REVIEW

The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis

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Apoptosis can be induced in response to hypoxia. The severity of hypoxia determines whether cells become apoptotic or adapt to hypoxia and survive. A hypoxic environment devoid of nutrients prevents the cell undergoing energy dependent apoptosis and cells become necrotic. Apoptosis regulatory proteins are delicately balanced. In solid tumours, hypoxia is a common phenomenon. Cells adapt to this environmental stress, so that after repeated periods of hypoxia, selection for resistance to hypoxia induced apoptosis occurs. These resistant tumours probably have a more aggressive phenotype and may have decreased responsiveness to treatment. The key regulator of this process, hypoxia inducible factor 1 (HIF-1), can initiate apoptosis by inducing high concentrations of proapoptotic proteins, such as BNIP3, and can cause stabilisation of p53. However, during hypoxia, antiapoptotic proteins, such as IAP-2, can be induced, whereas the proapoptotic protein Bax can be downregulated. During hypoxia, an intricate balance exists between factors that induce or counteract apoptosis, or even stimulate proliferation. Understanding the regulation of apoptosis during hypoxia and the mechanisms of resistance to apoptosis might lead to more specific treatments for solid tumours.

Mammalian cells have developed a range of adaptations to survive acute and even prolonged hypoxia. Hypoxia reduces the ability of a cell to maintain its energy level, because less ATP will be obtained from glycolysis than from oxidative phosphorylation. Cells will adapt by activating the expression of genes involved in metabolic adaptation, such as those involved in glycolysis. In addition, cell proliferation and angiogenesis will be stimulated, enabling better oxygenation of the tissue.[1] Severe hypoxia causes a high mutation rate, resulting in point mutations, which may be explained by reduced DNA mismatch repair activity resulting from decreased MLH1 and PMS2 concentrations, which are caused by hypoxia.[2] In addition, hypoxia induces genetic instability by the induction of fragile sites causing gene amplification.[3–5] Therefore, during severe hypoxia or anoxia, the cell initiates a cascade of events that leads to apoptotic cell death, thereby preventing the accumulation of cells with hypoxia induced mutations.[6]

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Hypoxia is a common phenomenon in solid tumours because impaired vascular function results in an inadequate blood supply. The supply of oxygen and nutrients is severely hampered by the malformed vessels. The combination of a lack of oxygen and a lack of nutrients causes energy deprivation. Low ATP concentrations in hypoxic tumour cells disable the apoptotic cascade and induce cell death by necrosis.[7] Severe hypoxia in the presence of energy stimulates cells to undergo apoptosis, whereas oxygen levels above 0.5% prevent cell death.[8 9] Therefore, tight regulation of cellular responses to the microenvironment is needed.

> ''During severe hypoxia or anoxia, the cell initiates a cascade of events that leads to apoptotic cell death, thereby preventing the accumulation of cells with hypoxia induced mutations''

Cells in rapidly growing tumours are intermittently, or sometimes constantly, exposed to hypoxic conditions. In severe or prolonged hypoxia, cells will initiate the process of programmed cell death. Some cells may adapt to the environmental stress, escape necrosis and apoptosis, and survive. These selected hypoxia resistant cells probably have a more aggressive phenotype.[10] Such tumour cells with a reduced sensitivity to apoptosis will be less responsive to anticancer treatment.[11]

The key regulator of the hypoxia response is the hypoxia inducible factor 1 (HIF-1). HIF-1 has a complex role. HIF-1 can induce apoptosis,[12 13] prevent cell death, or even stimulate cell proliferation.[14] This review will focus on the delicate balance between the adaptation of the cell to the hypoxic environment and self sacrifice of the cell by apoptosis, by which the accumulation of mutated cells is prevented.

HYPOXIA INDUCIBLE FACTOR 1

HIF-1 is involved in embryonic development,[15–17] tumour growth, metastasis,[18 19] and apoptosis.[12 20]

Abbreviations: Apaf-1, apoptotic protease activating factor-1; BNIP3, BCL-2/adenovirus E1B 19 kDa interacting protein 3; ES, embryonic stem; HIF-1, hypoxia inducible factor 1; HRE, hypoxia response element; IAP-2, inhibitor of apoptosis protein 2; IRES, internal ribosome entry site; Jab1, jun activation domain binding protein 1; JNK, c-Jun NH_2 terminal kinase; NF-κB, nuclear factor κB; ODD, oxygen dependent degradation; PI3K, phosphoinositide-3 kinase; ROS, reactive oxygen species; SAPK, stress activated protein kinase; VHL, von Hippel Lindau

HIF-1 is a heterodimer composed of the rate limiting factor HIF1α and the constitutively expressed HIF-1β.[21] HIF-1β is also called the aryl hydrocarbon receptor nuclear translocator. It heterodimerises with several other factors, such as the Ahr transcription factor.[22] HIF-1α is induced by hypoxia, and also by oncogenes, such as HER-2/neu, v-src, and ras, as reviewed by Semenza.[23] The induction of HIF-1 by hypoxia takes place at the protein level, because HIF-1α mRNA expression remains unchanged. During normoxia, HIF-1α protein is expressed but is unstable. Rapid degradation of HIF-1 by the proteasome results from its ubiquitination by the product of the Von Hippel Lindau tumour suppressor gene (VHL). In patients with loss of the VHL gene, HIF-1α and HIF-1 dependent genes, such as angiogenesis factors, are also expressed during normoxia.[24] Vascular tumours are often seen in these patients, who suffer from the von Hippel Lindau syndrome. The binding of HIF-1α to pVHL requires a modification of HIF-1α by proline hydroxylases in the oxygen dependent degradation domain (ODD) within the HIF-1α protein.[25–27] These enzymes are oxygen dependent and therefore HIF-1α cannot be hydroxylated during hypoxia. In those circumstances, HIF-1α accumulates and is translocated to the nucleus. Here it binds to HIF-1β to form the active transcription factor HIF-1.

Stabilisation of HIF-1α by the ODD domain is not only caused by prolyl hydroxylases. HIF-1α becomes unstable when bound to p53.[28] Jab1 (jun activation domain binding protein 1) directly interferes with the HIF-1α-p53 complex and leads to stabilisation of the HIF-1α protein during hypoxia.[29]

In addition to it regulation by stabilisation, HIF-1α is also regulated at the translational level. Recently, internal ribosome entry site (IRES) sequences were detected in the promoters of various hypoxia inducible genes, such as VEGF and HIF-1.[30 31] During hypoxia, the translation of classic cap dependent mRNA transcription is reduced, and only mRNA containing an IRES sequence will be translated. To become active, HIF-1α complexes with HIF-1β. The HIF-1 complex can bind to hypoxia response element (HRE; 5′-RCGTG-3′) sequences in the promoter of HIF-1 target genes to initiate gene expression.[32] Many genes regulated by HIF-1α are involved in several adaptive pathways including metabolism, angiogenesis, and survival to overcome hypoxic stress.[1] However, in the presence of different environmental factors HIF-1 is involved in apoptosis.[33]

APOPTOSIS

Cells encountering environmental stress can undergo apoptosis. The characteristics of apoptosis are chromatin condensation, membrane blebbing, phosphatidylserine exposure on the cell surface, cytoplasmic shrinkage, the formation of apoptotic bodies, and DNA fragmentation.[34] Apoptosis is an energy dependent process, in contrast to necrosis, which also occurs in the absence of ATP.[35] Apoptosis is regulated by a cascade of proteins called caspases. Caspases are the apoptosis executor proteins and are present as pro-forms in all cells. After cleavage, caspases become active and initiate pathways leading to apoptosis.

The signalling pathway leading to programmed cell death is fine tuned by positive and negative regulators, and a tight balance between these factors decides whether the cell undergoes apoptosis or survives. Proteins that can shift the balance towards survival are the antiapoptotic proteins Bcl-2 and Bcl-xL, whereas the proapoptotic proteins Bax, Bad, Bak, and Bid induce programmed cell death.[36]

''Apoptosis is regulated by a cascade of proteins called caspases''

An important regulator of apoptosis after DNA damage is the p53 protein. After DNA damage, p53 can induce the Bax and Bak proteins, which regulate the release of cytochrome C from the mitochondria, thereby initiating the cascade leading to apoptosis.[37]

After the induction of apoptosis by hypoxia, cytochrome C is released into the cytoplasm (fig 1). Cytochrome C binds to the apoptotic protease activating factor 1 (Apaf-1).[38] Apaf-1 activates caspase 9, which in turn cleaves caspases 3 and 6,[38 39] leading to cell death.

In addition to intrinsic apoptotic pathways, extrinsic pathways have been identified that can initiate and execute the cell death process. Apoptosis by extrinsic pathways is initiated by death ligands, such as the Fas ligand or tumour necrosis factor α,[40] leading finally to the activation of caspase 8 and caspase 3.[41]

HYPOXIA AND APOPTOSIS

Hypoxia can induce apoptosis by causing hyperpermeability of the inner mitochondrial membrane, which leads to the release of cytochrome C (fig 1). The most direct induction of hypoxia induced apoptosis is the inhibition of the electron transport chain at the inner membrane of the mitochondria (fig 1). The lack of oxygen inhibits the transport of protons and thereby causes a decrease in the membrane potential. The reduction of mitochondrial derived ATP causes activation of Bax or Bak, leading to cytochrome C release into the cytosol.[7] Hence, fibroblasts of mice lacking Bax and Bak genes are resistant to oxygen deprivation induced apoptosis.[35] In addition to energy deprivation, radical formation, in particular reactive oxygen species (ROS) generation, contributes to hypoxia induced apoptosis. It has been reported that the activation cascade resulting from ROS in human neuroblastoma cells is different from classic mitochondrial mediated apoptosis. In this case, the initiator caspase 9 is cleaved directly to the active form by caspases 3 and 12, without the involvement of cytochrome C in response to hypoxia.[42] It is only after cleavage of caspase 9 that mitochondrial permeability is increased, which then results in the activation of Apaf-1.[43]

A third mechanism by which hypoxia can induce apoptosis is the activation of c-Jun NH_2-terminal kinase (JNK), also termed stress activated protein kinase (SAPK). This mechanism has been reported in melanoma cells. JNK/SAPK is involved in the process of apoptosis,[44] because dominant negative mutants of JNK/SAPK inhibited hypoxia induced apoptosis. Wild-type JNK/SAPK had a slight proapoptotic effect. Inhibiting JNK/SAPK at normoxia had no effect on apoptosis.[45]

In contrast to the proapoptotic effects of hypoxia, cells can become resistant to apoptosis during hypoxia. Dong *et al* showed that cells treated with a strong apoptosis inducer, staurosporine, were less sensitive to apoptosis in severe hypoxia (near 0% oxygen) than when oxygen levels are normal.[46] Death resistance of hypoxic cells takes place on at least two levels: in the mitochondria and in the cytosol. In staurosporine treated cells, translocation of the proapoptotic protein Bax to the mitochondria was suppressed during hypoxia. Accumulation of Bax in the mitochondria caused the release of cytochrome C into the cytosol, which was strongly reduced in the hypoxic environment. This prohibited the cascade leading to cell death. Bax translocation was suppressed as a result of increased concentrations of the inhibitor of apoptosis protein 2 (IAP-2). Resistance to apoptosis was strongly abolished by decreased availability of IAP-2 caused by immunodepletion. IAP-2, together with the factors that prevent Bak translocation and preserve mitochondrial integrity, may facilitate cell survival during hypoxia.[46] Increased IAP-2 expression is induced by the

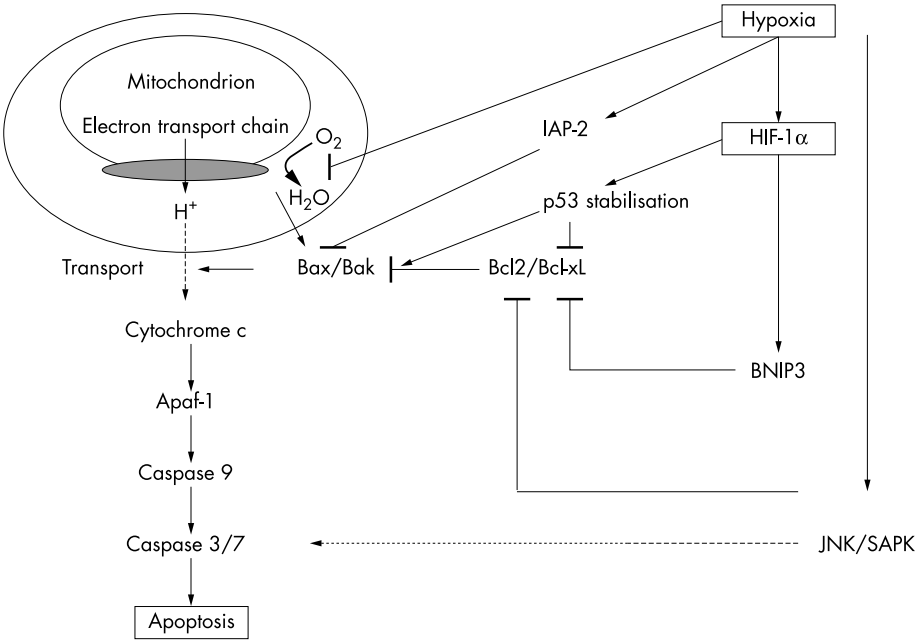

Figure 1 Schematic representation of signalling pathways induced by hypoxia leading to apoptosis. The involvement of HIF-1α is depicted in these pathways. The solid lines indicate a direct interaction, the dashed line an indirect interaction. Apaf-1, apoptotic protease activating factor-1; BNIP3, BCL-2/adenovirus E1B 19 kDa interacting protein 3; HIF-1, hypoxia inducible factor 1; IAP-2, inhibitor of apoptosis protein 2; JNK, c-Jun NH_2 terminal kinase; SAPK, stress activated protein kinase.

hypoxia induced transcription factor nuclear factor κB (NF-κB), and its induction is independent of HIF-1.[47] An alternative mechanism for increased IAP-2 synthesis may be the activation of IRES, which are present in the promoter of XIAP, another IAP gene.[48] As described above for HIF-1, the IRES may be responsible for increased translation of XIAP mRNA during hypoxia, thereby preventing the cell from undergoing apoptosis.[31]

In addition, VHL has been shown to have a protective role in chemical induced apoptosis in renal cells. Cells lacking VHL are sensitive to apoptosis, but reintroduction of VHL rendered the cells resistant to apoptosis. Prevention of apoptosis by VHL may act via Bcl-2 dependent pathways, because downregulation of Bcl-2 expression by antisense oligonucleotides in VHL positive cells made them sensitive to apoptosis.[49]

> ''Death resistance of hypoxic cells takes place on at least two levels: in the mitochondria and in the cytosol''

Both severe hypoxia and the presence of ATP are required to induce apoptosis. Oxygen levels above 0.5% prevent cells from undergoing apoptosis.[9] Cell survival under mild hypoxia is mediated by phosphoinositide-3 kinase (PI3K) and its downstream target Akt.[50] The PI3K/Akt signalling pathway is important for cell survival and proliferation because it prevents Bad from inhibiting the antiapoptotic activity of Bcl-xL. Akt induces NF-κB, which leads to cell survival.[50] The importance of the PI3K/Akt pathway is emphasised by the fact that tumours that overexpress HER-2/neu, thereby activating the PI3K/Akt pathway, become resistant to apoptosis. Resistance to apoptosis is mediated by the PI3K/Akt pathway and can be reversed by inhibitors such as LY294002 and wortmannin.[51]

HIF-1 AND APOPTOSIS

HIF-1α is involved in hypoxia induced apoptosis. Hypoxia in combination with hypoglycaemia reduces proliferation and increases apoptosis in wild-type embryonic stem (ES) cells, but not in ES cells with inactivated HIF-1α genes. The reduced rate of hypoxia induced apoptosis may explain why tumours from HIF-1α knockout ES cells grow faster than wild-type cells.[12] Two homologues of HIF-1α are known—HIF-2α and HIF-3α—and it was thought that HIF-2α or HIF-3α might substitute the function of HIF-1α in cells lacking HIF-1α. However, HIF-2α deficiency does not protect cells from hypoxia induced apoptosis as HIF-1α does.[52] Whether HIF-3α induces apoptosis has not yet been studied.

HIF-1α can induce apoptosis via two mechanisms. First, it can increase the stability of the product of the tumour suppressor gene p53. In environmental stress or DNA damage, p53 induces programmed cell death by regulating proteins such as Bax, or it can cause growth arrest, which is mediated by p21. Recently, it was shown that HIF-1α directly binds to the p53 ubiquitin ligase mdm2 both in vivo and in vitro, thereby stabilising p53.[53] However, another report showed a direct binding of p53 to the ODD domain of HIF-1α.[54] HIF-1α interacts with wild-type p53 but not with tumour derived mutant p53.[55] This may reflect a difference in behaviour of HIF-1α in physiological circumstances compared with a tumour environment.

Second, in hypoxic perinecrotic regions of tumours, the proapoptotic proteins BNIP3 (BCL2/adenovirus E1B 19 kDa interacting protein 3) and NIX, a BNIP3 homologue, are overexpressed at the transcriptional level.[56] BNIP3 is upregulated by hypoxia in human carcinoma cell lines, endothelial cells, and macrophages. Overexpression of BNIP3 in Rat-1 fibroblasts and breast cancer cells (MCF7) induces apoptosis by binding to and inhibiting the antiapoptotic proteins Bcl-2 and Bcl-xL.[57] Hypoxia induced apoptosis mediated by BNIP3 may be HIF-1α dependent because cells lacking HIF-1 cannot produce large amounts of BNIP3 and a reduced cell death rate is seen.[58] The BNIP3 promoter contains an HRE so that HIF-1 can induce the expression of this gene.[59] Apart from initiating apoptosis, BNIP3 may also be involved in inducing necrosis. The disruption of the mitochondria by BNIP3 is different from the action of other proapoptotic proteins: the cell death induced by BNIP3 resembles necrosis in fibroblasts.[60] BNIP3 may prove to be an important protein for the elimination of damaged cells by undergoing rapid cell death.

HIF-1α does not only induce, but may also prevent, apoptosis. In pancreatic cancer cell lines, high concentrations of HIF-1α were seen at normoxia, and these may have been caused by activation of the PI3K/Akt pathway, rather than

hypoxia. These cells showed more resistance to apoptosis caused by hypoxia and glucose deprivation than did cell lines with low HIF-1α expression at normoxia.[14] The reduced sensitivity to apoptosis was seen in acute hypoxia; the influence of chronic hypoxia was not studied. In addition, Chen *et al* recently reported that dominant negative HIF-1α rendered pancreatic cancer cells sensitive to apoptosis and growth inhibition by hypoxia and glucose deprivation.[61]

It can be concluded that HIF-1 plays a role in hypoxia induced apoptosis, but that the exact mechanism by which it acts is not yet clear. The effect of HIF-1 might be influenced by other factors, which may determine whether HIF-1 shifts the balance towards apoptosis or acts as an antiapoptotic factor.[62]

HYPOXIA, HIF-1, AND APOPTOSIS IN TUMOURS

Hypoxia is commonly found in solid tumours of various origins. Selection by hypoxia renders tumour cells resistant to hypoxia induced apoptosis.[63] These cells with a reduced apoptotic potential may also explain the resistance of many solid tumours to cancer treatment.[11]

In carcinomas of the uterine cervix, proliferation and the state of hypoxia appeared to be two independent predictors of outcome.[64] In the hypoxic cell compartment, cells are in growth arrest. However, small numbers of proliferating cells could be seen.[65 66] In one study, an increased overlap was seen between hypoxia and proliferating cells after radiotherapy in canine tumours.[67] In human soft tissue sarcomas, hypoxic tumours contained the fastest proliferating tumour cells.[68] The use of immunohistochemistry to investigate proliferation patterns and hypoxic profiles may identify clinically relevant cell populations in solid tumours with a more aggressive phenotype.[69]

The prognostic impact of hypoxia related apoptosis is not yet clear. The prognostic value may differ between various histological tumour types from different organs. For example, in squamous cell carcinomas of the uterine cervix, a high apoptotic index indicated poor prognosis,[70 71] whereas in cervical adenocarcinomas a high apoptotic index after treatment indicated a good prognosis.[72 73] One problem might be the difficult distinction between spontaneous apoptosis (a common phenomenon in tumours) and treatment induced apoptosis.

HIF-1α is involved in cell proliferation and apoptosis, and HIF-1α overexpression, as detected by immunohistochemistry, is often found in different solid tumours.[74 75] In lymph node negative patients with breast cancer, HIF-1α overexpression appeared to be a negative prognostic factor and correlated with increased proliferation as measured by Ki-67.[74 76] In oral squamous cell cancer, HIF-1α overexpression correlated with a low apoptotic index,[77] but in invasive breast cancer, the opposite correlation was described.[78] No relation between the proliferation of lung tumours and HIF-1 (α and β) was seen when the expression of cyclin A and the phases of the cell cycle were analysed in relation to the presence of HIF-1α. However, a correlation was seen between HIF-1 expression and the apoptotic index.[79] This correlation between HIF-1α expression and apoptosis was also seen in patients with non-small cell lung carcinomas, studying the proapoptotic factors caspase 3, Fas, and the Fas ligand.[80]

> ''Because hypoxia inducible factor 1 (HIF-1) is related to resistance to chemotherapy and radiotherapy, targeting HIF-1 may help improve antitumour treatment''

The relation between the antiapoptotic protein Bcl-2 and the overexpression of HIF-1 is controversial. In non-small cell lung cancer, HIF-1 expression showed a significant inverse association with Bcl-2 expression.[81] Bcl-2 overexpressing lung carcinomas have a relatively good survival[82]; however, in a breast carcinoma study the opposite was observed—HIF-1 and Bcl-2 had a strong positive correlation.[78] The differences may be explained by tissue specific regulation of hypoxia induced apoptosis. A more aggressive clinical behaviour was seen in tumours overexpressing HIF-1α, and this may be related to resistance to apoptosis. Because HIF-1 is related to resistance to chemotherapy and radiotherapy,[83 84] targeting HIF-1 may help improve antitumour treatment. In this case, the inhibition of HIF-1 may not be sufficient as a treatment, but targeting HIF-1 may decrease resistance to the conventional treatment.

Take home messages

- Severe and prolonged hypoxia may initiate apoptosis, whereas cells often adapt to acute and mild hypoxia and survive
- The key regulator of hypoxia induced apoptosis is hypoxia inducible factor 1 (HIF-1), which acts in combination with many other factors, and can either induce or inhibit apoptosis
- HIF-1 can initiate hypoxia mediated apoptosis by increasing the expression of Bcl-2 binding proteins (BNIP3 and NIX), thereby inhibiting the antiapoptotic effect of Bcl-2, or by stabilising wild-type p53—if the cell already has a p53 gene mutation, hypoxia induced apoptosis is prevented
- Hypoxia by itself can also prevent apoptosis by inducing the expression of the antiapoptotic protein IAP-2
- HIF-1 may also have an antiapoptotic function because cells with high amounts of HIF-1 are more resistant to hypoxia induced apoptosis
- Further investigations into the function of HIF-1 in the regulation of apoptosis by hypoxia are required because understanding the regulation of apoptosis during hypoxia and the mechanisms of resistance to apoptosis might lead to more specific treatments for solid tumours

CONCLUSION

Severe and prolonged hypoxia may initiate apoptosis, whereas under acute and mild hypoxia cells may adapt to this environmental stress and will survive. Fine tuning of the regulation of apoptosis by hypoxia is influenced by HIF-1 in combination with many other factors. The role that the key regulator of hypoxia, HIF-1, plays in this hypoxia mediated programmed cell death is not entirely clear yet. HIF-1 can initiate hypoxia mediated apoptosis by increasing the expression of Bcl-2 binding proteins—BNIP3 and NIX—thereby inhibiting the antiapoptotic effect of Bcl-2. A different mechanism of inducing apoptosis is by stabilisation of wild-type p53 by HIF-1. If the cell already has a p53 gene mutation, hypoxia induced apoptosis is prevented. However, hypoxia by itself can also prevent apoptosis by inducing the expression of the antiapoptotic protein IAP-2. HIF-1 may also have an antiapoptotic function because cells with high amounts of HIF-1 are more resistant to hypoxia induced apoptosis.

Although many studies have focused on the role of HIF-1 in angiogenesis, it is clear that the function of HIF-1 in the regulation of apoptosis by hypoxia deserves more attention.

Translating the in vitro data to the clinic is still rather complex because of the dual role of HIF-1. In addition, different cell types may influence the balance of apoptosis. A better understanding of the regulation of apoptosis by hypoxia in solid tumours may enhance insight into tumour behaviour and the effect of hypoxia on antitumour treatments.

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