 10 kDa protein (IP10) [214]. Furthermore, the group B streptococcus toxin, CM101 that selectively targets proliferating blood vessels has completed phase I trials with encouraging results [215].

3.2.2.2. Vascular targeting. The concept of 'vascular targeting' was championed many years ago [181,216] and has recently become a very active area of research. This concept refers to the use of agents that exploit vasculature features that are unique within the tumour. Several advantages of targeting the vasculature have been presented including: (i) potential efficacy against any solid tumour since the main target is the endothelial cell lining, (ii) lack of treatment-induced resistance, since endothelial cells are genetically stable, (iii) accessibility of the drug and target, and (iv) indirect killing of many thousands of tumour cells from vessel damage and subsequent nutrient deprivation. This approach would also result in killing of those cells that are at intermediate levels of hypoxia, resistant to classical therapies [217]. Five different approaches to vascular targeting have been attempted in clinical settings (see Table 1).

The specificity of hyperthermia and photodynamic therapy for vasculature is somewhat limited as is the accessibility of these modalities for a variety of tumour sites. Flavone acetic acid (FAA) has been shown to be active in a variety of murine tumours [218–220]. This activity was accompanied by the induction of tumour necrosis factor α (TNFα), blood flow changes and the induction of haemorrhagic necrosis. However, changes in blood flow were not observed in patients and therefore this agent was ineffective in clinical trials [221,222]. Its structural analogue, the 5,6-dimethylxanthenone 4-acetic acid (DMXAA) compound, appears to induce TNFα more strongly in tumours than in normal tissues

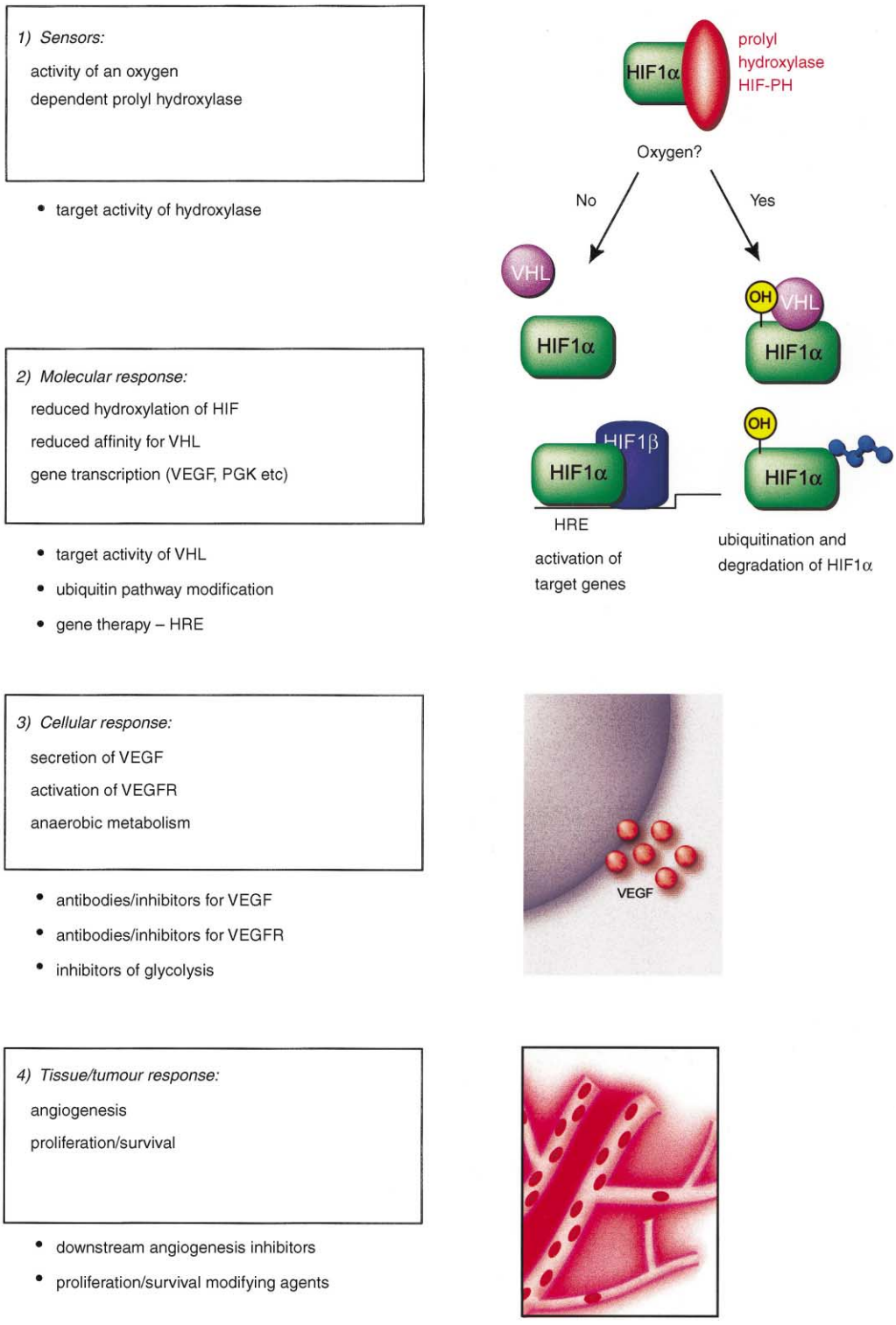

Fig. 1. Biological responses to hypoxia can be viewed in terms of four successive steps. The HIF-1 pathway serves as an example of such a response. The first step is to sense that oxygen is limiting and in the HIF-1 pathway this is carried out by an oxygen-dependent prolyl hydroxylase. The second step is the initiation of a molecular response through the activation of downstream signalling pathways. In this example, this results in the activation of several classes of genes as a result of stabilisation of the HIF-1α subunit. A cellular response occurs due to these changes in gene expression, in this case resulting in a switch to anaerobic metabolism and secretion of angiogenic factors. Finally, a tumour/tissue response occurs. In the HIF-1 pathway, this may be the induction of angiogenesis in the tumour micro-environment together with increased survival and proliferation of the tumour cells. Each of these steps in the biological response to hypoxia is an opportunity for targeting therapy as indicated below each box.

and to exert specific anti-tumour activity independently in humans [223,224]. DMXAA is presently being tested in a phase I trial both in the United Kingdom and New Zealand.

Various tubulin-interfering drugs have also been reported to provide anti-tumour activity through vasculature shutdown and the induction of haemorrhagic necrosis. This was demonstrated for the tubulin-binding drugs vincristine and vinblastine (both well known chemotherapeutics), colchicine, as well as the structurally similar compound homoharringtonine [218,220,225]. However, these effects were only observed at doses near the maximum systemically tolerable concentrations.

More recently, the combretastatin family of tubulin-binding compounds with more selective anti-tumour activity has been introduced [226]. CA-4P has been selected from this family for preclinical and clinical evaluation [219,227–232]. A single CA-4P dose of 1/3 to 1/10 of the maximum tolerable dose (rat or mouse experiments, respectively) results in rapid blood vessel damage, and subsequently tumour necrosis. The efficacy is somewhat tumour-dependent—being more effective in the mouse KHT sarcoma [230] and the WAG/Rij rat rhabdomyosarcoma [232] models than the mouse C3H mammary carcinoma [219]. Typically CA-4P results in central tumour necrosis, leaving a viable rim of cells on the edge of the tumour. CA-4P also appears to be much more effective in large tumours (>7 cm^3) compared with small (<1 cm^3) tumours [232]. The mechanism of action of CA-4P seems to result from a cell shape change that occurs in newly formed endothelial cells, resulting in blood vessel occlusion and total vascular shutdown [229,233]. Currently, a limited number of phase I clinical studies in the United States and the United Kingdom are examining the impact of CA-4P on tumour physiology, as well as general compliance and normal organ function.

3.2.3. Exploit the biological responses to hypoxia

The final strategy being pursued to target hypoxia is based on exploiting the recently understood biological responses to hypoxia. As described earlier, cells respond to hypoxia by modulating the expression of many genes. These changes in gene expression, in turn, cause a cellular and tissue response to hypoxia that affects both the cellular sensitivity to treatment and the processes of metastasis and angiogenesis. By targeting the early steps in the activation of these pathways, one may develop more specific and effective types of therapy.

Various biological responses to hypoxia can be viewed in a generalised sequence of four successive steps (see Fig. 1). The first step is carried out by an oxygen sensor—a protein that is capable of sensing and responding to reduced levels of oxygen. Activation of the sensor causes a molecular response consisting of the activation of downstream signalling pathways. This molecular response, in turn, leads to a cellular response, and finally a tissue or tumour response. In the past several years, we have learned much about one of the main hypoxic biological response pathways in mammalian cells—that involving the HIF-1 transcription factor. This pathway serves as a good example of this general response sequence and for how this knowledge can be translated into new cancer therapies.

Two recent reports suggest that the oxygen sensor in the HIF-1 pathway is a prolyl hydroxlyase [110,111]. This enzyme, designated HIF-PH, requires oxygen for its activity (hydroxylation of proline residues). In this example, the molecular response to hypoxia is initiated as a result of reduced hydroxylation of a proline residue in the HIF-1α subunit (P564). Reduced hydroxylation prevents the recognition of HIF-1α by the VHL ubiquitin ligase, thereby preventing ubiquitination. As a result, HIF-1 is stabilised and can transactivate its many targets, such as *EPO*, *VEGF* and *GLUT-1*. These changes in gene expression lead to a cellular response that may consist of increased glycolysis in the tumour cells or activation of endothelial cell proliferation and migration by binding of VEGF to its receptor. Finally, this leads to a tumour or tissue response that consists of increased angiogenesis, and to increased survival of tumour cells resulting from a switch to anaerobic metabolism [234].

The important part of this illustration is that a detailed biological understanding of this pathway offers a plethora of options for targeting cancer treatment to the tumour. For example, an attractive molecular treatment would be one based on augmenting the activity of the oxygen sensor itself. Since the multiple cellular and tissue effects stem from this one initial protein, it provides a very specific and potent treatment target. There are already many examples of research directed against the second level of this pathway. Several compounds designed to alter the activity of HIF-1 [235,236], VHL [237], or the ubiquitin system itself [238–240] are being explored in cancer treatment. At the level of the cellular response, antibodies and inhibitors of both VEGF and its receptor Flk-1 have been developed (as discussed under the anti-angiogenesis strategies). Recent reports suggest that inhibiting the ability of tumour cells to shift to glycolysis would also be advantageous [234,241]. Finally, targeting treatment to the cellular or tissue response of this pathway would consist of the more generalised anti-angiogenesis and hypoxia-targeted therapeutics (both discussed earlier). It is clear that as one moves downwards in this pathway from the oxygen sensor to the cellular and tissue responses, the targets become less specific in nature.

Elements of this pathway can be exploited as well as inhibited. For example, the DNA recognition sequence for the HIF-1 transcription factor is well described. This HRE can be inserted within gene therapy constructs, to

limit the expression of therapeutic proteins to hypoxic areas of tumours [242–245]. Dachs and colleagues [243] established the potential for tumour hypoxia to be exploited for targeted gene expression by showing that the HRE from the mouse *PGK-1* gene could be used to drive expression of heterologous genes within the mass of a solid tumour.

The HIF-1 pathway is relatively well understood and serves as a good example of how knowledge of the biological responses to hypoxia can translate into new therapies. However, there are numerous other molecular and cellular responses to hypoxia that are independent of HIF-1, perhaps each with unique oxygen sensors. Continued research into the basic molecular and cellular responses of hypoxia will undoubtedly contribute further to the development of novel hypoxia-based cancer therapies.

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