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Hypoxia modulated gene expression: angiogenesis, metastasis and therapeutic exploitation

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Abstract

Tumour hypoxia is the result of an imbalance in oxygen supply and demand. It is an adverse prognostic indicator in cancer as it modulates tumour progression and treatment. Many genes controlling tumour biology are oxygen regulated, and new ones are constantly added to the growing list of hypoxia-induced genes. Of specific importance are hypoxia-responsive transcription factors, as they can modulate the expression of numerous different genes. Similarly, growth factors which govern the formation of new blood vessels or which control blood flow are vitally important for both the maintenance of the primary tumour and metastases at distant sites. The purpose of this review is to present an update of selected issues regarding hypoxia-inducible gene expression and how this affects prognosis, angiogenesis and metastasis. It will conclude by discussing gene therapy as one possible means of exploiting tumour hypoxia for the treatment of cancer.

Keywords: Hypoxia inducible factor-1; Vascular endothelial growth factor; Nitric oxide synthase; Gene therapy

Most solid tumours larger than 1 mm^3 contain regions of low oxygen tension (hypoxia) due to an imbalance in oxygen supply and consumption. In this review, we intend to update hypoxia inducible gene expression, describe how this affects malignant progression and how it may be exploited as a tumour-specific condition. We will start by reporting on clinical tumour hypoxia and its impact on outcome, then discuss the most important oxygen-regulated genes, including transcription factors: hypoxia inducible factor-1 (*HIF-1*), specific oncogenes (*TP53*); angiogenic factors vascular endothelial growth factor (*VEGF*), platelet-derived endothelial cell growth factor (*PDECGF*) and vasoactive factors nitric oxide synthase (*NOS*) and proteins specific for metastasis: matrix metalloproteinase (*MMP*). The review will close by discussing hypoxia-regulated gene therapy as one possible way of exploiting tumour hypoxia.

1. Hypoxia in tumours

The angiogenic stimulus in tumours and rapid proliferation of tumour cells results in an abnormal and chaotic blood supply, which does not adequately or consistently supply the whole tumour with oxygen and nutrients. Since the late 1980s, when a computerised polarographic needle electrode system for oxygen measurement (Eppendorf) became commercially available, a convincing body of evidence has shown that reduced oxygen tensions are a common feature of both experimental and human solid tumours [1]. For example, in a study of human breast cancer, the median tumour oxygen partial pressure (pO_2) for all stages of the disease was found to be approximately 28 mmHg, compared with 65 mmHg for normal breast tissue. In the normal breast, pO_2 values less than 12.5 mmHg could not be detected, whereas pO_2 values less than 2.5 mmHg were detected in approximately one-third of the breast cancer cases [2]. Severe hypoxia has also been reported for head and neck cancer [3–6], cervical cancer [4,7,8] and melanomas [9]. These severely hypoxic regions can arise not only from diffusion limitations, resulting from large intercapillary distances, but also from temporal changes in blood flow [10,11] such that even the tumour endothelium is subjected to hypoxia [12,13]. Additionally, severe anaemia can lead to the development of tumour hypoxia [4,14].

Intratumour variability in oxygen measurements can be reduced by taking multiple measurements along several electrode tracks within the tumour [6]. However, intertumour variability was large in all the tumour types

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investigated, with only a weak or absent association between oxygenation and tumour size or other recorded parameters such as clinical stage, histology, extent of necrosis, etc. A minority of solid tumours, within a single tumour type, have been found to be well oxygenated. For instance, in squamous cell carcinomas of the head and neck, approximately 10% of the tumours had a median pO_2 comparable with that measured in normal tissues [15].

Cellular responses to radiation, chemotherapy and cytokines are modified by oxygen tension and this has led to clinical studies attempting to link pretreatment tumour oxygenation with treatment outcome (loco-regional control, survival or freedom from metastasis). A variety of methods aimed at overcoming radiotherapy-resistant hypoxic cells had already achieved moderate success, without any attempt to select patients for treatment on the basis of tumour oxygenation. This was most successful in cancers of the head and neck and bladder [16,17]. A link between pretreatment tumour hypoxia, as measured by oxygen electrodes, and poor treatment outcome was first shown in human cervical carcinomas [18] and soft tissue sarcomas [19]. More recent analyses, with longer follow-up times, have confirmed these results in soft tissue sarcomas, head and neck cancer and cervical cancer [3,20,21].

Interpreting results from these clinical studies has proved difficult. Classical radioresistance of hypoxic cells cannot explain the results entirely, especially as the extent of hypoxia seems to influence the outcome of treatment in patients with cervical cancer treated with surgery alone as well as with radiotherapy [18,22]. It has also been reported that primary tumour hypoxia predicts for the likelihood of distant metastases in soft tissue sarcoma [19] and cervical tumours [23] and that recurrent cervical tumours have significantly lower median oxygen partial pressure values than the primary disease [18,22]. Results such as these have led to the concept that tumour hypoxia is related to tumour progression. This is counter-intuitive, as severe hypoxia for extended periods must eventually lead to cell death. However, tumour cells can survive protracted periods of hypoxia and nutrient deprivation. It is also not clear as to whether hypoxia is an independent prognostic indicator. For instance, associated tumour characteristics such as re-oxygenation following transient hypoxia, glucose deprivation and an increase in lactate levels may also be important [24,25]. However, clinical and experimental evidence appear to be converging to suggest that tumour hypoxia acts to produce a more aggressive phenotype.

Hypoxic tumours are associated with poor treatment outcome.

A further complication to the clinical results arises from recent studies, which have sought to relate the amount of pretreatment tumour angiogenesis to treatment outcome. Several of these have found that high microvascular density (MVD) in so-called vascular hotspots (i.e. areas of the tumour with the largest number of microvessels, as identified by immunohistochemistry) is associated with poor treatment outcome [26]. At first sight, this is at variance with the hypoxia studies described above, as high intratumoural microvessel density is unlikely to be associated directly with hypoxia. However, histological studies only provide a 'snapshot' of the true dynamic state of the vasculature and associated tumour cell environment within tumours. Hypoxic tumours may also be the most pro-angiogenic, with the continual development of hypoxia, associated with tumour growth, stimulating angiogenesis, with a consequent re-oxygenation of previously hypoxic areas.

2. Hypoxic gene control

Hypoxia is recognised as a specific stimulus for gene expression. The critical genes modulated by hypoxia include transcription factors, growth factors, oncoproteins and glycolytic enzymes (Table 1). The effect of these gene products is to counteract the detrimental effects of low oxygen: angiogenic factors attract new vasculature to increase oxygenation, glucose transporters and glycolytic enzymes allow the switch to energy-saving glycolysis and oncoproteins give hypoxic tumour cells a growth advantage.

Hypoxia is a stress unrelated to ionising radiation or heat shock, but can be mimicked by carbon monoxide, desferrioxamine, and cobalt, nickel and manganese, probably due to their interference with the oxygen sensing process (for reviews see [43,44]). The effect of hypoxia on gene expression is under intense investigation. The control of erythropoietin (*EPO*), the main regulator of red blood cell production, was studied as a model to gain an understanding of how cells sense and respond to changes in oxygen tension. An oxygen-responsive enhancer sequence and its corresponding transcription factor were subsequently identified [32,45], but the exact mechanism of oxygen sensing is still unknown.

The cellular response to hypoxia consists of two main components, namely the HIF-1-dependent transcriptional regulation of stress response genes, and a hypoxia-dependent stabilisation of certain mRNAs.

2.1. Transcription factor: hypoxia inducible factor-1

The DNA binding activity of the transcription factor HIF-1 is upregulated by hypoxia [46] (Fig. 1). HIF-1 is a heterodimer consisting of the subunits HIF-1α and HIF-1β, both belonging to the basic-helix-loop-helix Per-aryl hydrocarbon receptor nuclear translocator-Sim (PAS) family of transcription factors [47]. HIF-1 is

Table 1
Genes induced by hypoxia

Gene	Function of protein	Regulated at level of: [Ref.]
Transcription factors		
HIF-1	Principal hypoxic transcription factor	
HIF-1α	Binding partner of HIF-1β	Protein stability [27]
AP-1	Stress responsive transcription factor	
jun	Binding partner of fos in AP-1	Transcription, mRNA stability [28]
NFκB	Stress responsive transcription factor	Release of IκB inhibitory unit [29]
TP53	Transcription factor, tumour suppressor, Regulator of hypoxia-induced apoptosis	Protein production? [30]
Growth factors		
EPO	Regulator of red blood cell production	Transcription, mRNA stability [31,32]
VEGF	Principal angiogenic growth factor in tumours, regulator of vessel permeability	Transcription, mRNA stability [33–35]
PD-ECGF/TP	Angiogenic growth factor, enzyme activates 5-fluorouracil (5-FU)	Protein production? [36]
NOS	Messenger molecule, regulator of vasodilation, cytotoxic defence	
ecNOS	Regulator of blood flow	Transcription? [37] Constitutive? [38]
iNOS	Regulator of blood flow, cytotoxic defence	Transcription [39–41]
ET-1	Vasoconstrictor	Transcription [42]

iNOS, inducible NOS; Et-1, endothelin 1; AP-1, activator protein 1; ecNOS, endothelial cell NOS; HIF-1, hypoxia-inducible factor-1; NFκB, nuclear factor κ B; Epo, erythropoietin; VEGF, vascular endothelial growth factor; PDECGF, platelet-derived endothelial cell growth factor; TP, thymidine phosphorylate; NOS, nitric oxide synthase.

common to all mammalian cells tested to date and was also detected in all human tissues and organs assayed [48–50]. Recently, 179 clinical tumour specimens were analysed for the HIF-1α protein subunit and 13/19 tumour types were found to overexpress it [51]. HIF-1α correlated with aberrant p53 accumulation and cell proliferation in these clinical samples. Preneoplastic lesions showed increased HIF-1α whereas benign tumours did not; in fact 29% of primary breast cancer and 69% of breast cancer metastasis overexpressed the protein. Furthermore, increased levels of HIF-1α protein in human prostate cancer cell lines correlated with increased metastatic potential [52].

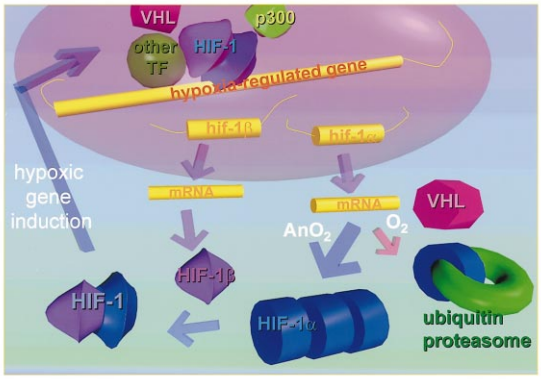

Fig. 1. Gene regulation of and by the hypoxia-inducible factor 1 (HIF-1). HIF-1 is regulated at the post-translational level by the degradation of HIF-1α via the ubiquitin proteasome in air (O_2), and under hypoxia (AnO_2) by complexing with HIF-1β thereby changing the conformation and stability of the α subunit. Active HIF-1 binds to the recognition site HRE in combination with the p300 coactivator and other transcription factors (TF), resulting in transcriptional activation of hypoxia-regulated genes. The von Hippel Lindau (VHL) protein appears to be involved both in targeting HIF-1α to the proteasome and in HIF-1′s DNA binding activity.

HIF-1α probably correlates with tumour progression.

HIF-1β is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT) which is required for the transcriptional response to xenobiotics. During the xenobiotic response, ARNT binds to its other ligand, the aryl hydrocarbon receptor (AHR, or dioxin receptor). HIF-1α and AHR compete for ARNT, with HIF-1α showing a higher affinity for ARNT than AHR [53].

Additional association partners of HIF-1α under hypoxic conditions have been identified, including the general transcription activator p300 (CBP) [54], the molecular chaperone HSP90 [53] and, recently, the von Hippel Lindau (VHL) tumour suppressor [55]. It appears that efficient hypoxia-induced transcription requires a multiprotein complex of HIF-1, p300 and other transcription factors [56]. The activation of HIF-1α is a multistep process of hypoxia-dependent nuclear import, which is dependent on the nuclear localisation sequence in the carboxy terminus, de-repression of the activation domain and finally, recruitment of the p300 coactivator [57]. HSP90 was shown to interact with HIF-1α only in air, yet appeared essential for HIF-1 activation under hypoxia [58].

von Hippel Lindau disease is a hereditary cancer predisposition resulting in highly angiogenic tumours. VHL appears to target HIF-1 for proteolysis, since constitutive HIF-1α stabilisation and activation were detected in VHL mutant cells [55]. The VHL protein was shown to be contained in the hypoxic HIF-1–DNA binding complex. On the other hand, RNA–protein complexes, which stabilise certain mRNAs under hypoxia, were shown to be constitutively elevated in cells lacking a functional VHL protein [59]. Intriguingly, an endogenous and abundant HIF-1α antisense (aHIF), arising from the downstream untranslated region of HIF-1α mRNA, was identified in tumours lacking VHL (specifically non-papillary clear cell renal carcinomas) [60]. In VHL competent cells, aHIF was markedly induced by hypoxia resulting in a temporal decline in HIF-1α mRNA *in vitro*. The authors speculated that aHIF may represent an autofeedback mechanism, which appears to be VHL responsive.

The transcriptional co-activators, steroid receptor coactivator (SRC-1), which harbours histone acetyl transferase activity, and TIF-2, a transcription intermediary factor, were recently shown to also interact with HIF-1α to potentiate hypoxic activation [61]. Several HIF-1α-like proteins have been identified, including endothelial PAS-1 (EPAS1) [62], and HIF-1α-like factor (HLF) [63].

Some controversy existed about the activation and expression of the HIF-1 subunits, but it is generally accepted now that HIF-1β is not oxygen sensitive. HIF-1α is hypoxia-regulated at the post-translational level (Fig. 1). The amount of HIF-1α protein increases during hypoxia and is degraded rapidly during re-oxygenation in intact cells [27]. HIF-1α is polyubiquinated in air, which targets it to the ubiquitin–proteasome degradation pathway, whereas ubiquination is reduced under hypoxia [64,65]. In addition, the hypoxic increase in HIF-1α protein depends on a redox-dependent increase in protein stabilisation which in turn originates from a change in conformation upon dimerisation with HIF-1β. HIF-1α and HIF-1β bind in the cytosol and dimerisation is required for stable association with the nuclear compartment [66].

Several studies using HIF-1 mutant cells have shown that HIF-1 has a profound effect on tumour biology. HIF-1β-defective mouse hepatoma cells showed absent or reduced responses to hypoxia-regulated genes *in vitro* and *in vivo* [67]. Significantly greater intravascular distances and reduced vascular density were observed in mutant compared with wild-type tumour xenografts. In association with these changes, mutant tumours grew more slowly than wild-type, and more cells needed to be implanted to achieve tumour growth [68]. The mutant xenografts were more radiosensitive than the wild-type, and could not be sensitised by the radiosensitiser misonidazole or by fractionated radiotherapy, as opposed to the wild-type tumours [69]. Tumour hypoxia of the two xenograft models was similar, indicating a difference in viable hypoxic cells. This was supported by the observations that the HIF-1 mutant cells displayed a reduced tolerance to glucose deprivation and prolonged anoxia *in vitro*. Tumours grown from HIF-1α-defective embryonic stem cells also displayed reduced hypoxia responsiveness and abnormal vascularity, but the growth rate of very large tumours was reported to be accelerated and the hypoxic fraction was increased compared with the wild-type tumours [70].

Hypoxia may also have an effect on more global gene regulation, such as methylation. The core HIF-1 binding site (5′-CGTG-3′) contains a CpG island, and methylation was shown to abolish gene activation [71].

2.2. *Tumour suppressor:* TP53

p53 Has a dual role of transcription factor and tumour suppressor. p53 has been implicated in tumour progression and is absent or mutated in more than half of human cancers. A consequence of hypoxia in cells is the induction of programmed cell death. Hypoxia-induced apoptosis was found to be reduced in cells lacking p53, and small numbers of cells lacking p53 could outgrow similar p53 wild-type cells under hypoxia *in vitro* [72]. Furthermore, highly apoptotic regions strongly correlated with hypoxic regions in p53 wild-type tumours, but not in tumours grown from p53-deficient cells. It therefore appears that tumour hypoxia can select for variants that have lost their apoptotic potential *in vivo*.

Hypoxia selects for loss of p53.

The interaction of p53 with the transcription factor HIF-1 is intriguing. High levels of p53 were shown to inhibit HIF-inducible transcription via the coactivator p300 [73]. However, p53 upregulation by hypoxia was still detected in HIF-1α mutant cells, indicating that upregulation of HIF-1α was not sufficient for hypoxic induction of p53 [74]. Recently, it has been demonstrated that p53 promoted the ubiquination and degradation of HIF-1α, and that the loss of p53 resulted in an increase in HIF-1α protein [75]. This interaction may contribute to the angiogenic switch via VEGF during tumorigenesis.

2.3. *Angiogenic factor: vascular endothelial growth factor (VEGF)*

Angiogenesis, the sprouting of new blood vessels from the pre-existing capillary bed, consists of distinct steps including hyperpermeability and mobilisation of the endothelium, cellular proliferation, canalisation of a solid capillary bud and production of periendothelial stroma.

VEGF (or vascular permeability factor) is probably the most important angiogenic growth factor in

tumours. VEGF induces endothelial cell proliferation and is therefore important in wound healing, tumour growth and metastasis. Its expression is increased during exposure to hypoxia and mRNA levels return to background levels during re-oxygenation *in vitro* [33]. Most importantly, VEGF was shown to be induced in tumour areas in close proximity to necrotic foci in gliomas [33,76]. Parallel with VEGF expression in the tumour cells, its receptors, flt-1 and flk-1/KDR, were upregulated in the surrounding endothelial cells [76,77]. VEGF accumulation was also increased in metastatic melanomas compared with primary lesions, and infiltrating inflammatory cells in all tumours analysed expressed VEGF [78]. Tumour progression of normal colon to colon carcinoma correlated with increased VEGF expression, indicating that VEGF is upregulated prior to an invasive phenotype [79].

However, VEGF expression and its effect on tumour progression are still controversial. VEGF staining was not prognostic in 156 patients with squamous head and neck carcinoma [80]. Also, VEGF expression and protein did not correlate with microvessel density or other histopathological features in hepatocellular carcinoma, whereas mvd correlated with disease-free survival [81]. Serum VEGF levels were increased after clinical surgery for lung metastases [82]. It has been proposed that VEGF plays a role in the growth of dormant micrometastases, and that a postoperative increase in VEGF levels may disrupt the suppression of angiogenesis.

Endothelin-1 (ET-1), a potent vasoconstrictor under HIF-1 control [42], was shown to interact with VEGF. VEGF enhanced pre-pro *ET-1* mRNA and ET-1 secretion *in vitro*, and ET-1 enhanced VEGF expression and secretion [83].

Recent findings questioned the universal regulation of VEGF by hypoxia. It was demonstrated that severe hypoxia was not necessarily adjacent to necrosis in glioma xenografts, and that VEGF expression and protein staining did not correspond to regions of radiobiological hypoxia [84]. Analysis of clinical samples using the hypoxia marker pimonidazole also did not correlate or co-localise with VEGF in squamous carcinoma of head and neck, and of the cervix [85]. Interestingly, pimonidazole staining co-localised strongly with a molecular marker for differentiation, involucrin [86].

Effect of VEGF on tumour progression is controversial.

The hypoxia-mediated response was shown to depend on hypoxia regulated element (HRE) sequences in the 5′ and 3′ regions of the *VEGF* gene [87,88] (Fig. 2). Hypoxia-regulated protein production does not only rely on increased transcription, but also on increased mRNA stability [34,89]. A correlation was shown between the presence of RNA–protein complexes and an increase in mRNA stability [90]. The 3′ untranslated region (UTR) of *VEGF* was found to contain five AU-rich hypoxia-inducible RNA–protein binding sites [35]. In VHL mutant cells, this RNA–protein complex was constitutively elevated resulting in stabilised *VEGF* mRNA [59]. A RNA-binding protein, HuR, has been identified and shown to specifically bind to an AU-rich element in the *VEGF* 3′UTR [91]. The steady-state levels of HuR, however, did not change under hypoxia. It has been demonstrated that not only the 3′UTR, but also the 5′UTR contained destabilising elements, which need to co-operate for normoxic instability and hypoxic stability of the mRNA [92].

VEGF also appears to be regulated at the translational level by hypoxia. The 5′UTR of the *VEGF* mRNA, being very GC-rich, forms a complicated structure which is incompatible with efficient ribosomal scanning, This region was shown to contain a functional internal ribosomal entry site (IRES) which allows translation in a cap-independent manner and represents an advantage under hypoxia, where overall translation is reduced and competition for initiation factors is high [93–95].

2.4. Angiogenic factor: platelet-derived endothelial cell growth factor (PDECGF/TP)

PDECGF/TP, a potent endothelial mitogen and chemotactic agent, was shown to be identical to the enzyme thymidine phosphorylase (TP), which regulates steady-state thymidine levels in cells and converts the chemotherapeutic agent 5-fluorouracil (5-FU) to a cytotoxic agent. It was demonstrated that PDECGF/TP could confer resistance to hypoxia-induced apoptosis via the degradation products of thymidine [96]. Studies done *in vitro* indicated that PDECGF/TP protein production and enzyme activity were increased under hypoxia in a human breast cancer cell line [36]. Immuno-

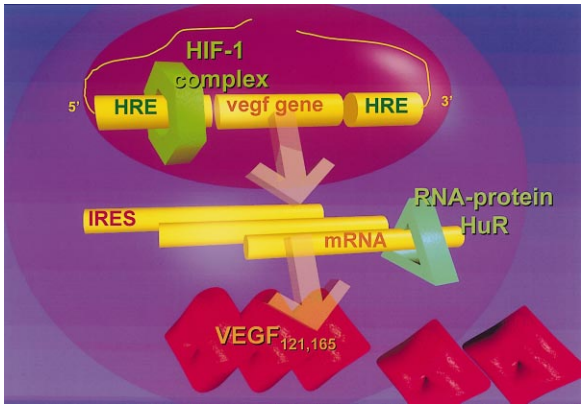

Fig. 2. Gene regulation of the vascular endothelial growth factor by hypoxia. Vascular endothelial growth factor (VEGF) production under hypoxia is regulated at the transcriptional level, via 3′ and 5′ HIF-1 recognition sites, at the post-transcriptional level, via stabilisation of the mRNA with the HuR RNA binding protein, and at the translational level, via an internal ribosome entry site (IRES).

histochemical staining of xenografted tumours showed focal expression in hypoxic regions, which became widespread when the blood supply to the tumour was restricted. Breast carcinoma cells transfected with PDECGF/TP showed an increased growth rate *in vivo* with a larger proportion of well oxygenated cells compared with untransfected tumours [97]. High expression of this angiogenic factor correlated with poor prognosis in non-small cell lung cancer [98], and with vascular density in primary breast cancer [99].

2.5. Vasoactive agent: nitric oxide synthase

Nitric oxide (NO) is an important messenger molecule, acting as a physiological vasodilator and neurotransmitter. In addition, when produced at high concentrations by stimulated macrophages, it acts as a cytotoxic defence molecule. NO produced by endothelial cells is involved in at least two important functions of the endothelium, that is maintenance of blood flow via vasodilation, induced by smooth muscle relaxation, and maintenance of an antithrombotic surface via the inhibition of platelet aggregation, both via the release of cyclic guanosine monophosphate (cGMP). There is accumulating evidence that NO-producing pathways are upregulated in a large subgroup of animal and human solid tumours and that NO is a major controlling factor of tumour blood flow (see [100,101] for reviews). At least three different isoforms of nitric oxide synthase (NOS) have been identified: the calcium-dependent forms, neuronal NOS (nNOS or NOSI) and endothelial cell NOS (ecNOS or NOSIII), and the calcium-independent form, immunological or inducible NOS (iNOS or NOSII). All three isoforms have been identified in tumours, although the exact isoenzyme complement is very variable between different studies and between different tumour types.

Hypoxia can increase the gene expression of the NOS isoforms but some conflicting data has been reported. The 5′ flanking region of *iNOS* was found to contain a functional HRE sequence [40,41]. Increased transcription of *iNOS* under chronic hypoxia was reported in tumour endothelial cells and tumour parenchyma cells *in vitro*, as well as in rat brain slices *ex vivo* and rat lungs *in vivo* [39,102–104].

Transcription of *ecNOS* is also influenced by oxygen tension, but results are contradictory. In bovine aortic endothelial cells, coronary microvessel and arterial endothelial cells, *ecNOS* transcription was increased by hypoxia *in vitro* [105,106]. However, decreased *ecNOS* mRNA and protein has been found in several bovine and human endothelial cells [38,107,108].

The precise effects of hypoxia on NOS gene expression require clarification; discrepancies between different reports may well be related to differences in the extent and duration of hypoxia, as well as varying responses between tissues [109]. Short-term hypoxia causes physiological and reversible modulation of vascular tone and blood flow, whereas chronic hypoxic stress results in irreversible remodelling of the vasculature and surrounding tissues [110], suggesting that gene expression may be different under the two conditions.

In vivo, NO production under hypoxia may be very low even if there is increased gene and protein expression of NOS, because of the requirement for co-factors and molecular oxygen for enzyme activity [111]. nNOS can couple its generation of NO to oxygen over the whole physiological range [112].

Evidence is accumulating for endogenously produced NO in tumours having a tumour growth-promoting role. Clinically, NOS activity has been positively correlated with solid tumour growth and progression [113–115]. In experimental tumours, systemic NOS inhibition was shown to be associated with a slowing of growth in experimental tumours [116,117]. Also, tumour cells transfected with *iNOS*, at clinically relevant levels, produced faster and better vascularised tumours than their untransfected counterparts [118].

The metastatic process is also influenced by NO. Transfection of mouse melanoma cells with the *iNOS* gene virtually abolished the incidence of lung metastases in a mouse model in which the cells were injected intravenously into the recipient animals [119]. However, a later paper suggested that a low to moderate expression of *iNOS* directly correlated with metastatic ability and only high levels are sufficiently toxic to suppress tumour growth and metastasis [120]. In a mouse mammary tumour model, the expression of *cNOS* by tumour cells was positively correlated with invasive and metastatic abilities and the invasion-stimulating effects of NO were related to an upregulation of matrix metalloproteinases and a downregulation of their natural inhibitors [121]. Endothelial cells in distant organs from the primary tumour also affect the incidence of metastasis by modulating the adherence of circulating cancer cells and NO can affect this process [122].

The role of NO production in tumour growth and metastasis is therefore still poorly understood. However, the evidence, so far, points to a growth-promoting effect of endogenous NO levels. NO also interacts with p53 [123]. It was suggested that NO-induced selection of mutant p53 clones contributes to a growth-promoting effect of NO [120,124]. Growth promotion is likely to be related to vascular roles of NO. Competitive inhibition of NOS, by systemic administration of analogues of L-arginine, results in a decrease in blood flow to experimental tumours [125–127], which is sufficient to cause a decline in energy status [128].

NO is probably growth-promoting in tumours.

Angiogenesis associated with wound healing is impaired in *ecNOS*-deficient mice [129] and there is increasing evidence that promotion of angiogenesis

plays a major role in the tumour growth-promoting effects of NO [130]. There is also evidence indicating that ecNOS acts downstream from VEGF in the angiogenic process [131,132]. *In vitro* studies have shown that VEGF-induced endothelial cell migration and proliferation can be strongly attenuated by NOS inhibition [133,134]. VEGF-induced activation of its flk-1/KDR receptor appears to activate ecNOS [135] and VEGF has been reported to increase both mRNA and NOS protein in endothelial cells in culture [131].

VEGF not only acts by activating ecNOS and upregulating its expression but NO itself can regulate the production of VEGF via HIF-1 [136]. Other studies have shown that NO and carbon monoxide (CO) can suppress the hypoxia-induced production of *VEGF* mRNA with reduced binding of HIF-1 to its binding site on the *VEGF* promoter HRE [137]. Interestingly, there are parallels between the biological actions of CO and NO; CO is a vasodilator and acts as a signalling molecule via cGMP. The enzyme haemoxygenase (HO) is the main biological source of CO and the inducible form, HO-1, can be upregulated by hypoxia [138]. The regulation of NO and CO production are intimately linked with an increase in NO levels causing increased transcription of the *HO-1* gene [139,140]. HO-1 may play a protective role following ischaemic damage of normal tissue by promotion of angiogenesis [138]. The tumour microenvironment is conducive to the expression of HO-1 and may contribute to the maintenance of tumour blood flow and angiogenesis [141].

2.6. Metastasis: matrix metalloproteinase (MMP) and urokinase-type plasminogen activator receptor (uPAR)

Several critical steps are involved in metastasis following tumour growth at the primary site: intravasation to the vasculature, adherence to the endothelial basement membrane at a secondary site, invasion, proliferation to give micrometastasis followed by angiogenesis and tumour growth.

MMPs (collagenase) are closely associated with a metastatic phenotype *in vitro* and *in vivo*. The MMP enzyme activity can be inhibited by complexing with the tissue inhibitor of metalloproteinases (TIMP). Tumour cells exposed to hypoxia have been shown to transiently increase their metastatic potential, especially when allowed to recover in air prior to implantation [142]. Mild *in vitro* hypoxia increased collagen production, decreased MMP-2 activity and increased TIMP-1 protein and mRNA in human proximal tubular epithelial cells [143]. The role of membrane type 1 matrix metalloproteinase (MT1-MMP), a membrane-type MMP, is to activate MMP2. Clinical samples of human breast tumours showed that only when MT1–MMP co-localised with PDECGF/TP in stroma, not in tumour cells, did it present a prognostic indicator [144].

MMP-9 showed no consistent increase following hypoxia *in vitro*, even though its promoter contained consensus AP-1 and NFκB sites [145]. uPAR is involved in the invasion of the extracellular matrix. Mild hypoxia was shown to increase *uPAR* mRNA, cell surface uPAR protein and invasion through matrix, but to decrease secreted uPAR *in vitro* [146].

3. Therapeutic exploitation of hypoxia

Hypoxia is generally perceived as a major hindrance to therapy. However, severe hypoxia is also a condition specific to solid tumours and therefore exploitable. Hypoxia-targeted gene therapy is one possible strategy to exploit tumour hypoxia which may not only target the primary tumour, but also distant metastases. Gene therapy of cancer aims to treat the malignant disease using genetic material. For a gene therapy strategy three separate issues need to be considered: delivery of a gene to the tumour, regulation of gene expression once in the tumour and the therapeutic efficacy of the genes delivered.

Hypoxic cells are known to have a reduced metabolic rate, reduced transcription and translation, and often show a cell cycle arrest. It was therefore necessary to establish the feasibility of delivering DNA under tumour conditions. Across a range of oxygen tensions and mammalian cell lines it was shown that even anoxic (0% O_2) cells could be transfected *in vitro*. Transfection efficiencies under hypoxia varied depending on the level of hypoxia, the cell line and the gene product/promoter used [147].

In an initial study of hypoxia-regulated gene expression, a triplicate HRE (originating from the phosphoglycerate kinase-1 (*PGK-1*) gene) inserted into a range of heterologous promoters was used to control the production of a marker gene by the level of hypoxia *in vitro* [148]. A correlation between decreased oxygen in the gas phase and increased reporter protein production was demonstrated, with a further increase in marker protein during re-oxygenation following hypoxia. Tumour cells stably transfected with the HRE-controlled marker gene, which were grown as xenografts in nude mice, showed focal expression of the marker gene in regions bordering necrosis. The use of a combined immunohistochemical stain and the Comet assay, which identifies hypoxic cells on a single cell basis, showed that the synthetic HRE-containing promoter could control gene expression under hypoxic conditions *in vivo* [148].

Since these initial studies were performed a range of improved constructs, including retroviral and adenoviral constructs, have been made, reporting increased levels of gene induction of up to the level of the strong viral cytomegalovirus (CMV) promoter [149,150]. When five copies of the VEGF HRE were combined with the *VEGF* 3′ mRNA destabilising region it did not result in a further improvement of hypoxia responsiveness [151].

Previously the cytosine deaminase encoding gene from *Escherichia coli* was utilised for gene-directed enzyme prodrug therapy [148]. The introduction of the bacterial gene into mammalian cells can sensitise them to the prodrug 5-fluorocytosine (5-FC), which is converted to the cytotoxic agent 5-FU. Human tumour cells transfected with hypoxia-regulated cytosine deaminase exhibited an increase in enzyme activity and sensitivity to 5-FC following hypoxia compared with normoxia. However, cells treated under anoxia could not be sensitised, probably because these cells were not actively dividing. Most enzyme–prodrug combinations inhibit DNA synthesis and need cell proliferation for their action.

Enzyme–prodrug systems for hypoxia-targeted gene therapy are therefore being developed, including bacterial and human enzymes [152,153]. The plant enzyme horseradish peroxidase (HRP) and the plant hormone indole-3-acetic acid (IAA) were utilised as an enzyme/prodrug combination for gene therapy [154]. Human T24 bladder carcinoma cells transfected with the HRP-encoding gene displayed increased cell kill after treatment with IAA. The HRP/IAA combination was equally effective under anoxic conditions [155].

As an interesting alternative, macrophages transduced with hypoxia-regulated genes have been analysed for their use as a delivery system for gene therapy [156]. Adenovirally transduced macrophages, either using a marker gene or the human cytochrome *P450 2B6* gene, were shown to infiltrate tumour spheroids *in vitro*. Cytochrome P450 2B6, usually located in the liver, is known to activate cyclophosphamide to a toxic metabolite. Macrophages were shown to be good vehicles for this enzyme–prodrug combination since they were not killed by the activated drug. CYP450 2B6 expression in transduced macrophages was increased under hypoxia, and tumour cell kill in macrophage-infiltrated spheroids depended on the presence of cyclophosphamide.

4. Conclusions

Hypoxia has been shown to be a significant factor in tumour progression and cancer treatment. Important genes that govern angiogenesis, vasocontrol and invasion are modulated by hypoxia resulting in more aggressive tumours with poor prognosis. Gene therapy targeted to hypoxic cells in tumours is an exciting proposition which still needs to be verified in the clinic.

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