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The molecular response of mammalian cells to hypoxia and the potential for exploitation in cancer therapy

GU Dachs and IJ Stratford

Medical Research Council, Harwell, Didcot, OX11 0RD, UK

Summary In this review, reports of the increased expression of selected genes in response to hypoxia have been summarised. The best studied mammalian hypoxia response systems are those of the erythropoietin (Epo) and the vascular endothelial growth factor (VEGF) genes, which will be described in some detail. Other genes discussed here include those encoding growth factors, cytokines, transcription factors, metabolic enzymes and DNA repair enzymes. Short DNA sequences (hypoxia response elements) governing the increased gene expression in response to hypoxia have been discovered in the vicinity of most of these genes. The review will end by analysing the possibility of exploiting tumour hypoxia via the use of hypoxia response elements for gene therapy of cancer.

Keywords: hypoxia; erythropoietin; vascular endothelial growth factor; gene therapy; hypoxia inducible factor 1;

Aggressive, fast-growing tumours frequently outstrip the growth of the available vascular supply, which results in areas of low oxygen tension, as well as acidic and nutrient-starved regions in tumours. Cells within this aberrant environment can often remain viable and are generally radio- and chemoresistant. Following therapy these surviving cells can repopulate and cause a relapse of the cancer. On the other hand, severe hypoxia is a condition unique to tumours, and is therefore potentially exploitable.

Oxygen levels of normal tissue and tumours have been determined, but the exact concentration in the microenvironment of an individual cell can only be interpolated. Direct measurements have been done in human tumours using a needle oxygen probe. They showed a range of median oxygen concentrations from 1.3% to 3.9% (oxygen partial pressure of 10 to 30 mmHg), whereas concentrations measured in normal tissue ranged from 3.1% to 8.7% (24 to 66 mmHg) (for a review see Vaupel, 1993). More importantly, readings of less than 0.3% O_2 (2.5 mmHg) were frequent (up to 82% of readings taken) in solid tumours, but scarce in normal tissues. This depended on the type and grade of the tumour, as well as the tissue type. Cells treated under anoxic conditions are approximately threefold more resistant than fully aerated cells to the cytotoxic effects of radiation. Radiosensitivity increases rapidly with increased oxygen concentration, with half-maximum radiosensitivity occurring at about 0.3% O_2 (for a review see Hall, 1988).

The effect of hypoxia on prokaryotic and eukaryotic cells has been analysed for many years, particularly in measuring responses to therapy. However it is only recently that the biochemical changes induced by hypoxia in cells have been evaluated at the molecular level. The purpose of this review is to describe the current knowledge of these hypoxia induced biochemical changes. Since most of the molecular analysis has been from the standpoint of respiration and physiology, rather than radiobiology, hypoxia is often regarded as low oxygen tension. In this paper the term hypoxia is used to describe a gas phase oxygen concentration of 1% O_2 unless indicated otherwise.

In order to gain an understanding of how cells sense and respond to changes in oxygen tension, we have chosen to review the biochemistry of representative proteins that reflect the diversity of the responses available to cells when subjected to hypoxia. In conclusion we analyse how such response systems, which clearly will be operational in tumours, provide a novel target for therapeutic attack and exploitation.

Correspondence: GU Dachs

Erythropoietin

The best studied mammalian hypoxia response system is that of the erythropoietin (Epo) gene. Epo is a glycoprotein hormone, expressed mainly in the kidney and fetal liver, which is the main regulator of red blood cell production. It binds to its specific receptor on erythroid progenitor cells to induce cell growth, differentiation and to prevent apoptosis. The upregulation of Epo by anaemia and hypoxia (usually 1% O_2) has been exhaustively studied. Accumulation of Epo mRNA following hypoxia is caused by both an increased rate of transcription and increased message stability (Goldberg *et al.*, 1991).

Epo production is also induced by cobalt (10-100 μM), nickel (100-400 μM) and manganese (50-600 μM), but is not increased by zinc (50-600 μM), iron (50-500 μM), cadmium (1-500 μM), tin (10-100 μM), hydrogen peroxide (100 μM), heat shock (42°C for 6-24 h), α-interferon (2×10^3 to 2×10^4 U ml^{-1}) or cyanide (Goldberg *et al.*, 1988; Goldberg and Schneider, 1994). The authors also reported that carbon monoxide (10%) and the iron chelator desferrioxamine (dfx) (130 μM), as well as the inhibition of haem synthesis, reduced the hypoxia-mediated induction of Epo. These results indicate that the Epo hypoxia response mechanism does not simply react to non-specific cellular stress but contains a specific oxygen sensor. This sensor is believed to be a haem protein that changes conformation according to its redox state, since (a) the metals that induce Epo production could simulate hypoxia by substituting for iron in the porphyrin ring, but due to their low binding affinity for oxygen they effectively lock the haem protein in the deoxy-form, and (b) carbon monoxide could block hypoxic induction by substituting for oxygen and thereby locking it in the oxy-form.

In contrast to the results reported by Goldberg *et al.* (1988), Wang and Semenza (1993*a*) showed that dfx induced Epo gene expression in air. It was confirmed that pretreatment of the cells with dfx before exposure to 1% O_2 or concurrent treatment of cells with dfx and low oxygen reduced the upregulation of Epo mRNA. The iron chelator alone however did not act as an inhibitor of gene expression. Dfx may chelate free iron which would prevent ferrous iron from binding to the porphyrin ring of the putative haem sensor and thereby induce signalling properties other than the

intact sensor in its deoxy form. Alternatively, it is possible that prolonged treatment with dfx simply has a negative effect on cellular metabolism since it was observed that the overall recovery of mRNA was reduced (Wang and Semenza, 1993*a*). But, it has been reported that cells treated with dfx were protected against lethal cell injury caused by hypoxia, subsequent reoxygenation and hydrogen peroxide (Paller and Hedlund, 1994; Lefebvre and Buccalderon, 1995).

Also, contrary to Goldberg *et al.* (1988), Fandrey *et al.* (1994) reported a decrease of hypoxia-mediated Epo production following treatment with hydrogen peroxide (500 μM), although at a higher concentration. The inhibitory effect of the higher peroxide concentration could be overcome by co-incubation with catalase. Endogenous production of peroxide depends on the pericellular oxygen tension, being lower under hypoxia than oxia. The authors speculated, that a haem protein as part of the oxygen-sensing system might generate the easily diffusible hydrogen peroxide, which under normoxia would suppress, but under hypoxia would allow the Epo gene expression.

Iron homeostasis is modulated by oxygen levels, which might effect the proposed haem oxygen sensor. The production of the major iron-binding protein ferritin is induced by hypoxia in rat oligodendrocytes and a human oligodendroglioma cell line (Qi *et al.*, 1995). Ferritin synthesis is also increased by either the release of iron from transferrin due to the a reduction of the intracellular pH or by the addition of exogenous iron. Hypoxia-mediated induction of ferritin could be blocked either by the chelator dfx or by the prevention of intracellular acidification. The authors suggested that the interference of free iron homeostasis might be an early event in oxygen sensing.

Epo synthesis is also modulated by nitric oxide (NO) and cyclic guanosine 3′,5′-monophosphate (cGMP) (Ohigashi *et al.*, 1993). NO acts as a signalling molecule that induces vasodilation, whereas the inhibition of NO synthase (NOS) activity causes vasoconstriction. NO causes relaxation of vascular smooth muscle cells by elevating intracellular cGMP levels. Treatment of ex-hypoxic polycythaemic mice with L-NAME, an NOS inhibitor, decreased Epo levels in the blood significantly. Epo levels in these mice were increased following administration of the NO donor sodium nitroprusside. *In vitro* cGMP levels were elevated in hypoxic (1% O_2) relative to aerobic cells. cGMP levels could be elevated further by nitroprusside or authentic NO but blocked by L-NAME. It had been shown previously that guanylate cyclase contains a haem moiety that could bind NO, resulting in the production of cGMP (Goldberg *et al.*, 1987). Hypoxia-mediated induction of Epo in cell lines was shown to be inhibited by L-NAME, LY 83583 (a guanylate cyclase inhibitor) or Rp-8-Bromo-cGMPS (a cGMP dependent protein kinase inhibitor) (Ohigashi *et al.*, 1993). The authors concluded that NO and cGMP may co-ordinately interact in regulating hypoxia-mediated Epo production. It has been postulated that the oxygen sensor may be a haem-containing protein. It is therefore interesting to note that NO synthase contains a haem moiety (Klatt *et al.*, 1992) and is induced by hypoxia (Pohl and Busse, 1989; Hwang *et al.*, 1994; Xue *et al.*, 1994).

Madan and Curtin (1993) defined the minimal DNA region necessary for hypoxia response of the Epo gene as a 24 base pair (bp) sequence downstream to the coding region. The isolated hypoxia enhancer could drive transcription of a luciferase reporter gene regardless of orientation, distance or whether a homologous or heterologous promoter was used. The Epo gene has a composite response to hypoxia mediated in part by the enhancer and to a lesser degree by *cis*-acting sequences within the promoter. The hypoxia-mediated induction could be increased by reducing the distance between the enhancer and the gene, and further by inserting multiple copies of the 24 bp sequence (Pugh *et al.*, 1994*a*).

The 3′ flanking region of Epo also contains binding sites for the general transcription factors AP and Sp1, as well as binding sites for the tissue specific regulatory proteins A-activator, DBP, HNF and fbr C/EBP (Leehuang *et al.*, 1993). Studies using transgenic mice defined 5′ and 3′ regions within 9 kb of the coding region and the first intron that are required for tissue specific induction by hypoxia of Epo in the liver (Madan *et al.*, 1995). Sequences needed for the induction in the kidney are located within 22 kb upstream of the gene.

Further analysis by Pugh *et al.* (1994*b*) of the 3′ region of the Epo gene in different cell lines revealed a sequence downstream of the enhancer that up- or downregulates the hypoxia response, depending on whether the cell lines produce Epo or not. Galson *et al.* (1995) analysed the *in vitro* binding of 11 orphan nuclear receptors to the nuclear hormone receptor consensus sequences within the Epo 3′ enhancer and promoter regions. They showed that hepatic nuclear factor 4 (HNF-4), TR2-11 and EAR3/COUP-TF1 bound selectively to the hexanucleotide elements of the hormone receptor sequence. Non Epo-producing cells transfected with a construct containing the luciferase gene under the control of the minimal Epo enhancer and promoter could be induced a further eightfold in response to hypoxia (1% O_2) by co-transfecting them with a construct containing the gene encoding HNF-4. This increased induction could be eliminated by the introduction of an EAR3/COUP-TF1-containing construct into the transfectants. The authors concluded that the HNF-4 protein plays an inducer role and the COUP protein family has a suppressor role in hypoxia-specific production of Epo. It is likely that HNF-4 may interact directly with the hypoxia inducible factor 1 (HIF-1, see next section) to elicit the hypoxia response, since the regulatory regions are in close proximity and both are required for optimum induction.

Hypoxia inducible factor 1

Beck *et al.* (1993) identified a 120 kDa protein that is upregulated by hypoxia (1% O_2) and binds to the Epo hypoxia enhancer sequence. This nuclear transcription factor was identified as hypoxia inducible factor 1 (HIF-1) and found to be common to a variety of Epo-producing and non-producing mammalian cells (Wang and Semenza, 1993*b*). Both the HIF-1 protein and the HIF-1 DNA binding activity are upregulated by hypoxia. The induction of HIF-1 and Epo mRNA could be prevented by an inhibitor of transcription (actinomycin D) and also by a protein kinase inhibitor (2-aminopurine), showing that both *de novo* transcription and phosphorylation are required in the signal pathway (Wang and Semenza, 1993*c*). The kinetics of HIF-1 association and dissociation with its recognition site are rapid with a $t_{1/2}$ for both of less than 1 min.

HIF-1 was subsequently found to bind to hypoxia enhancer regions of a range of genes encoding glycolytic enzymes, including aldolase A, phosphoglycerate kinase 1, enolase 1, lactate dehydrogenase A, pyruvate kinase M and phosphofructokinase L (Semenza *et al.*, 1994). The HIF-1 transcription factor functions as a heterodimer consisting of HIF-1α and HIF-1β, which are basic-helix-loop-helix proteins (Wang *et al.*, 1995). The expression of both subunits is upregulated by hypoxia and the proteins degrade rapidly upon reoxygenation. The HIF-1 protein has since been purified (Wang and Semenza, 1995). The 120 kDa HIF-1α and the 91–94 kDa HIF-1β subunits were found to be in direct contact with the DNA.

Vascular endothelial growth factor

The production of vascular endothelial growth factor (VEGF, VPF) is regulated by hypoxia in a fashion similar to that of Epo. VEGF mRNA levels are increased during exposure to hypoxia and return to background levels during reoxygenation (Shweiki *et al.*, 1992). The oxygen concentrations used for *in vitro* experiments ranged from 1% O_2 to catalyst-induced anoxia, with increased expression of VEGF with decreased oxygen tension. It is also upregulated in response to cobalt, nickel and manganese, it is inhibited by

carbon monoxide and it requires protein synthesis for the hypoxia-responsive induction (Goldberg and Schneider, 1994). Recently it was shown that VEGF is upregulated by another condition common to tumours, namely glucose deficiency (Shweiki *et al.*, 1995). Increased VEGF expression was observed in monolayer cultures and multicellular spheroids exposed to either low oxygen or decreased glucose. Tissue culture cells simultaneously exposed to both forms of stress did not increase VEGF synthesis, presumably due to a reduced metabolic rate brought about by the deleterious effects of the combination of these conditions.

VEGF specifically targets endothelial cells to induce proliferation. It is therefore important in wound healing and tumour growth, since both need the sprouting of new blood vessels (angiogenesis). VEGF is a dimeric glycoprotein whose structure resembles that of two other angiogenic growth factors, namely- platelet derived growth factor (PDGF) (Plate *et al.*, 1992*a*) and placenta growth factor (PLGF) (Gleadle *et al.*, 1995). Different variants of VEGF have been identified as alternative splice forms of the mRNA, with $VEGF_{165}$ and $VEGF_{121}$ being the secreted splice forms of the protein. All major isoforms of VEGF are upregulated by hypoxia. Very importantly, VEGF was found to be upregulated *in vivo* in tumour areas in close proximity to necrotic foci (Shweiki *et al., 1992; Plate et al.*, 1992*b*). In biopsies from glioblastomas, which are the most common and most malignant brain tumours in humans, increased levels of VEGF mRNA were detected in a narrow strip along necrotic regions of the tumour cells. Capillary bundles were seen alongside these VEGF expressing cells. Plate *et al.* (1992*b*, 1993) analysed this phenomenon further and found that concurrent with VEGF expression in the tumour cells, its cognate receptors, Flt-1 and Flk-1 (KDR/Flk), were upregulated in the surrounding endothelial cells. The VEGF receptors Flt-1 and Flk-1 are highly homologous tyrosine kinase receptors which also function as PDGF receptors. Flt-1 was found to be upregulated in tumour endothelial cells, but not in the normal brain endothelium.

The expression of VEGF and its receptors is also upregulated in normal lung tissue by *ex vivo* hypoxic perfusion (ventilating with nitrogen for 2 h) of isolated lungs (Tudor *et al.*, 1995). Similar to Epo, VEGF production is modulated by NO, but apparently differently. The NO donor sodium nitroprusside decreased and the inhibitor of NO synthesis L-NAME increased synthesis of both VEGF and its receptors in normoxic and hypoxic lung tissue. This is opposite to the effects seen on Epo production (Ohigashi *et al.*, 1993) and might be due to the different oxygen concentrations used or the diversity of sensing mechanisms available for controlling expression of the different genes.

Ladoux and Frelin (1993) examined previous sequencing data (Tischer *et al.*, 1991) and discovered a sequence in the 3′ region of the human VEGF gene that is highly homologous to 12 bp of the hypoxia responsive elements near the EPO gene. Minchenko *et al.* (1994) analysed the functional hypoxia-mediated response of the regulatory elements in the 3′ and the 5′ flanking regions of the gene. The Epo-like downstream enhancer is contained within a 160 bp fragment located 60 bp from the polyadenylation site. The upstream enhancer sequences are structurally unrelated to the Epo enhancer and are contained within 100 bp located 800 bp from the start of the coding region. There is no apparent difference in the hypoxia or cobalt mediated response of the two enhancer elements. A binding site for the transcription factor AP-1, which is a dimer consisting of the fos and jun proteins, was found in the 5′-flanking region of VEGF. The expression of members of the fos and jun family of proteins was found to be upregulated in response to low oxygen (1% O_2) (Goldberg and Schneider, 1994). However, studies using dexamethasone as an inhibitor of AP-1 activity showed that the binding of AP-1 is not required for hypoxia-mediated induction of VEGF (Finkenzeller *et al.*, 1995).

The signal transduction pathway that leads to the induction of VEGF by hypoxia (gassing with 95% N_2, 5% CO2) has been studied (Mukhopadhyay *et al.*, 1995). VEGF induction could be inhibited by either an inhibitor of protein tyrosine kinase or a dominant negative mutant form of the kinase c-Src or Raf-1, which acts downstream of Src signalling. The kinase activity of c-Src was increased by the exposure to hypoxia, but other members of the Src family of tyrosine kinases (Fyn and Yes) were not effected. VEGF induction by hypoxia is impaired in *c-src* mutant cells and c-Src overexpression in transfected cell lines causes an increase in VEGF mRNA under hypoxia. It was therefore concluded that a tyrosine kinase cascade, including c-Src, forms part of the hypoxia response of mammalian cells.

Other growth factors and cytokines

Although VEGF is most studied, the response of other growth factors and cytokines to hypoxic stress has been examined. Fibroblasts exposed to hypoxia showed enhanced proliferation in response to serum and growth factors such as epithelial growth factor (EGF) (Storch and Talley, 1988). The EGF receptor (EGF-R) is a transmembrane glycoprotein containing an extracellular ligand-binding domain and an intracellular tyrosine kinase domain (Ullrich and Schlessinger, 1990). Both the mRNA and the protein levels of EGF-R were upregulated by hypoxia ($< 0.2\%$ O_2) in three human squamous carcinoma and two normal cell lines (Laderoute *et al.*, 1992*a*). A further increase in the receptor proteins was detected when the cells were allowed to reoxygenate following hypoxia.

Another important angiogenic growth factor is the basic fibroblast growth factor (bFGF). Although hypoxia (1.8% O_2) inhibits the synthesis of bFGF in endothelial cells, it induces bFGF production and release in macrophages (Kuwabara *et al.*, 1995). The acidic FGF (aFGF) and the platelet-derived growth factor (PDGF) proteins were upregulated simultaneously with bFGF in the macrophages. It was suggested that their expression was regulated via the same oxygen-sensing haem protein as Epo, since their production was also increased by the exposure to nickel and cobalt. However, unlike VEGF, bFGF synthesis was not induced by glucose deprivation (Shweiki *et al.*, 1995).

Treatment of vascular smooth muscle cells with mild hypoxia (2.5% O_2) was shown to increase the expression of VEGF marginally, whereas the combined treatment with mild hypoxia and either bFGF or transforming growth factor β (TGF-β) showed a marked synergistic effect on VEGF production (Stavri *et al.*, 1995). The interaction of the two growth factors with VEGF indicates that angiogenesis could be induced both by the direct action of each of the factors as well as the indirect synergistic process.

Hypoxia has been shown to modulate the synthesis of TGF-α and -β. The two isoforms have very different effects on cell proliferation. TGF-α exhibits mitogenic properties, whereas TGF-β is known as an inhibitor of endothelial mitogenesis *in vitro*, yet acts as an angiogenic factor *in vivo* (for a review see Bicknell and Harris, 1991). Hypoxia induced TGF-β in the rat brain in an oxygen concentration and time-dependent manner (Klempt *et al.*, 1992). TGF-α, a ligand for EGF-R, was found to be upregulated in multicellular spheroids, but that was dependent on cell density rather than the hypoxic stress found in the centre of the spheroids (Laderoute *et al.*, 1992*b*).

Another angiogenic factor with significant sequence homology to VEGF, the placenta growth factor (PLGF), was shown to be upregulated by oxygen deprivation (1% O_2) as well as treatment with cobalt or dfx (Gleadle *et al.*, 1995). PLGF can form homodimers comprised solely of PLGF subunits as well as functional heterodimers with VEGF (DiSalvo *et al.*, 1995).

The production of the anti-inflammatory cytokine interleukin 6 (IL-6) and its mRNA was found to be upregulated by hypoxia *in vitro* (1.6–2% O_2) in endothelial cells and *in vivo* (breathing 6% O_2) in mice (Yan *et al.*, 1995). Besides its role in inflammation, IL-6 also promotes vasorelaxation and

induces cell proliferation of smooth muscle cells (Ohkawa *et al.*, 1994). A region of less than 225 bases 5′ of the IL-6 start codon was needed for the hypoxia response. A nuclear factor IL-6-site within this sequence could bind the nuclear factor IL-6 (NF-IL-6) and was sufficient for the hypoxia-mediated induction of a marker gene. The CCAAT-enhancer-binding protein β (C/EBP-β), which is a member of the NF-IL-6 family of DNA binding proteins, showed increased binding to the NF-IL-6 site following hypoxia. Using gel retardation assays it was shown that HIF-1 was not involved in the regulation of IL-6.

Transcription factors

Several hypoxically induced transcription factors have been identified, which include the proto-oncogenes *jun* and *fos* (Ausserer *et al.*, 1994; Goldberg and Schneider, 1994), the tumour suppressor gene p53 (Graeber *et al.*, 1994), the nuclear factor κB (NF-κB) (Koong *et al.*, 1994; Yao and O'Dwyer, 1995) and the heat shock transcription factor HSF (Benjamin *et al.*, 1990). The increased levels of *c-jun* mRNA following hypoxia (<0.01% O_2) were found to result from both message stabilisation and transcriptional activation (Ausserer *et al.*, 1994). However, the induction of *c-jun* gene expression by hypoxia did not result in or from increased AP-1 DNA binding activity, which is unlike the response to radiation. Similarly, unlike the effect of DNA-damaging agents, p53 activity was not required for the hypoxia-induced G_1- phase checkpoint (0.02% O_2) (Graeber *et al.*, 1994).

NF-κB is an enhancer-binding protein originally found in human T cells which is involved in the activation of a number of stress-inducible genes. NF-κB functions as a heterodimer consisting of the 50 kDa and 65 kDa subunits (p65 or relA) (Perkins *et al.*, 1992). The induction of NF-κB by cellular stresses does not require *de novo* protein synthesis, since its activation results from the release of the inhibitory subunit, IκBα. Koong *et al.* (1994) showed that exposure to hypoxia (0.02% O_2) results in the degradation of IκBα and the increase in DNA-binding of NF-κB followed by gene transactivation. The activation of NF-κB was found to be due to the phosphorylation of tyrosine residues within the inhibitory subunit.

Treatment of cells with harmful agents, such as heat and hypoxia, causes the accumulation of misfolded or damaged proteins. Heat shock proteins (HSPs) have been identified as chaperones which aid in the correct folding of proteins. This set of proteins is distinct from that including Epo and VEGF, since the latter are not induced by heat shock. HSPs and c-fos are induced *in vivo* by hyperthermia and ischaemic hypoxia (Binienda and Scallet, 1994). However, the induction of the HSPs, especially HSP 70, by 42°C heat shock prior to hypoxia *in vivo* did not protect the animals from ischaemic renal injury (Joannidis *et al.*, 1995).

Transcriptional activation of HSP 70 following heat shock is controlled by the nuclear transcription factor HSF (or HSTF) (Benjamin *et al*, 1990). HSF was also found to be induced by both hypoxia (0.0002% O_2) and reoxygenation *in vitro* and *in vivo*, but at a slower rate to the induction by heat (2 h *vs* 1 h) (Giaccia *et al.*, 1992). Protein synthesis was not needed for the induction of HSF activity following either form of stress. Functional HSF binding sites have been found in the vicinity of the mouse c-*fos* and the haem oxygenase genes (Colotta *et al.*, 1990; Shibahara *et al.*, 1987). The transcription factor binds to specific regulatory regions 5′ of the HSP 70 coding region. Activated HSF binding was very specific for its recognition site, as shown by gel shift and site-directed mutagenesis assays. It has been postulated that changes in cytoplasmic concentrations of cations, caused by ATP depletion, may change the conformation of HSF, resulting in its activation. Alternatively it has been postulated that misfolded proteins caused by cellular stress induce the activation of HSF (Price and Calderwood, 1993).

Metabolic and DNA repair enzymes

The set of proteins induced by hypoxia (<0.01% O_2) had similar molecular weights to the main glucose regulated proteins (Heacock and Sutherland, 1986). Two of the oxygen regulated proteins (ORP 80 and ORP 100) were found to be identical to the glucose regulated proteins GRP 78 and GRP 94, and ORP 33 was identified as haem oxygenase 1 (HO-1) (Murphy *et al.*, 1991). Excess haem and haem-containing proteins are degraded by HO to ultimately produce the antioxidant bilirubin. Unlike Epo and VEGF, HO-1 was found to be inducible by UV-A radiation, which causes oxidative stress (Keyse and Tyrrell, 1990). The increase in steady state mRNA levels of HO-1 observed following oxidative stress was due to an increase in the transcription rate and not due to a prolonged half life of the RNA. HO-1 was found to be induced *in vivo* in endothelial cells by methaemoglobin (a form of haemoglobin which does not bind oxygen because the ferrous iron of the haem is oxidised to the ferric state), but not by oxyhaemoglobin (Balla *et al.*, 1995). Functional binding sites of the transcription factors NF-κB and AP-2 were found in close proximity to the HO-1 promoter (Lavrovsky *et al.*, 1994).

Endothelial cells (ECs) show an increased tolerance to hypoxia as compared with most other mammalian cells. A unique set of proteins is induced by hypoxia in EC which is different from the set induced by heat shock or glucose stress (Graven *et al.*, 1994). A 36 kDa protein which is induced by hypoxia (3% and 0% O_2) in EC was identified as the glycolytic enzyme glyceraldehyde- 3-phosphate dehydrogenase (GAPDH). The induction is due to both an increase in transcription rate as well as post-transcriptional regulation. A search of the GAPDH DNA sequence for potential Epo- like hypoxia responsive elements did not reveal homologous regions. It is important to note, that the expression of GAPDH is apparently not upregulated in other mammalian cells, where it is often used as a non-inducible control in transcriptional analyses.

Exposure of mammalian cells to severe hypoxia can result in programmed cell death (apoptosis). Interestingly, normal rat fibroblasts exposed to catalyst-induced anoxia maintain full viability for at least 2 days and show no sign of apoptosis (Stoler *et al.*, 1992). Extraction of DNA using a method that releases proteins from cytoplasmic pockets, however, liberated an endonuclease which degraded the chromosomal DNA into a 200 bp ladder. This sequestered endonuclease activity is induced threefold by severe hypoxia (anaerobic cabinet). Fragmented DNA can first be detected after 16 h and DNA degradation continues to increase up to 48 h of anoxia. Of the rat tumour cell lines tested, only those expressing high levels of the VL30 retrotransposon showed anoxia-inducible endonuclease activity.

The expression of the human DNA repair enzyme HAP1 is upregulated in response to hypoxia (1% O_2 or gassing with 95% N_2, 5% CO_2) (Walker *et al.*, 1994; Yao *et al.*, 1994*a*). HAP1 (APE/Ref-1) is a bifunctional nuclear enzyme involved in the repair of apurinic/apyrimidinic sites in DNA and in the reduction of oxidised proto-oncogene products, such as the members of the jun and fos proteins. The induction of genes by hypoxia therefore could result both from transactivating factors that bind to the AP-1 recognition site and from the redox activity of HAP1 which enhances their affinity for the AP-1 element (Yao *et al.*, 1994*a*). Cells stably transfected to express HAP1 antisense RNA were found to be extremely sensitive to a range of DNA damaging agents (such as methyl methanesulphonate and hydrogen peroxide, but excluding UV radiation) and to the exposure to hypoxia and hyperoxia (1% and 100% oxygen, respectively) (Walker *et al.*, 1994). The promoter of the HAP1 gene was dissected and gel mobility shift assays showed that the universal transcription factor Sp1 could bind to its recognition site in the promoter region (Harrison *et al.*, 1995). Interestingly, Sp1 had also

been shown to function synergistically with NF-κB, which is induced by hypoxia, to activate a viral promoter (HIV-1) (Perkins *et al.*, 1993).

Some secondary response genes are also under hypoxia-mediated control. The enzyme DT-diaphorase [DTD, NAD(P)H quinone oxidoreductase] is involved both in the detoxification of xenobiotics and the reductive activation of some quinone anti-cancer drugs. The expression of DTD is upregulated maximally after 8 h of hypoxia (gassing with 95% N_2, 5% CO_2) followed by 24 h of reoxygenation (Yao *et al.*, 1994*b*). The mRNA level and enzyme activity of DTD were elevated concurrently. Since the 5′ flanking region of the gene contains a consensus AP-1 binding site and the expression of the genes encoding the components of AP-1 has been shown to be upregulated by the exposure to hypoxia, it seemed likely that the hypoxia response of the DTD encoding gene was AP-1 mediated (Yao and O'Dwyer, 1995). However, it was found that AP-1 binding did not follow the time course of the DTD hypoxia induction and is therefore probably not involved in the induction of DTD by hypoxia. In contrast, an NF-κB binding site in the same DNA region was shown to be involved in the upregulation of DTD by hypoxia.

The enzyme xanthine oxidase has been shown to activate cancer prodrugs, especially the quinone-based agents (Gustafson and Pritsos, 1992). The activities of xanthine dehydrogenase and xanthine oxidase (XD/XO) in fibroblasts and endothelial cells are regulated by hypoxia (Hasan *et al.*, 1991; Hassoun *et al.*, 1994). Increased expression in response to low oxygen (3% O_2) was found to be due to an increased rate of transcription, rather than improved message stability. Exposure of the cells to hyperoxia caused a decrease in XD/XO expression when compared to normoxia.

The hypoxia response elements of the phosphoglycerate kinase 1 (PGK-1) and the lactate dehydrogenase A (LDH-A) genes are similar to the Epo enhancer (Firth *et al.*, 1994). The physiological response of PGK-1 and LDH-A resembles that of Epo in that they are upregulated by cobalt, but not cyanide or heat shock, and that protein synthesis is required for the response. A further increase in gene expression in response to hypoxia could be achieved by using concatamers of the enhancer, which in the case of PGK-1 only consists of 18-bp. We have utilised the PGK-1 enhancer sequence in the context of its homologous promoter and two heterologous promoters (of the thymidine kinase gene and the 9-27 gene) and demonstrated hypoxia-mediated regulation of a reporter gene *in vitro* and in experimental tumours *in vivo* (O_2 concentrations ranged from 2% O_2 to catalyst-induced anoxia) (Dachs *et al.*, 1995).

Therapeutic exploitation

In order to test the use of the hypoxia-responsive elements in gene therapy, DNA constructs, which encode the CD2 surface marker under the transcriptional control of promoters containing the PGK-1 hypoxia responsive element, were introduced into the human fibrosarcoma cell line HT1080 (Dachs *et al.*, 1995). The resulting stable transfectants were tested for their response to severe hypoxia (0.001% O_2 and catalyst-induced anoxia) and different levels of oxygen (0.1, 1, 2, 5 and 20% O_2). The increase in CD2 production depended on the length and severity of hypoxia. Following severe hypoxia, CD2 expression in the transfected cell lines increased further during subsequent aeration, whereas, following less severe hypoxic conditions, CD2 expression peaked immediately after hypoxia. CD2 expression was not induced following exposure to 5% or 20% oxygen. Expression of CD2 was not seen in the parental cell line in response to hypoxic conditions. Transfectants grown as xenografts in nude mice showed localised expression of CD2. Further, it could be demonstrated, using single cell electrophoresis coupled to immunoflourescence, that specifically the hypoxic tumour cells had increased CD2 expression. The observed increases in gene expression *in vitro* and *in vivo* demonstrate the potential for the exploitation of this system in future cancer therapy.

Upregulation of therapeutically relevant genes could have implications in the treatment of cancer. The artificial introduction of a gene under the control of a hypoxia-responsive enhancer could result in the overproduction of the gene product specifically in the hypoxic regions of the body, which would make expression tumour specific. Bioreductive agents, designed to selectively kill hypoxic cells, need to be converted to toxic drugs by reductase enzymes. It is known that increased activity of reductase enzymes correlates with increased toxicity of bioreductive drugs (Robertson *et al.*, 1994; Patterson *et al.*, 1995). Hence, it is envisaged that the upregulation of the gene encoding a reductase could improve the therapeutic potential of the bioreductive drugs. Our current aim therefore is to exploit the physiological differences between tumours and normal tissues for gene therapy approaches to cancer treatment.

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