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Hypoxia-inducible factors and hypoxic cell death in tumour physiology

Andrea L Bacon and Adrian L Harris

Hypoxic up-regulation of hypoxia-inducible factors (HIFs) during tumourigenesis presents an interesting paradox with respect to their role in tumour growth. Hypoxia-inducible factor 1 (HIF-1) plays a key role in the adaptive response to hypoxia, trans-activating many genes whose protein products are involved in pathways of angiogenesis, glucose metabolism and cell proliferation, thus facilitating tumour progression. However, it is also emerging that up-regulation of HIF-1 trans-activates anti-proliferative and pro-apoptotic genes (such as BNIP3, NIX and IGFBP3). This makes it unclear as to whether HIF-1 up-regulation provides a selective advantage or disadvantage to neoplastic progression under hypoxia. In addition, vagaries in the hypoxic microenvironment of the tumour such as pH changes, presence of reactive oxygen species and energy availability in the form of adenosine triphosphate (ATP), appear to influence the function of HIF-1 and up-regulated pathways and affect susceptibility to undergo hypoxic cell death. It is apparent that hypoxic cancer cells must be able to select against HIF-1 mediated cell death signals in order to survive and progress towards malignancy. Hypoxia-induced HIF-1 may in itself serve to select for increased malignancy by exerting pressure in the form of anti-proliferative signals that must be escaped. Understanding the mechanisms by which HIF-1 induces cell death and the manner in which the tumour cell can overcome such signals, is critical for our understanding of cancer progression and the development of effective therapeutics.

Keywords: apoptosis; BNIP3; hypoxia-inducible factor; necrosis

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Hypoxia and cancer

The ability of cells to adapt to periods of hypoxia is important for their survival in both physiological and pathophysiological states. Regions of hypoxia are known to exist within many tumours and the extent of tumour hypoxia is regarded as an important prognostic factor influencing neoplastic aggression, resistance to therapy and overall patient survival (1–4).

Cells undergo a number of adaptive physiological responses to hypoxia, the earliest being the transition from aerobic to anaerobic metabolism (5). Glycolytic energy metabolism results in increased glucose consumption, increased lactic acid production and a lowered intracellular pH. (6). One of the key factors in mediating cell survival and tumour progression is the hypoxically regulated production of growth factors that induce new blood vessel formation (angiogenesis). However, in various clinical entities such as ischemic disease and organ transplantation, cell death due to hypoxia is well documented (7, 8). Hypoxic regions of solid tumours are also often highly necrotic (9, 1) suggesting that there is a dichotomy between cell growth promoting factors and cell death within hypoxic cells.

Hypoxia-inducible factors mediate the hypoxic response

At the molecular level, cellular adaptation to hypoxia is mediated by the hypoxia-inducible factors (HIFS) which are members of the basic helix loop helix-PAS family of transcription factors (12). PAS being an acronym of the first three proteins identified as containing this polypeptide motif that is approximately 260 amino acids in length and contains two direct repeats of 60 amino acids (the *period* gene product of fruit flies, the *aryl* hydrocarbon receptor nuclear transporter and the *single-minded* gene product of fruit flies). HIFs are heterodimers of two

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subunits: an oxygen sensitive alpha subunit (HIF-1α, HIF-2α and HIF-3α) and a constitutively expressed β subunit (HIF-1β also known as the aryl hydrocarbon receptor nuclear translocator, ARNT) (13–15). Under normoxic conditions, the HIF-α subunits are rapidly degraded by the proteosome after being targeted for ubiquitination in a process that is dependent on the Von Hippel Lindau tumour suppressor protein (VHL) (16) (Fig1). The interaction between HIF-α and VHL is regulated through hydroxylation of two proline residues (Pro-402 and Pro-564 in HIF-1α), by three prolyly hydroxylases, known as prolyl hydroxylase-domain proteins 1–3 (PHD1-3) (17, 18). The identification of these prolyl hydrolases has provided a direct link between the regulation of HIF-αs and molecular oxygen since these enzymes have an absolute requirement for di-oxygen as a co-substrate (18). Under hypoxic conditions, the PHD enzymes are no longer active and the unmodified prolyl HIF-α no longer interacts with VHL and subsequently accumulates (Fig 1).

HIF heterodimers, have been demonstrated to bind to a 6bp [5′-ACGTG(C/G)-3′] hypoxia-responsive element (HRE) that is functional as a transcriptional enhancer in hypoxia-responsive genes (19). The hypoxic induction of the HIFs serves to co-ordinately activate the expression of downstream target genes (Fig 1). Inactivation of HIF-1α or HIF-2α for example, by RNA interference (RNAi), results in the down regulation of transcription of such target genes (20).

Key messages

- **Hypoxia-inducible factors play a critical role in the hypoxic response in cancers.**
- **Hypoxic up-regulation of the HIFs mediates the transactivation of proteins that can function to positively and negatively regulate tumour growth.**
- **Hypoxic cell death is influenced both by the up-regulation of cell death and anti-proliferative factors and also vagaries in the microenvironment including changes in pH, reactive oxygen species and ATP availability.**

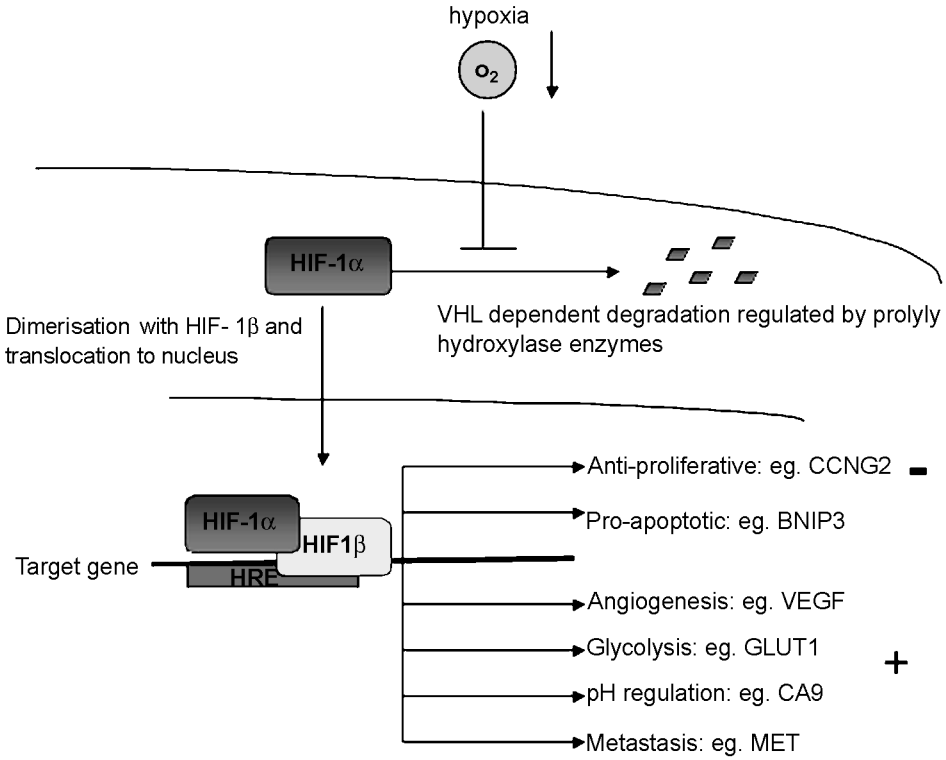

Figure 1. Mechanism of HIF-1 target gene activation under hypoxia. Under normoxia HIF-1α undergoes rapid Von Hippel Lindau protein (VHL)-dependent proteosomal degradation. The interaction between VHL and the proteosome is regulated through hydroxylation of two proline residues on HIF-1α by three prolyly hydroxylases (PHD1-3). Under hypoxia, the PHD enzymes are no longer active; the unmodified prolyl HIF-1α no longer interacts with VHL and accumulates. HIF-1 translocates to the nucleus where it trans-activates target genes *via* hypoxia-regulated elements (HREs) located in their promoters. Classes of proteins are up-regulated that have both a positive (+) and negative (−) effect on cell growth. CCNG2 = cyclin-G2; BNIP3 = BCL2 19 kda interacting protein; VEGF = vascular endothelial growth factor; GLUT1 = glucose transporter 1 (SLC2A1 = solute carrier family 2, member 1); CA9 = carbonic anhydrase 9; MET = c-met protooncogene product.

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Differential functions of HIF-1α, HIF-2α and HIF-3α

HIF-1α HIF-2α and HIF-3α appear to have different biological functions and one of the current challenges is to unravel the specific roles of each in the hypoxia-mediated response. The HIF RNAi study mentioned above highlighted some of the functional differences between HIF-1α and HIF-2α (20). Whereas the response of hypoxia-inducible genes was critically dependent on HIF-1α alone in breast epithelial and endothelial cell lines, in renal carcinoma cells regulation was HIF-2α dependent (20). That HIF-1α and HIF-2α have different biological functions is also apparent from mice knockout studies (21–25). Whereas Hif-$1\alpha^{-/-}$ mice develop both cardiac and vascular malformations, Hif-$2\alpha^{-/-}$ mice undergo vasculogenesis but present remodelling problems later in development. More recently DNA microarray analysis of cells expressing HIF-2α and HIF-1α and of HEK293 cells over expressing stabilized HIF-1α and HIF-2α has revealed differential roles in hypoxic gene regulation. While HIF-2α up-regulated more genes than had been anticipated, it was interesting to note that HIF-1α but not HIF-2α stimulated glycolytic gene expression in both endothelial and epithelial cells. This study indicated for the first time that HIF-1α and HIF-2α have unique trans-activation targets (26).

HIF-3α has been reported to comprise 6 splice variants three of which are hypoxically regulated (13). In comparison to HIF-1α and HIF-2α there has been relatively little study of HIF-3α expression, regulation and function, nonetheless, of significant interest is that certain HIF-3α splice variants may be able to block the HIF mediated hypoxic response. Transfection of HIF-3α-1 into COS cells suppresses HRE driven gene transactivation when the expression of ARNT is limited (27). Furthermore, in mice, a splice variant of HIF-3α called IPAS (inhibitory PAS domain protein) has been demonstrated to have a dominant negative effect on HIF mediated gene control (28).

HIF-1α: a pro- or anti-apoptotic factor?

There is controversy surrounding the role of the HIFs, in particular the HIF-1 mediated hypoxic response in tumourigenesis. Reports have suggested, that HIF-1 acts as both a positive and negative regulator of tumour growth (Fig 1).

Many of the target genes trans-activated by HIF-1, produce proteins that either increase oxygen delivery or allow metabolic adaptation to reduced oxygen availability. Such responses are key to cell survival under hypoxia. HIF-1 has been suggested to mediate tumour progression *via* specific target genes that encode angiogenic factors (such as erythropoietin and vascular epidermal growth factor VEGF), glucose transporters and glycolytic enzymes (such as glucose transporter 3 GLUT3) (4, 12, 29). More recently, an angiogenesis independent hypoxia-induced pathway has been identified that links tumour hypoxia to increased malignancy. Pennacchietti et al. demonstrated that hypoxia trans-activates the *MET* proto-oncogene *via* a HIF-1α dependent HRE element in the promoter. Hypoxic up regulation of MET was shown in a cell line panel, cultured in normoxic and hypoxic conditions and also in hypoxic areas of human cancer xenografts induced in nude mice (30). MET up-regulation resulted in sensitivity to hepatocyte growth factor (HGF) stimulation leading to induction of invasive growth (30).

Studies of HIF-1α null embryonic stem (ES) cell derived tumours and H-ras derived fibrosarcomas have further indicated that HIF-1 acts as a positive regulator of tumour growth (21, 31). Maxwell et al. also demonstrated that xenografts derived from the mouse hepatoma cell line HEPA-1 grew more quickly than those derived from a mutant strain of HEPA-1 that lacks ARNT and thus does not form HIF-1, again suggesting that HIF-1 is a necessary factor for tumour growth (32). Of significant interest is the recent study by Erez et al. who revealed that ectopic expression of mouse PHD1 suppresses HIF-1α in a lung carcinoma cell line. This inhibition of HIF-1α by PHD1 led to a reduction in tumour growth that was correlated with increased necrosis and marked decrease in micro-vessel density (33).

Mechanistically, it can easily be extrapolated that the positive regulation by HIF-1α is mediated by trans-activation of growth promoting genes, most notably those involved in angiogenesis. In reality it may not be so simple and there is evidence that HIF-1 mediated tumour expansion can occur independently of obvious HIF-1 targets such as VEGF (31).

The controversy over the growth-promoting role of HIF-1 has arisen from a number of studies that present HIF-1 as a general mediator of cell death (34, 35). Embryonic stem cells in which the gene encoding HIF-1α has been disrupted do not undergo apoptosis in response to hypoxia, as is the case for their parental counterpart (34) and Chinese hamster ovary cells lacking proper HIF-1α expression are also resistant to hypoxia-inducible apoptosis (34). A particularly relevant study by Mack et al. examined the effects of VHL inactivation in ES cells. While VHL loss resulted in constitutive activation of HIF-1α, $VHL^{-/-}$ tumours were smaller than controls supporting the notion that HIF-1α is a negative regulator of tumour cell growth. However, this growth reduction was attributed to a proliferation deficiency in $VHL^{-/-}$ cells rather than increased apoptosis (36).

Blouw et al. demonstrated that HIF-1 can have

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anti- or pro-proliferative effects on astrocytoma progression depending on the location of the tumour (37). HIF-1α knockout tumours injected into mice subcutaneously, showed decreased proliferation, decreased tumour growth, decreased vessel density, increase in necrotic areas and no capacity for invasion compared to HIF-1α wild type tumours. However HIF-1α knockout tumours in the brain showed an almost opposite phenotype, notably increased proliferation, tumour growth and vessel density and greatly increased capacity for invasion compared to wild type astrocytomas injected into the brain. Some of these differences in phenotype were attributed to the differences in the vascular microenvironment of the subcutaneous and intracranial locations. This study highlights the complexity of the HIF-1 mediated hypoxia-signalling cascade and indicates that the microenvironment of the tumour, in addition to specific up-regulated pathways, may significantly affect the role of HIF-1 in tumourigenesis.

Pro-apoptotic genes up-regulated by hypoxia

Expression profiling using differential display and micro-array analyses has been key in identifying additional genes that are directly trans-activated by HIF-1 (38–41). Many are pro-apoptotic and anti-proliferative (Table 1), providing new insight into mechanisms by which HIFs and in particular HIF-1 may be able to activate cell death and growth arrest (42).

Stabilisation of P53

Hypoxia is known to induce p53 dependent apoptosis (43, 44). p53 is a potent transcription factor that can regulate target genes that activate cell death (e.g., *BAX, NOXA, PUMA* and *PERP*) or cause growth arrest (e.g., *p21*) (45). In addition p53 can be stabilised by HIF-1α (46). An et al. demonstrated that p53 stabilisation in hypoxia is dependent on the presence of HIF-1α since it is not observed in HIF-1α$^{-/-}$ cells. However, p53 stabilisation is not dependent on the transcriptional activity of HIF-1 since it is still observed in cells deficient for ARNT (HIF-1β) (46). The mechanism of stabilisation appears dependent on the phosphorylation status of HIF-1α (47). Under prolonged exposure to hypoxia two major forms of HIF-1α; de-phosphorylated and phosphorylated, are induced. While phosphorylated HIF-1α binds to ARNT, the de-phosphorylated HIF-1α mediates apoptosis by binding to and stabilizing p53 (47).

Although stabilisation of p53 is observed under conditions of severe hypoxia, it should be considered that moderate levels of hypoxia, while still capable of inducing HIF-1α, do not induce either p53 stabilisation or p53 gene expression (48).

BNIP3, NIX and NOXA

BNIP3, NIX and NOXA are all BH3-only members of the BCL2 family of proteins that are directly regulated by HIF-1 and are capable of inducing cell death. BNIP3 homodimerises and antagonises the activity of pro-survival proteins (49, 50). Although it contains a BH3 domain, it is the C-terminal transmembrane domain of BNIP3 that is essential for membrane targeting and promotion of apoptosis. BNIP3 expression is normally undetectable in most organs including the heart, but can be induced by hypoxia in many cell types (38, 51). BNIP3 mRNA and protein has been demonstrated to accumulate dramatically in response to hypoxia in a wide range of cell lines (38, 51). Of important clinical significance, this up-regulation of BNIP3 is also detected in clinical material from human breast tumours where, BNIP3 mRNA is expressed at higher levels compared to normal breast tissue (38). This up-regulation of BNIP3 under

Table 1. Hypoxia-inducible proteins involved in anti-apoptotic and anti-proliferative pathways.

Hypoxia-inducible protein	Function	Reference
P53	Pro-apoptotic, tumour suppressor	(46, 35, 43, 44)
BNIP3	Pro-apoptotic	(20, 49–52)
NIX (BNIP3L)	Pro-apoptotic homologue of BNIP3	(20)
CCNG2	Cell cycle inhibition	(39)
BHLHB (STRA13/DEC1)	Growth arrest and terminal differentiation	(39)
TGM2	Protection against some apoptotic stimuli	(39)
BIK	Pro-apoptotic	(40)
IGFBP-3	Pro-apoptotic	(40, 57)
NOXA	Pro-apoptotic	(55)

P53 = tumour suppressor protein 53; BNIP3 = BCL2 19 kda interacting protein; NIX = BCL2 19 kda interacting protein like; CCNG2 = cyclin-G2; BHLHB = Basic helix loop helix containing class B 2; TGM2 = transglutaminase 2; BIK = BCL2 interacting killer; IGFBP-3 = Insulin-like growth factor binding protein 3; NOXA = NADPH oxidase activator.

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hypoxia is mediated by HIF-1 *via* the HRE element located in its promoter region (51, 52).

NIX (nip like protein X) is the homologue of BNIP3 and like BNIP3 is up-regulated in carcinoma lines under hypoxia and expressed at higher levels in breast carcinoma compared to normal adjacent breast material (38). However, while high expression of BNIP3 in ductal carcinoma in situ (DCIS) of the breast correlates with high grade necrotic lesions with invasive potential, no such correlation has been observed with the expression of NIX (20). This observation suggests that the downstream effects of hypoxically up-regulated BNIP3 and NIX are likely to be different.

More recently a third BH3-only domain family member has been implicated in the hypoxic response. NOXA was identified as a hypoxically regulated pro-apoptotic gene by subtractive hybridization (53). Hypoxic induction of NOXA is also mediated by HIF-1 *via* an HRE element located in its promoter. Suppression of endogenous NOXA with antisense oligonucleotides protected cells against hypoxic cell death supporting the notion that NOXA has a central role in the apoptotic response to hypoxia.

The challenge now is not only to identify whether other similarly functioning BH3-only family members exist, but also to elucidate the functional roles of these proteins in hypoxic cell death, determine the mechanisms by which cell death is signalled and reconcile these with a model of tumour growth and progression.

Mechanistic differences between BNIP3 and NOXA signalling are already suggested by the fact that BNIP3 relies on the trans-membrane domain and not the BH3 domain to exert cell death (54) whereas the BH3 domain is crucial for cell death by NOXA (55). Furthermore NOXA facilitates cell death by the generation of reactive oxygen species (ROS) which does not appear to be the mechanism of action in BNIP3 signalling (53).

IGFBP-3

Insulin-like growth factor (IGF) binding protein–3 (IGFBP-3) blocks IGF action and inhibits cell growth but furthermore it has been shown that IGFBP-3 can also induce apoptosis independently of p53 and IGF (42). Its hypoxic up-regulation has been reported in a number of expression studies where it is widely hypoxically inducible in human glioblastoma cells, HEPG2 cells, normal cervical and dermal keratinocytes, normal stromal fibroblasts and transformed keratinocytes and bladder tumours (40, 41, 56, 57). In some cases fold induction was greater than that of VEGF and mediation of up-regulation by HIF-1 has also been demonstrated (40, 56). Inactivation of IGFBP-3 by methylation in non-small cell lung cancers and association of this phenotype with poor prognosis suggests one mechanisms to bypass anti-tumour effects (58).

Effects of the microenvironment on hypoxically-induced cell death: pH, ROS, ATP, reoxygenation

The most studied of these microenvironmental effects on cell death include effects from pH, reoxygenation and the influence of free radicals or reactive oxygen species (ROS).

pH

Adaptation to hypoxia is accompanied by decreased intracellular pH as a result of cells switching from respiration to glycolytic energy metabolism, with increased glucose consumption and lactic acid production. pH change is known to be a major cause of cell death under hypoxia. The HIF-1 inducible protein CA9 (carbonic anhydrase 9) has been proposed to have a role in regulating cellular pH under hypoxia and consistent with this hypothesis, inactivation of CA9 results in reduced cell growth (59). In cardiac myocytes, acidosis is necessary to induce cell death under hypoxia (8), and this cell death is mediated by BNIP3 (60). However, while BNIP3 mRNA was up-regulated in these cells under hypoxia it was clearly demonstrated that acidosis was required in order to activate apoptosis (60). Thus the acidity of the cellular environment under hypoxia likely contributes to the propensity to undergo cell death. Studies such as this underscore the notion that up-regulation of a protein does not necessitate functional activity and stresses the need to understand downstream roles of hypoxically regulated proteins in the relevant tumour types and microenvironments.

Reoxygenation and reactive oxygen species

Hypoxic cell death is also influenced by the presence of free radicals or reactive oxygen species (ROS). ROS are well known to accumulate after hypoxic reoxygenation leading to extensive cell death (see (61) and references within). ROS have also been shown to cause hypoxic cell death in an epithelial cell line by activation of caspase 9 in a cytochrome C independent manner (53). A number of insights have emerged linking HIFS directly with the production of these ROS. HIF-2α knockout mice suffer from a syndrome, the prominent sites of pathology being those representing tissues that are particularly sensitive of oxidative stress. Interestingly the absence of HIF-2α is associated with an enhanced generation of ROS in

these mice, leading to the suggestion that HIF-2α may have a role in protecting against ROS. The HIF-1 inducible pro-apoptotic protein NOXA has also been demonstrated to signal cell death *via* the generation of ROS (53).

Necrosis, apoptosis and aponecrosis

Changes in the microenvironment can also determine the mechanism of hypoxic cell death in terms of influencing the relative contribution of apoptosis and necrosis. It is widely becoming accepted that apoptosis and necrosis do not necessarily represent two distinct mechanisms and that there is a likely gradation through phenotypes of necrosis, aponecrosis to classical caspase-dependent apoptosis, and that this may be highly dependent on the prevailing conditions in the microenvironment in addition to that attributed to differential gene expression (62).

In a study of rat phaeochromocytoma cells (PC12) subjected to hypoxic conditions, both apoptosis and necrosis were detected by electron microscopy (63). Other cell types also exhibited necrosis and apoptosis although the relative levels of each varied (63). *In-vitro* hypoxia-induced cell death has been reported to take place by apoptosis in E1A and H-Ras transformed cell lines, several non-glioma tumour cell lines and non-transformed cells. However, *in vivo* highly necrotic regions are frequently observed within solid tumours and these areas correlate with areas of hypoxia and HIF-1 expression (9–11).

HIF-1-mediated up regulation of proteins that are involved in triggering apoptosis or necrosis would be consistent with hypoxia-dependent apoptotic and necrotic phenotypes and the relative expression or selection against such proteins, could go some way to explain differences in cell death phenotypes observed under hypoxia. While stabilisation of p53 under severe hypoxia may contribute to tumour apoptosis other candidates such as BNIP3 may contribute to a more necrotic phenotype. Velde et al. demonstrated that despite being a member of the BCL2 family, BNIP3-mediated cell death is independent of classical apoptotic markers (49).

The relative contribution from hypoxically induced pro-cell death proteins will likely be compounded by influences from environmental factors. Under conditions of acidosis, hypoxically induced BNIP3 appears to trigger an apoptotic rather than necrotic mechanism of cell death. In cardiac myocytes, hypoxia acidosis regulated BNIP3 caused DNA fragmentation and mitochondrial pore transmembrane permeability while classical markers of necrosis such as early plasma membrane permeability and decreased ATP levels were not observed (60).

One of the most striking examples of direct influence of the microenvironment on cell death mechanisms is demonstrated by the effect from ATP under hypoxia. A number of studies have observed that as energy deprivation is increased under hypoxia the mechanism of cell death switches from apoptosis to necrosis suggesting that apoptosis and necrosis represent different outcomes of the same pathway (64, 65). The dependence of apoptosis on glycolytic ATP is attributed to energy dependent activation of caspases. In rat cardiac myocytes, apoptosis progressively replaced necrosis as the major form of cell death under hypoxia as glucose concentration and concordantly ATP concentration in the cell media increased. This switch was independent of cellular acidification (65). Similar effects from ATP levels on cell death were also observed in human malignant glioma cells under hypoxia (64).

Selection against hypoxic cell death

That the induction of HIF-1 results in the up-regulation of proteins that activate cell death pathways as well as those whose function is crucial for cell survival, seemingly creates a paradox. Does the up-regulation of HIF-1 during tumour progression provide a selective advantage or disadvantage? HIF-1 induction will only be advantageous if cancer cells can effectively escape any pro-apoptotic signals (Fig 2). Indeed such selective pressure may be the driving force to select cells that are resistant to cell death and thus are ultimately more aggressive. In addition to elucidating the mechanisms of hypoxically induced cell death signals, it is critically important to determine those mechanisms by which a cancer cell may select against them.

At each point in the initiation, promotion and progression of cancer there is constant selection for cells bearing particular and changing combinations of genetic and epigenetic alterations that lead to progressive dis-regulation of the normal mechanisms controlling cell growth (29, 66).

The transformed genome contains many mutations and there will be selection for those that confer a growth advantage. Hypoxia likely exerts pressure to select for cells that have mutations in genes contributing to pathways of cell death. Indeed cells exposed to hypoxia and low pH have a diminished capacity for DNA repair which may result in genetic instability (67). *p53* mutations occur in more than 50% of all human cancers (68) and the higher malignancy of hypoxic tumours has been attributed to the ability of hypoxia to select for cells that are more resistant to apoptosis by virtue of their acquisition of *p53* mutations (43, 69). However, *p53* mutations alone may not be sufficient to overcome all of the pro-apoptotic signals activated by hypoxia

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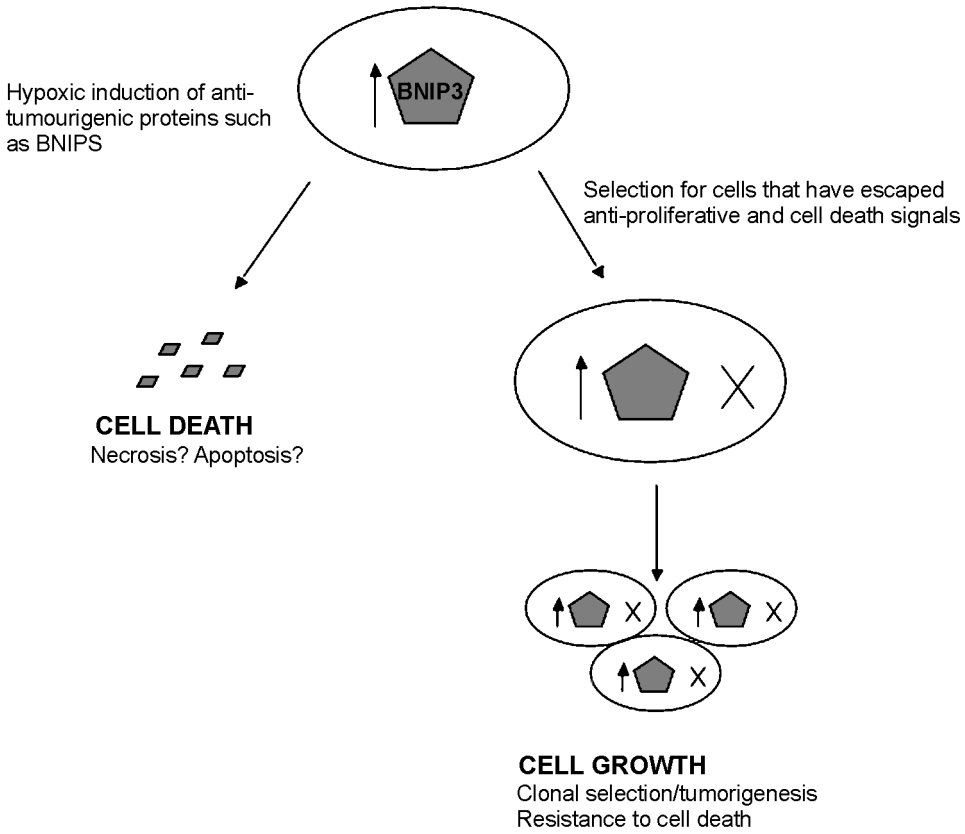

Figure 2. Selection against hypoxically inducible anti-tumourigenic proteins. Up-regulation of anti-apoptotic and anti-proliferative proteins under hypoxia will result in cell death and growth arrest. However, the up-regulation of such proteins, which include BNIP3 may provide selective pressure to overcome such signals. For example BNIP3 must likely be inactivated to allow clonal selection of cancer cells that are resistant to BNIP3 activated cell death. Successive waves of inactivation of hypoxically induced anti-tumourigenic proteins and subsequent clonal selection may be envisaged to result in an increasingly aggressive, death resistant, tumour.

especially those that may be able to activate cell death independently of p53. It will be of significance to determine whether tumour specific inactivating mutations are detected in other HIF dependent pro-apoptotic genes. Inactivation of IGFPB3 by methylation in hepatocellular carcinomas and lung cancers has already been demonstrated (58, 70). Similar findings for *BNIP3*, *NIX*, and *NOXA* or the identification of functionally inactivating mutations may not only suggest their functional importance in such cancers but also demonstrate the selective mechanisms responsible for overcoming the pro-apoptotic pathways that they activate.

Growth factors are able to provide survival responses to cells (71, 72), and it has recently been demonstrated that epidermal growth factor (EGF) and insulin-like growth factor (IGF) can protect epithelial cells from BNIP3 induced cell death (52). Adrenomedullin, a hypoxically induced growth factor, similarly protected cells from hypoxic cell death (73). Since many tumours express growth factors and their corresponding receptors, these may serve to immediately protect cells from hypoxically induced cell death signals such as BNIP3.

Summary

In conclusion, the continued increase in HIF-1 transactivational targets being identified highlights the complexity of the cellular hypoxia adaptation response. It is becoming apparent that HIF-1 cannot be regarded solely as a promoter of cell survival under hypoxic conditions. Instead, that it activates a wide spectrum of molecular pathways that both facilitate and hinder neoplasia under hypoxic conditions. For hypoxic cancer cells, the balance between the relative contributions of proliferative and cell death signals will ultimately determine survival, and understanding selective mechanisms by which escape of cell death is achieved will not only improve our understanding of tumourigenesis but also highlight areas that are amenable for therapeutic intervention or offer prognostic value.

In addition, dissection of the relative contributions from HIF-1, HIF-2, and HIF-3 will be paramount to fully understanding the complexities of the hypoxic response in different cell types and with changes to the microenvironment.

Therapeutic approaches to block growth factor

pathways such as antagonists of IGF, transforming growth factor alpha (TGFα) and hepatocyte growth factor (HGF) may additionally harness the hypoxia-driven apoptotic pathways to induce cell death. Similarly methylation inhibitors are in clinical trials and may reactivate epigenetically silenced pro-apoptotic genes.

References

1. Hockel M, Schlenger K, Hockel S, Aral B, Schaffer U, Vaupel P. Tumor hypoxia in pelvic recurrences of cervical cancer. Int J Cancer 1998;79:365–9.
2. Stadler P, Becker A, Feldmann HJ, Hansgen G, Dunst J, Wurschmidt F, et al. Influence of the hypoxic subvolume on the survival of patients with head and neck cancer. Int J Radiat Oncol Biol Phys 1999;44:749–54.
3. Knowles HJ, Harris AL. Hypoxia and oxidative stress in breast cancer. Hypoxia and tumourigenesis. Breast Cancer Res 2001;3:318–22.
4. Harris AL. Hypoxia – a key regulatory factor in tumour growth. Nat Rev Cancer 2002;2:38–47.
5. Dang CV, Semenza GL. Oncogenic alterations of metabolism. Trends Biochem Sci 1999;24:68–72.
6. Gatenby RA, Gawlinski ET. The glycolytic phenotype in carcinogenesis and tumor invasion: insights through mathematical models. Cancer Res 2003;63:3847–54.
7. Kajstura J, Cheng W, Sarangarajan R, Li P, Li B, Nitahara JA, et al. Necrotic and apoptotic myocyte cell death in the aging heart of Fischer 344 rats. Am J Physiol 1996;271:H1215–28.
8. Webster KA, Discher DJ, Kaiser S, Hernandez O, Sato B, Bishopric NH. Hypoxia-activated apoptosis of cardiac myocytes requires reoxygenation or a pH shift and is independent of p53. J Clin Invest 1999;104:239–52.
9. Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 2001;93:266–76.
10. Beasley NJ, Leek R, Alam M, Turley H, Cox GJ, Gatter K, et al. Hypoxia-inducible factors HIF-1alpha and HIF-2alpha in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected patients. Cancer Res 2002;62:2493–7.
11. Tomes L, Emberley E, Niu Y, Troup S, Pastorek J, Strange K, et al. Necrosis and hypoxia in invasive breast carcinoma. Breast Cancer Res Treat 2003;81:61–9.
12. Semenza GL. Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 1999;15:551–78.
13. Maynard MA, Qi H, Chung J, Lee EH, Kondo Y, Hara S, et al. Multiple splice variants of the human HIF-3 alpha locus are targets of the von Hippel-Lindau E3 ubiquitin ligase complex. J Biol Chem 2003;278:11032–40.
14. Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, et al. Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. Blood 1998;92:2260–8.
15. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci USA 1995;92:5510–4.
16. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999;399:271–5.
17. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. Science 2001;294:1337–40.
18. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, et al. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 2001;107:43–54.
19. O'Rourke JF, Dachs GU, Gleadle JM, Maxwell PH, Pugh CW, Stratford IJ, et al. Hypoxia response elements. Oncol Res 1997;9:327–32.
20. Sowter HM, Ferguson M, Pym C, Watson P, Fox SB, Han C, et al. Expression of the cell death genes BNip3 and NIX in ductal carcinoma in situ of the breast; correlation of BNip3 levels with necrosis and grade. J Pathol 2003;201:573–80.
21. Ryan HE, Lo J, Johnson RS. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. EMBO J 1998;17:3005–15.
22. Kotch LE, Iyer NV, Laughner E, Semenza GL. Defective vascularization of HIF-1alpha-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. Dev Biol 1999;209:254–67.
23. Peng J, Zhang L, Drysdale L, Fong GH. The transcription factor EPAS-1/hypoxia-inducible factor 2alpha plays an important role in vascular remodeling. Proc Natl Acad Sci USA 2000;97:8386–91.
24. Compernolle V, Brusselmans K, Acker T, Hoet P, Tjwa M, Beck H, et al. Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat Med 2002;8:702–10.
25. Tian H, Hammer RE, Matsumoto AM, Russell DW, McKnight SL. The fresponsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev 1998;12:3320–4.
26. Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC. Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Mol Cell Biol 2003;23:9361–74.
27. Hara S, Hamada J, Kobayashi C, Kondo Y, Imura N. Expression and characterization of hypoxia-inducible factor (HIF)-3alpha in human kidney: suppression of HIF-mediated gene expression by HIF-3alpha. Biochem Biophys Res Commun 2001;287:808–13.
28. Makino Y, Cao R, Svensson K, Bertilsson G, Asman M, Tanaka H, et al. Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature 2001;414:550–4.
29. Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 2003;9:677–84.
30. Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell 2003;3:347–61.
31. Ryan HE, Poloni M, McNulty W, Elson D, Gassmann M, Arbeit JM, et al. Hypoxia inducible factor-1alpha is a positive factor in solid tumor growth. Cancer Res 2000;60:4010–5.
32. Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, et al. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci USA 1997;94:8104–9.
33. Erez N, Milyavsky M, Eilam R, Shats I, Goldfinger N, Rotter

 ISSN 0785-3890

V. Expression of prolyl-hydroxylase-1 (PHD1/EGLN2) suppresses hypoxia-inducible factor-1alpha activation and inhibits tumor growth. Cancer Res 2003;63:8777–83.
34. Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, et al. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 1998;394:485–90.
35. Halterman MW, Miller CC, Federoff HJ. Hypoxia-inducible factor-1alpha mediates hypoxia-induced delayed neuronal death that involves p53. J Neurosci 1999;19:6818–24.
36. Mack FA, Rathmell WK, Arsham AM, Gnarra J, Keith B, Simon MC. Loss of pVHL is sufficient to cause HIF dysregulation in primary cells but does not promote tumor growth. Cancer Cell 2003;3:75–88.
37. Blouw B, Song H, Tihan T, Bosze J, Ferrara N, Gerber HP, et al. The hypoxic response of tumors is dependent on their microenvironment. Cancer Cell 2003;4:133–46.
38. Sowter HM, Ratcliffe PJ, Watson P, Greenberg AH, Harris AL. HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. Cancer Res 2001;61:6669–73.
39. Wykoff CC, Pugh CW, Maxwell PH, Harris AL, Ratcliffe PJ. Identification of novel hypoxia-dependent and independent target genes of the von Hippel-Lindau (VHL) tumour suppressor by mRNA differential expression profiling. Oncogene 2000;19:6297–305.
40. Koong AC, Denko NC, Hudson KM, Schindler C, Swiersz L, Koch C, et al. Candidate genes for the hypoxic tumor phenotype. Cancer Res 2000;60:883–7.
41. Denko NC, Fontana LA, Hudson KM, Sutphin PD, Raychaudhuri S, Altman R, et al. Investigating hypoxic tumor physiology through gene expression patterns. Oncogene 2003; 22:5907–14.
42. Rajah R, Valentinis B, Cohen P. Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-beta1 on programmed cell death through a p53- and IGF-independent mechanism. J Biol Chem 1997;272:12181–8.
43. Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 1996; 379:88–91.
44. Graeber TG, Peterson JF, Tsai M, Monica K, Fornace AJ, Jr, Giaccia AJ. Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. Mol Cell Biol 1994;14:6264–77.
45. Schuler M, Green DR. Mechanisms of p53-dependent apoptosis. Biochem Soc Trans 2001;29:684–8.
46. An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM. Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. Nature 1998;392:405–8.
47. Suzuki H, Tomida A, Tsuruo T. Dephosphorylated hypoxia-inducible factor 1alpha as a mediator of p53-dependent apoptosis during hypoxia. Oncogene 2001;20:5779–88.
48. Wenger RH, Camenisch G, Desbaillets I, Chilov D, Gassmann M. Up-regulation of hypoxia-inducible factor-1alpha is not sufficient for hypoxic/anoxic p53 induction. Cancer Res 1998; 58:5678–80.
49. Vande Velde C, Cizeau J, Dubik D, Alimonti J, Brown T, Israels S, et al. BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. Mol Cell Biol 2000;20:5454–68.
50. Ray R, Chen G, Vande Velde C, Cizeau J, Park JH, Reed JC, et al. BNIP3 heterodimerizes with Bcl-2/Bcl-X(L) and induces cell death independent of a Bcl-2 homology 3 (BH3) domain at both mitochondrial and nonmitochondrial sites. J Biol Chem 2000;275:1439–48.
51. Bruick RK. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. Proc Natl Acad Sci USA 2000;97:9082–7.
52. Kothari S, Cizeau J, McMillan-Ward E, Israels SJ, Bailes M, Ens K, et al. BNIP3 plays a role in hypoxic cell death in human epithelial cells that is inhibited by growth factors EGF and IGF. Oncogene 2003;22:4734–44.
53. Kim JY, Park JH. ROS-dependent caspase-9 activation in hypoxic cell death. FEBS Lett 2003;549:94–8.
54. Chen G, Ray R, Dubik D, Shi L, Cizeau J, Bleackley RC, et al. The E1B 19K/Bcl-2-binding protein Nip3 is a dimeric mitochondrial protein that activates apoptosis. J Exp Med 1997; 186:1975–83.
55. Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, et al. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science 2000;288:1053–8.
56. Lal A, Peters H, St Croix B, Haroon ZA, Dewhirst MW, Strausberg RL, et al. Transcriptional response to hypoxia in human tumors. J Natl Cancer Inst 2001;93:1337–43.
57. Tazuke SI, Mazure NM, Sugawara J, Carland G, Faessen GH, Suen LF, et al. Hypoxia stimulates insulin-like growth factor binding protein 1 (IGFBP-1) gene expression in HepG2 cells: a possible model for IGFBP-1 expression in fetal hypoxia. Proc Natl Acad Sci USA 1998;95:10188–93.
58. Chang YS, Wang L, Liu D, Mao L, Hong WK, Khuri FR, et al. Correlation between insulin-like growth factor-binding protein-3 promoter methylation and prognosis of patients with stage I non-small cell lung cancer. Clin Cancer Res 2002;8: 3669–75.
59. Parkkila S, Rajaniemi H, Parkkila AK, Kivela J, Waheed A, Pastorekova S, et al. Carbonic anhydrase inhibitor suppresses invasion of renal cancer cells in vitro. Proc Natl Acad Sci USA 2000;97:2220–4.
60. Kubasiak LA, Hernandez OM, Bishopric NH, Webster KA. Hypoxia and acidosis activate cardiac myocyte death through the Bcl-2 family protein BNIP3. Proc Natl Acad Sci USA 2002; 99:12825–30.
61. Saitoh Y, Ouchida R, Miwa N. Bcl-2 prevents hypoxia/reoxygenation-induced cell death through suppressed generation of reactive oxygen species and upregulation of Bcl-2 proteins. J Cell Biochem 2003;90:914–24.
62. Zhivotovsky B. Apoptosis, necrosis and between. Cell Cycle 2004;3:64–6.
63. Shimizu S, Eguchi Y, Kamiike W, Itoh Y, Hasegawa J, Yamabe K, et al. Induction of apoptosis as well as necrosis by hypoxia and predominant prevention of apoptosis by Bcl-2 and Bcl-XL. Cancer Res 1996;56:2161–6.
64. Steinbach JP, Wolburg H, Klumpp A, Probst H, Weller M. Hypoxia induced cell death in human malignant glioma cells: energy deprivation promotes decoupling of mitochondrial cytochrome c release from caspase processing and necrotic cell death. Cell Death Differ 2003;10:823–32.
65. Tatsumi T, Shiraishi J, Keira N, Akashi K, Mano A, Yamanaka S, et al. Intracellular ATP is required for mitochondrial apoptotic pathways in isolated hypoxic rat cardiac myocytes. Cardiovasc Res 2003;59:428–40.
66. Nowell PC. The clonal evolution of tumor cell populations. Science 1976;194:23–8.
67. Yuan J, Narayanan L, Rockwell S, Glazer PM. Diminished DNA repair and elevated mutagenesis in mammalian cells exposed to hypoxia and low pH. Cancer Res 2000;60: 4372–6.
68. Levine AJ. p53, the cellular gatekeeper for growth and division. Cell 1997;88:323–31.
69. Yu JL, Rak JW, Coomber BL, Hicklin DJ, Kerbel RS. Effect of p53 status on tumor response to antiangiogenic therapy. Science 2002;295:1526–8.
70. Hanafusa T, Yumoto Y, Nouso K, Nakatsukasa H, Onishi T,

Fujikawa T, et al. Reduced expression of insulin-like growth factor binding protein-3 and its promoter hypermethylation in human hepatocellular carcinoma. Cancer Lett 2002;176:149–58.
71. Kumar CC. Signaling by integrin receptors. Oncogene 1998; 17:1365–73.
72. Furstenberger G, Senn HJ. Insulin-like growth factors and cancer. Lancet Oncol 2002;3:298–302.
73. Oehler MK, Norbury C, Hague S, Rees MC, Bicknell R. Adrenomedullin inhibits hypoxic cell death by upregulation of Bcl-2 in endometrial cancer cells: a possible promotion mechanism for tumour growth. Oncogene 2001;20:2937–45.

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