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Review: Implications of In Vitro Research on the Effect of Radiotherapy and Chemotherapy Under Hypoxic Conditions

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Key Words. Hypoxia • Cell culture • Irradiation • Chemotherapy • Resistance

LEARNING OBJECTIVES

After completing this course, the reader will be able to:

1. Argue why hypoxic regions in solid tumors often contain viable cells that are intrinsically more resistant to treatment with radiotherapy or chemotherapy.
2. Describe how exposure of cells or cell lines to hypoxic conditions has major implications on multiple intracellular pathways.
3. Evaluate the importance of investigating chemoradiotherapy combinations in vitro under both normoxic and hypoxic conditions.

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ABSTRACT

As it is now well established that human solid tumors frequently contain a substantial fraction of cells that are hypoxic, more and more in vitro research is focusing on the impact of hypoxia on the outcome of radiotherapy and chemotherapy. Indeed, the efficacy of irradiation and many cytotoxic drugs relies on an adequate oxygen supply. Consequently, hypoxic regions in solid tumors often contain viable cells that are intrinsically more resistant to treatment with radiotherapy or chemotherapy. Moreover, efforts have been made to exploit hypoxia as a potential difference between malignant and normal tissues.

Nowadays, a body of evidence indicates that oxygen deficiency clearly influences some major intracellular pathways such as those involved in cell proliferation, cell cycle progression, apoptosis, cell adhesion, and others. Obviously, when investigating the effects of radiotherapy or chemotherapy or both combined under hypoxic conditions, it is essential to consider the influences of hypoxia itself on the cell.

In this review, we first focus on the effects of hypoxia per se on some critical biological pathways. Next, we sketch an overview of preclinical and clinical research on radiotherapy, chemotherapy, and chemoradiation under hypoxic conditions. ***The Oncologist*** 2007;12:690–712

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Introduction

It is now well established that human solid tumors frequently contain a substantial fraction of cells that are hypoxic. In 1955, the histological studies of human lung adenocarcinomas by Thomlinson and Gray provided the first real indication that hypoxia does exist in tumors [1]. Since then, studies have demonstrated that hypoxic cells are a common feature in most solid tumors, including malignant brain tumors [2], melanomas [3], soft tissue sarcomas [4], prostate cancer [5], cervical cancer [6], invasive breast cancer [7], and non-small cell lung cancer (NSCLC) [8].

The physiology and the microenvironment of solid tumors are very different from those of normal tissues. While the oxygen tension (pO_2) in normal tissue ranges, depending on the tissue type, between 10 and 80 mmHg, tumors frequently contain regions with a $pO_2 < 5$ mmHg [6]. This oxygen deficiency can be either chronic or acute.

Chronic hypoxia is often referred to as "diffusion-limited" hypoxia, arising from large intervascular distances, beyond the diffusion limit of oxygen (i.e., approximately >150 μm). However, the origins of chronic hypoxia are more complex: compared with normal tissue vessels, the tumor microvasculature shows characteristic structural and functional abnormalities. Tumor blood vessels display a highly irregular vascular geometry with arteriovenous shunts, blind ends, lack of smooth muscle or enervation, and incomplete endothelial linings [9]. Furthermore, the abundant proliferation of tumor cells results in a disturbed balance between oxygen supply and demand [10]. Moreover, recent studies suggest that the relative lack of arteriolar input into tumors creates severe longitudinal pO_2 gradients within the vessels themselves [11]. All of these features contribute to the fact that a great portion of cancer cells is situated in chronically hypoxic regions [12].

In addition, spontaneous fluctuations in tumor blood flow can produce temporary regions of acute hypoxia ("perfusion-limited" hypoxia) [13]. These fluctuations are thought to result from transient occlusion and narrowing of vessels and arteriolar vasomotion [14].

Obviously, an important question to answer is: In what proportion are both types of hypoxia, acute and chronic, present in human tumors? In the past, chronic hypoxia has always been considered as the most important factor. However, studies have now demonstrated that microregional fluctuations in erythrocyte flow, consistent with transient, perfusion-driven changes in oxygenation, are also a common feature of human malignancies [15]. A study of four human melanoma xenografts even revealed that the fraction of acutely hypoxic cells was higher than the fraction of chronically hypoxic cells in three of the four tumors that were investigated [16]. Consequently, it has to be taken into account that both types of hypoxia occur commonly in human tumors.

Hypoxia influences tumor biology and physiology in paradoxically opposing ways: on the one hand, the oxygen deficiency on its own is cytotoxic, as it triggers a direct stress in the cells. On the other hand, the efficacy of radiation and many cytostatic drugs is directly linked with adequate oxygen tension. Consequently, hypoxic regions in solid tumors often contain viable cells that are intrinsically more resistant to treatment with radiotherapy or chemotherapy.

Increased attention is being given to the combination of radiotherapy with other treatment modalities to improve the therapeutic effect. Because hypoxia is a direct cause of therapeutic resistance and both radiotherapy and chemotherapy are significantly influenced by the presence of hypoxia, it is very important to analyze the effect of new combinations under hypoxic conditions in preclinical research. However, an unequivocal in vitro hypoxia model has not been described so far.

In this review, we first sketch an overview of the methods currently used for establishing hypoxia in vitro. Next, the influence of hypoxia on critical biological pathways is discussed, as well as the impact of oxygen deficiency on radiotherapy and chemotherapy.

Procedures to Investigate Hypoxia In Vitro

When attempting to classify the in vitro research on hypoxia, a first important criterion is the level of oxygen deprivation that is generated. Broadly speaking, two major subdivisions can be distinguished, that is, (a) moderate hypoxia, whereby a pO_2 of $\pm 1\%$ is established, and (b) extreme hypoxia or anoxia, whereby the pO_2 is 0.1% or even less. Experimentally, cellular hypoxia is mostly established by the use of environmentally controlled chambers or incubators in which the oxygen concentration in the gas phase is held at a constant level [17].

Besides the degree of hypoxia, a second factor that differs among the in vitro experiments is the duration of the hypoxic conditions. This parameter can range from 15 minutes to several days and is obviously linked with the method used and the level of oxygen deficiency that is induced. Very short exposures of 1 hour or even less are mostly examined in combination with extremely low oxygen tensions (95% N_2, 5% CO_2) [18–20]. Short, acute exposures up to 24 hours are most of the time carried out under anoxic conditions as well [21, 22]. However, sometimes acute moderate hypoxia is also investigated [23, 24].

Experimental setups in which the supply of oxygen is kept low for a longer period of time are less frequent. The induction of chronic hypoxia is mostly performed under

moderate hypoxic conditions [25, 26]. Chronic anoxia has been investigated also [27, 28], but Weinmann et al. [29] observed a retardation of cell growth and induction of apoptosis under anoxic (<0.1% O_2) but not under hypoxic (1% O_2) conditions in the cell lines they examined.

In addition to the level and the duration of hypoxia, a third parameter that can vary among different in vitro studies on hypoxia is the procedure for measuring hypoxia-induced cell death. In most studies, the clonogenic survival, that is, the colony-forming capacity of cells following exposure to hypoxia, is estimated [20, 30, 31]. Other studies have reported the use of nonclonogenic, colorimetric procedures. Skvortsova et al. [32] and Hay et al. [33] evaluated cell viability using the sulforhodamine B assay. Another colorimetric procedure used to assess cell survival after exposure to hypoxia is the microtetrazolium assay [18, 25, 34]. Still other investigators use the trypan blue exclusion procedure to estimate the percentage of viable cells following hypoxia [21, 35].

Influences of Hypoxia on Critical Biological Pathways

Influences of Hypoxia on the Cell in Culture

The intracellular pathways concerning the response to hypoxia still need to be further explored. Nevertheless, it is obvious that oxygen deficiency clearly influences proliferation rate, cell cycle progression, apoptosis, etc., and ongoing research focuses on the biomolecular mechanisms involved (Fig. 1). To investigate the effect of radiotherapy, chemotherapy, or combination treatment under hypoxic conditions in vitro, it is essential to keep in mind the influences of hypoxia itself on the cell in culture.

Hypoxia and Cell Proliferation

Concerning the influence of hypoxia on the proliferation pattern, a study of four human tumor cell lines (two melanoma and two squamous cell carcinoma cell lines) reported that, with all four cell lines, the population doubling time was 24 hours or less under normoxic conditions. However, in anoxic cultures (<0.03% O_2), only one doubling took place during an up to four times longer observation time [28].

In addition, it was described that exposure of several solid tumor cell lines (A549, HCT 15, and NCI H460) and T-lymphoma cells to extreme hypoxia (<0.1% O_2) induced growth arrest in all the cell lines examined. In contrast, moderate hypoxia (1% O_2) for 24–72 hours did not affect the proliferation rate [29]. In light of these findings, Koch et al. [25] suggested that lowering the oxygen tension below 1% O_2 would preclude a meaningful analysis of the efficacy of chemotherapeutic agents because of the lack of viable cells.

Prabhakaran et al. [36] used a modified "sandwich model" to examine the effect of hypoxia on neuroblastoma cells in vitro. Those experiments confirmed decreasing fractions of proliferating cells with increasing duration of hypoxia. It was furthermore suggested that these viable but hypoxic and nonproliferating tumor cells are of particular interest, because it is presumed that they comprise a substantial fraction of the cells within clinical solid tumors [37].

The effect of oxygen deprivation on cell proliferation is obviously linked to the influence of hypoxia on cell cycle progression. Krtolica and Ludlow [38] indeed reported that the proliferative capacity of ovarian carcinoma cells upon reoxygenation after a 24-hour exposure to severe hypoxia varied between 20% and 65% of that measured for normoxic control cultures. They noticed, furthermore, that hypoxic conditions resulted in 80%–90% inhibition of DNA synthesis. By flow cytometric analysis of the cellular DNA content, it was shown that the reduced proliferation was associated with an arrest of the cell cycle progression [38].

In order to better understand the molecular mechanisms leading to a diminished proliferation rate during hypoxia, the activity of hypoxia inducible factor 1 (HIF-1) was measured. HIF-1 is considered as one of the major transcription factors affecting gene regulation under reduced oxygen tension. It is a heterodimeric transcription factor consisting of two distinct subunits, that is, HIF-1α (the oxygen-regulated subunit) and HIF-1β (which is expressed constitutively) [39], and is known to mediate changes in the expression of more than 30 genes.

Goda et al. [40] argued that, in two primary differentiated cell types, HIF-1α is a major regulator of hypoxia-induced growth arrest. This arrest would be correlated with a HIF-1α–dependent increase in the expression of the cyclin-dependent kinase inhibitors p21 and p27. Although Gardner et al. [41] agreed that p27 is a key regulator of hypoxia-induced growth arrest, they reported that the hypoxic induction of p27 appeared to be HIF-1α-independent. Thus, the role of p21 and p27 in hypoxia-induced growth arrest is not yet fully understood, especially because Green et al. [42] suggested that, though upregulated by hypoxia, p21 and p27 did not affect growth arrest in immortalized fibroblasts.

In conclusion, it is clearly suggested that the proliferation rate of tumor cells under hypoxic conditions in vitro is lower than the proliferation rate of the same tumor cells under normoxic conditions. The fraction of proliferating cells is decreased most strongly with extreme hypoxia and with increasing duration of hypoxia.

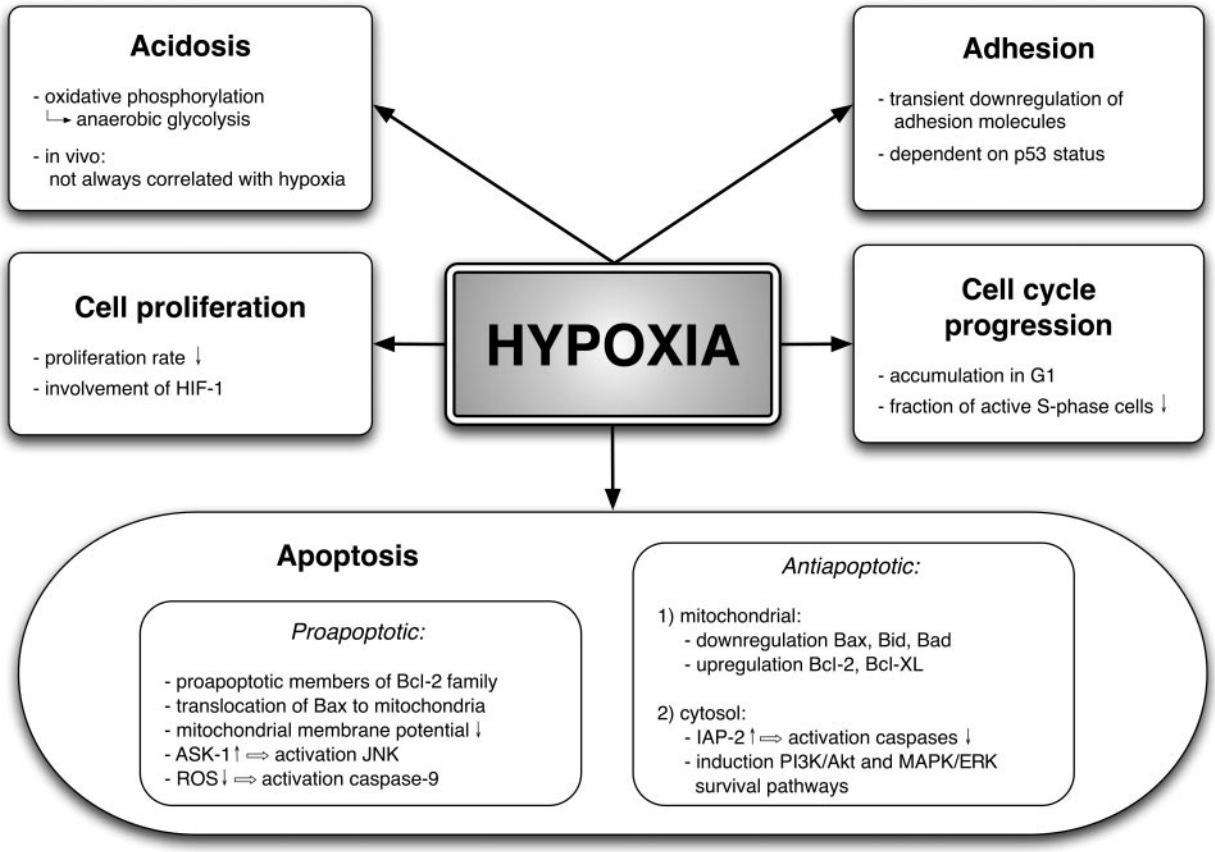

Figure 1. Overview of the influence of hypoxia on the cell in culture.

Abbreviations: ASK-1, apoptosis signal-regulating kinase 1; ERK, extracellular signal–regulated kinase; HIF-1, hypoxia inducible factor 1; IAP-2, inhibitor of apoptosis protein 2; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species.

Hypoxia and Cell Cycle Progression

As mentioned above, exposure to hypoxia mediates a striking effect on cell cycle progression. Amellem et al. [43, 44] revealed that mammalian cells in general tend to accumulate in the G_1 phase of the cell cycle during prolonged hypoxia. It has been reported that this accumulation results from three processes: (a) retinoblastoma protein (pRb)-mediated cell cycle arrest in mid-G_1; (b) activation of an oxygen-sensitive restriction point in late G_1, close to the G_1/S border; and (c) inhibition of DNA replication.

It was furthermore demonstrated that cells in G_2, mitosis, or early G_1 by the onset of hypoxia progress to the pRb-mediated checkpoint in mid-G_1 during continuation of hypoxic exposure. In contrast, pRb-incompetent cells or cells that have already passed this mid-G_1 checkpoint continue cell cycle progression until they are blocked in the oxygen-sensitive restriction point close to the G_1/S boundary. Moreover, cells in the S phase, when rendered hypoxic, are immediately arrested and are inactivated after only a few hours of oxygen deprivation. In general, cells residing in the S phase at the time of hypoxic conditions are much more sensitive to the lethal effects of hypoxia than cells in any other stage of the cell cycle. Consequently, following prolonged severe hypoxia, most clonogenic cells are arrested in one of the two restriction points in G_1 [31, 43, 44].

Interestingly, Zölzer and Streffer [28] reported that the fraction of active S-phase cells tended to decline under hypoxic conditions ($<0.03\%$ O_2). However, only in p53 mutant cell lines was this associated with an increase in the percentage of inactive S-phase cells. It was suggested that these inactive and quiescent S-phase cells arise because of the general breakdown of the cellular energy metabolism during hypoxia. In p53 wild-type cells, hypoxia would involve an activation of the G_1 checkpoint (in which the p53 protein is involved), so these cells would be preferentially blocked in G_1. In p53 mutant cells, this control is lost and cells would consequently enter the S phase without any delay or block. However, because of the energy depletion induced by oxygen deficiency, some of these cells would be unable to complete replication. They consequently stop cell cycle progression within the S-phase compartment, resulting in inactive S-phase cells [28].

In agreement with the observations of Zölzer and Streffer [28], Amellem et al. [45] noticed that cells are able to enter the S phase during anoxia (0.01–0.13% O_2), although they cannot complete DNA synthesis.

In addition, Green et al. [42] noticed that, in immortalized mouse embryo fibroblasts, DNA replication rapidly falls down at extremely low oxygen tensions (0.01% O_2), while at 0.1% O_2 replication is not significantly impaired. They support the hypothesis that inactivation of two critical enzymes (ribonucleotide reductase and dehydroorotate dehydrogenase) in nucleoside biosynthesis during hypoxia induces depletion of deoxynucleotide precursors necessary for DNA synthesis. This depletion in turn results in a shutdown of the replication [42]. Concerning this issue, Loffler [46] reported no correlation between pyrimidine metabolism and reduction of the proliferation rate at suboptimal O_2 levels (5%–7% O_2) in Ehrlich ascites tumor cells. At a critical O_2 concentration (<1% O_2), however, it was shown that the lack of pyrimidine deoxynucleotides substantially impairs cell cycle progression.

Furthermore, hypoxia seems to induce growth arrest by regulating the activity of several crucial cell cycle regulatory proteins. During hypoxia, a reduction in cyclin A, D, and E abundance is noticed. In addition, pRB-directed phosphatase activity was found to be greater in hypoxic cells than in aerobic cells, thus resulting in hypophosphorylation of pRB [38, 47]. At the molecular level, it has also been demonstrated that HIF-1α functionally counteracts Myc, thereby inhibiting cell cycle progression [48].

Besides the perturbations of the cell cycle distribution during hypoxia, Koritzinsky et al. [31] also investigated the effect of prolonged severe hypoxia followed by reoxygenation. They observed that tumor cells arrested in G_1 during anoxic conditions (<0.0004% O_2) progress very synchronously through the first cell cycle following reoxygenation. In addition, after reoxygenation, an accumulated cell population in the G_2/M phase was seen [31], wherein two different subpopulations of cells could be distinguished. The first subgroup consisted of cells arrested in G_1 during hypoxia that are delayed in G_2 after resuming cell cycle progression, possibly because hypoxia is associated with an elevated level of DNA damage [31]. The second subpopulation contained cells blocked in the S phase during hypoxia that remained permanently arrested in G_2 after reoxygenation [31, 49].

In conclusion, all these studies show that hypoxia induces G_1 arrest and can result in a reduced fraction of active S-phase cells. However, it has been reported that cell cycle effects such as growth arrest and cell cycle block would mainly be present under conditions of severe hypoxia (<0.1% O_2), but not under moderate hypoxic conditions (1% O_2) [29].

Hypoxia and Apoptosis

In addition to the effects on proliferation and cell cycle progression, low oxygen concentrations influence the mechanism of apoptosis or programmed cell death. The process of apoptosis is very complex and is regulated by a cascade of proteins, called caspases. This signaling pathway is fine-tuned by numerous positive (proapoptotic) and negative (antiapoptotic) proteins, so a delicate balance among these factors decides whether a cell undergoes apoptosis or survives [50].

Hypoxia interferes with several components of this mechanism, thereby either promoting or inhibiting cell death. Indeed, the effect of hypoxia on apoptosis is contradictory: besides the proapoptotic effects of oxygen deficiency, cells can become resistant to apoptosis during hypoxia.

The induction of apoptosis by hypoxia has been described by several groups, who showed that exposure to extreme hypoxia was associated with internucleosomal DNA fragmentation [18, 21]. It was found that moderate levels of hypoxia (1% O_2) did not induce a significant rise in apoptotic activity, while the number of apoptotic cells did increase after exposure to severe hypoxia (<0.1% O_2) [29, 51]. Moreover, cells subjected to anoxia underwent programmed cell death in a time-dependent way [18].

Although the pathways underlying apoptosis during oxygen deprivation are still largely unknown, several contributing components have been identified.

First, proapoptotic members of the Bcl-2 family seem to be involved in the initiation of apoptosis in response to oxygen deficiency. It has been reported that during hypoxia, the proapoptotic protein Bax is translocated from the cytosol to the mitochondria, resulting in cytochrome c release and caspase activation [52]. More recently, this has been confirmed by other observations that designate HIF-1 induction as the basis of Bax upregulation [53].

Second, McClintock et al. [54] suggested that hypoxia-induced apoptosis is associated with a decrease in the mitochondrial membrane potential. Hypoxic cells would be unable to maintain the membrane potential because of the decreased import of glycolytic ATP. As a consequence, cytochrome c would be released into the cytosol, followed by activation of caspase-9.

A third way by which hypoxia can induce apoptosis is by influencing mitogen-activated protein kinase (MAPK) signaling pathways. Hypoxia seems to mediate a transient increase in the activity of apoptosis signal-regulating kinase 1 (ASK-1), a MAP kinase kinase kinase (MAPKKK) that

triggers cascades leading to the phosphorylation and activation of c-Jun N-terminal kinase (JNK) [55]. This hypoxia-induced JNK activation plays, at least in melanoma cells, a critical role in apoptosis regulation, both in vitro and in vivo [56].

Finally, it has been suggested that oxygen deprivation stimulates reactive oxygen species (ROS) generation at complex III of the electron transport chain in the mitochondria [57]. In human neuroepithelioma cells, ROS would directly activate caspase-9. The activated caspase-9 would, in turn, disrupt the mitochondrial diffusion limit of cytochrome c, resulting in an increased release of cytochrome c and consequently apoptosis [58]. Recently, Ho et al. [59] confirmed that apoptosis, induced by hypoxia/reoxygenation in A431 squamous carcinoma cells, would likely be established through the intrinsic caspase cascade, because only caspase-9 and caspase-3, but not caspase-8 were activated. However, they reported that, under hypoxia, less ROS accumulated, while there was a significant increase in the intracellular ROS level upon reoxygenation. Therefore, they suggested that ROS would be important for the induction of apoptosis by reoxygenation after hypoxia, but not during exposure to hypoxic conditions [59].

In contrast to its proapoptotic effects, oxygen deprivation can also induce a significant resistance to apoptosis. This adaptive response of hypoxic tumor cells takes place at two levels, that is, in the mitochondria and in the cytosol.

At the mitochondrial level, downregulation of the proapoptotic proteins Bax, Bid, and Bad has been reported in a wide range of tumor cell lines under hypoxic conditions ($<0.1\%$ O_2 to 2% O_2). An HIF-1–dependent pathway would mediate the downregulation of Bid, whereas downregulation of Bax and Bad occurs independently of HIF-1 [60].

Moreover, accumulation of Bax in the mitochondrial membrane would be suppressed by hypoxia (1% O_2), with a concomitant reduction in cytochrome c release [61]. In addition, upregulation of Bax inhibitors, such as the antiapoptotic proteins Bcl-2 and Bcl-X_L, during hypoxia, would attenuate the function of Bax through protein–protein interactions [27, 62]. Among others, von Hippel-Lindau gene products seem to promote a striking upregulation of Bcl-2 expression following chemically induced hypoxia, thereby protecting tumor cells from apoptosis [35].

In the cytosol, apoptosis resistance is demonstrated specifically at the level of caspase activation. Severe hypoxia (95% N_2, 5% CO_2) strongly induces the transcription of inhibitor of apoptosis protein-2 (IAP-2) by HIF-1–independent mechanisms. Consequently, IAP-2 interacts directly with caspases, thereby suppressing their activation [61, 63]. Moreover, hypoxia (1% O_2) seems to be associated with reduced caspase-3 activity [64].

Finally, the hypoxia-dependent protection from apoptosis correlates with the induction of the phosphatidylinositol 3-kinase (PI3K)/Akt and the MAPK/extracellular signal–regulated kinase (ERK) survival pathways. PI3K/Akt is an important downstream mediator of many receptor tyrosine kinase signaling pathways involved in cell proliferation, migration, and inhibition of apoptosis. During hypoxia (1% O_2), it is phosphorylated and thus activated. Similarly, MAPK/ERK, which regulates proliferation in response to several growth factors, is also phosphorylated under hypoxic conditions [64, 65].

The role of HIF-1α in apoptosis is still a subject of discussion. HIF-1α shows both pro- and antiapoptotic effects, depending on its phosphorylation status. During moderate hypoxia, phosphorylated HIF-1α dimerizes with HIF-1β to transactivate various genes required for adaptation to the hypoxic conditions. However, under more severe hypoxia, the dephosphorylated form of HIF-1α stabilizes p53, leading to upregulation of Bax and further proapoptotic mechanisms [66, 67].

In conclusion, a delicate balance between pro- and antiapoptotic responses exists during reduced oxygen tension, but the molecular basis of the decisions made by the tumor cells still needs to be elucidated. In addition, apoptosis is not the only mode of oxygen deprivation–induced cell death. Extreme hypoxia possibly stimulates cells to initiate apoptosis. However, at a later stage, other forms of cell death, probably including necrosis, gain significance [68, 69].

Hypoxia and Adhesion

Another factor that has to be taken into account is that oxygen deficiency decreases cellular adhesion. Indeed, it has been reported that exposure to hypoxia is associated with a substantial downregulation of cell adhesion molecules, including E-cadherin and integrins, at the surface of human cancer cells in vitro [68, 70]. This downregulation causes reduced adhesion to extracellular matrix components, such as vitronectin and fibronectin, which in turn results in cell detachment [17]. Furthermore, it has been noticed that the ability of tumor cells to preserve adhesion during extreme hypoxia depends on their p53 status. While p53 wild-type cells detach and lyse rapidly under hypoxic conditions, p53 mutant cells remain attached, with unchanged morphology [28]. Interestingly, hypoxia induces only a transient reduction in the expression of adhesion molecules, and after reoxygenation, the expression and the cell–cell adhesion recover [70].

Hypoxia and Acidosis

Finally, we would like to highlight the importance of having knowledge of the pH of the culture medium when exposing cells to hypoxia in vitro.

This information is important since hypoxia can be associated with low pH. Under hypoxic conditions, cells have to compensate for the loss in energy generation resulting from the inhibition of oxidative pathways. Therefore, a switch from oxidative phosphorylation (Krebs cycle) to anaerobic glycolysis occurs. As a consequence of this switch, both the consumption of glucose and the production of lactic acid increase, and this may result in lactic acidosis. However, cells can also lower their level of energy production, and if so, glucose consumption and acidosis will not change rapidly. Indeed, HIF-1 not only stimulates glycolysis [71], it also suppresses mitochondrial function and oxygen consumption by directly transactivating the gene encoding pyruvate dehydrogenase kinase (PDK-1). PDK-1 inactivates pyruvate dehydrogenase, an enzyme of the Krebs cycle [72, 73].

Skoyum et al. [74] suggested that hypoxic tumor cells differ in their ability to avoid severe energy deprivation. These differences would be mainly attributable to differences in the capacity of cells to reduce energy-requiring processes rather than to differences in the capacity to generate energy by anaerobic glycolysis.

Schmaltz et al. [75] reported that lowering the pH would be a more important factor in tumor cell death than hypoxia. The results of their investigations indicated that the trigger for apoptosis in hypoxic cultures is acid accumulation (likely lactic acidosis) rather than hypoxia per se.

In addition, acidosis may have implications for treatment with chemotherapeutic agents. Because of the decreased extracellular pH, the cellular pH gradient is significantly reduced, possibly inducing changes in cellular drug accumulation and toxicity [76–78]. Consequently, when performing experiments under hypoxic conditions, it might be useful to monitor the pH of the culture medium.

Interestingly, in vivo experiments have revealed that hypoxia is not always correlated with a decrease in interstitial pH. Moreover, transient hypoxia can occur in a region with a normal pH [75, 79]. Therefore, it might be important to keep the pH neutral during in vitro research under hypoxic conditions, for example, by supplementing the culture medium with $NaHCO_3$ [16, 80].

Influences of Hypoxia on Tumor Biology

Although this review mainly focuses on in vitro research on the influence of hypoxia on cellular pathways, it is clear that hypoxia has a profound effect on tumor biology too. Therefore, in this section, we also highlight the relationship between hypoxia and some critical biological pathways in vivo.

Hypoxia and Tumor Growth

Though oxygen supply is considered as a key variable determining cell proliferation, the exact intracellular and extracellular dynamics that govern tumor growth in vivo remain poorly understood. Moreover, a study by Cairns et al. [14] showed that neither chronic nor acute hypoxic treatment (5%–7% O_2) affected tumor growth compared with control animals (normal laboratory air) after i.m. injection of murine fibrosarcoma cells into mice.

As mentioned above, HIF-1 is involved in the regulation of proliferation during hypoxic exposure. In several xenograft assays, manipulation of HIF-1 activity by genetic or pharmacological means has shown marked effects on tumor growth. Nevertheless, the exact role of HIF-1 in tumor biology is still controversial, because the results of various tumor models are contradictory. For example, different xenograft studies showed a tumor growth inhibitory effect by blocking HIF-1. Stoeltzing et al. [81] reported that inhibiting HIF-1α by stable overexpression of dominant-negative mutant HIF-1α resulted in inhibition of gastric tumor growth in vivo. Another study demonstrated that blockage of HIF-1 interaction with its coactivators p300 and cAMP-response element binding protein reduced the s.c. growth of human colon cancer HTC116 cells [82]. Furthermore, Höpfl et al. [83] injected wild-type (HIF-1$\alpha^{+/+}$) or HIF-1α–deficient (HIF-1$\alpha^{-/-}$) embryonic stem cells s.c. in nude mice. They showed that the resulting HIF-1$\alpha^{+/+}$ tumors grew significantly faster than HIF-1$\alpha^{-/-}$ tumors. In line with these studies, an in vivo experiment by Kondo et al. [84] suggested that overexpression of HIF-1α increased the tumor growth of renal cancer cells in nude mice. In contrast, Savai et al. [85] concluded that, in subcutaneous tumors derived from HIF-1α–overexpressing A549 cells, tumor growth was negatively affected as a result of decreased proliferation and increased apoptosis.

So, inherent differences among tumor models (e.g., cell types, sites of tumor growth, methods used to inhibit or enhance HIF-1α activity) have probably made the effect of HIF-1α on tumor growth difficult to interpret. Even in clinical studies, the role of HIF-1α in tumor biology is hard to unravel. For example, a study performed on patients with head and neck cancers revealed slower tumor growth in high HIF-1α–expressing tumors [86]. However, most clinical cancer studies described that overexpression of HIF-1α was associated with greater mortality [87, 88], making HIF-1α a potential target for anticancer therapy.

Hypoxia and Apoptosis

As extensively discussed previously, reduced oxygen tension implies a delicate balance between pro- and antiapoptotic signaling in vitro. This is also seen in vivo; for example, an inverse correlation between the hypoxic marker pimonidazole and the proapoptotic Bcl-2 family proteins Bid and Bax was observed when HCT116 cells were grown as tumor xenografts in nude mice [60].

However, there still remains controversy regarding the potential of HIF-1 in altering apoptosis in the tumor microenvironment. Studies using tumors derived from HIF-1–deficient embryonic stem cells have yielded conflicting data. Stoeltzing et al. [81] found no significant differences in the mean number of apoptotic cells between tumors derived from HIF-1$\alpha^{-/-}$ or HIF-1$\alpha^{+/+}$ cells. In contrast, Ryan et al. [89] suggested that the slower growth of HIF-1α–deficient tumors was partly attributable to a greater rate of apoptosis. Carmeliet et al. [90] observed the opposite effect, as they showed that hypoxia-induced apoptosis was consistently lower in HIF-1$\alpha^{-/-}$ tumors than in HIF-1$\alpha^{+/+}$ tumors. They suggested that apoptosis in vivo would be hypoxia-driven, because fluorescence microscopy showed that most apoptosis-positive fields were also positive for the hypoxia marker EF5. In summary, HIF-1α, probably, may promote both tumorigenesis and apoptosis in vivo, depending on the specific circumstances.

Hypoxia and Angiogenesis

The growth of new capillary blood vessels, or angiogenesis, is required for extended tumor growth and metastasis. This makes it an important control point in the progression of cancer: the transformation from a quiescent to an invasive phenotype is invariably accompanied by the acquisition of angiogenic properties ("angiogenic switch") and vascularization of the tumor [91]. For this reason, tumor angiogenesis has been the subject of extensive investigations.

It is generally believed that tumor angiogenesis is driven by hypoxia. The relationship between the two is often considered as a matter of supply and demand: ineffectively vascularized tumor tissue becomes hypoxic, stimulating neoangiogenesis to improve the influx of oxygen [92]. Indeed, a correlation between markers of hypoxia (e.g., vascular endothelial growth factor [VEGF]) and low vessel density has been reported [93].

Accumulating evidence indicates that hypoxia and the key transcriptional system HIF are the major triggers for new blood vessel growth. A large number of genes involved in different steps of angiogenesis are induced by HIF. One of the most important of such genes is *VEGF*, a primary regulator of the formation of new blood vessels. During hypoxia, *VEGF* transcription is activated by HIF [94] and its mRNA stability is increased [95]. In addition, a functional internal ribosome entry site in the 5′ untranslated region ensures efficient translation is maintained under hypoxia [96].

Further examples of HIF-induced angiogenic genes include those encoding other growth factors, such as angiopoietins and their receptors [97], as well as genes involved in matrix metabolism, including those encoding matrix metalloproteinases [98], plasminogen activator receptors and inhibitors [99], and collagen prolyl hydroxylase [100]. The net result of the activation of these genes is the stimulation of increased vessel formation and remodeling to provide adequate oxygen delivery to hypoxic tissues.

Although a straightforward relationship between hypoxia and angiogenesis is logically pleasing, some preclinical and clinical data show that tumor hypoxia and angiogenesis do not always go hand in hand. For example, Raleigh et al. [101] failed to show a spatial colocalization between hypoxia and VEGF protein expression in immunostained sections from patients with cervical or head and neck cancer.

Thus, the pathogenesis of tumor hypoxia probably is much more complicated than the supply–demand paradigm suggests. First, hypoxia might develop in tumors through mechanisms unrelated to inadequacies in tumor vascular supply. For example, the high oxygen consumption rates in tumors undergoing active proliferation might stimulate angiogenesis by lowering pO_2 [92]. Moreover, tumors can generate high concentrations of certain endogenous antiangiogenic compounds, such as angiostatin and endostatin [102]. As a result, hypoxic stimulation of proangiogenic signaling might not be sufficient for inducing angiogenesis.

In summary, although poor oxygenation is a strong stimulus for tumor angiogenesis, the story does not end there and multiple factors complicate hypoxia–angiogenesis interplay.

Hypoxia and Metastasis

It has now been firmly established that hypoxia has important roles in tumor progression and several clinical studies have linked tumor hypoxia directly to metastasis. The first study suggesting that the development of metastatic disease is associated with low oxygen tension in the primary tumor comes from Brizel et al. [103]. They showed that patients with hypoxic soft tissue sarcomas (defined as median pO_2 $<$10 mmHg) had a significantly higher risk for lung metastasis and lower disease-free survival rate than patients with less hypoxic tumors. This was confirmed by Nordsmark et al. [4], who also identified hypoxia as an independent factor predisposing patients with soft tissue sarcomas to metastatic spread. More recently, Fyles et al. [6] reported that node-negative cervical cancer patients with hypoxic tumors

(percentage of pO_2 readings <5 mmHg >50%) had a significantly higher 3-year cumulative incidence of distant metastases than patients with better-oxygenated tumors.

While a body of evidence suggests a role for hypoxia in tumor dissemination, the multiple mechanisms involved in hypoxia-induced metastasis are still under investigation.

First, angiogenesis is largely implicated in the process of tumor metastasis. The local shedding of tumor cells into the circulation has been shown to start only after the tumor has become vascularized by new vessels. More generally, the rate of tumor metastasis is related to the induction of proangiogenic factors such as VEGF and interleukin-8 [104, 105].

Second, it has been suggested that hypoxia may enhance metastasis by increasing genetic instability in the tumor microenvironment, as oxygen deficiency is associated with increased DNA damage, enhanced mutagenesis, gene amplification, chromosomal rearrangements, and functional impairments in DNA repair pathways (reviewed by Bindra and Glazer [106]).

Third, it has been hypothesized that hypoxia creates a selective pressure for tumors, allowing the expansion of cell subpopulations that have lost their apoptotic potential, in particular cell variants that have acquired p53 mutations [107].

In addition, hypoxia may induce changes in gene expression. It is interesting that many genes involved in extracellular matrix remodeling are induced by hypoxic exposure. For example, several members of the plasmin pathway are upregulated when exposed to hypoxia. Rofstad et al. [108, 109] reported that hypoxia promoted lymph node metastasis in human melanoma xenografts by upregulation of the urokinase-type plasminogen activator receptor (uPAR) and increased binding of uPA. This resulted in increased proteolytic activity adjacent to the tumor cells, which facilitated tumor cell migration toward lymphatic vessels.

Matrix metalloproteinases (MMPs) have also been implicated in metastatic progression, because MMPs can degrade all constituents of the basement membrane as well as structural components of the stroma. Several in vitro and in vivo studies have linked hypoxia with the regulation of expression and/or activity of members of the MMP family [110–112]. However, some of these reports are contradictory; for example, Himelstein and Koch [113] were unable to demonstrate a consistent hypoxia-mediated increase in MMP-9 protein, RNA, or transcriptional activity, suggesting that MMP-9 expression was not directly affected by exposure to hypoxia in vitro. Interestingly, a recent analysis of human breast tumors showed a strong correlation between hypoxic microenvironments and MMP-2 activation [114]. All together, it is suggested that hypoxia can modify the invasive capability of cells in part by shifting the balance between MMPs and their inhibitors, favoring increased MMP activity.

Finally, as described earlier, another landmark of invasion is the downregulation of cell adhesion molecules such as E-cadherin by hypoxia. An intriguing question is how hypoxia represses E-cadherin. Recently, Erler et al. [115] showed that the extracellular matrix protein lysyl oxidase (LOX) is consistently overexpressed in hypoxic human breast and head and neck tumor cells. Furthermore, in an orthotopic model of breast cancer, they showed that inhibition of LOX expression significantly reduced formation of lung metastases and liver metastases, indicating a role for LOX in controlling metastatic growth and dissemination [115].

In summary, while many elegant studies have demonstrated that hypoxia enhances metastatic potential, the critical issue for the future is to identify pertinent genes and proteins that are involved in this process in order to develop novel hypoxia-targeted therapy.

Impact of Hypoxia on Radiotherapy Response

Hypoxia and Radiotherapy Resistance

A positive correlation between intracellular pO_2 and the efficacy of radiotherapy has been described for a long time. The difference in radiation sensitivity between well-oxygenated and hypoxic cells, defined as the oxygen enhancement ratio (the ratio of doses to produce the same level of cell killing under hypoxic versus aerobic conditions) usually amounts to 2.5–3.0 for human cells [116].

Study of this phenomenon has revealed that it is the presence of oxygen at the time of irradiation that causes radiation sensitivity rather than any metabolic effect or intrinsic property such as transmembrane transport, DNA repair capacity, etc. [12]. The reason why low oxygen tension is associated with radioresistance relies on the fact that cell killing by ionizing radiation is caused by damage to the DNA. Either direct ionization or reaction of the radiation with hydroxyl radicals produced by radiolysis of nearby water molecules results in the origin of DNA radicals. Oxygen, a molecule with a very high electron affinity, reacts extremely fast with the free electrons of these radicals, thereby fixing the free radical damage. However, in the absence of oxygen, reducing species such as sulfhydryl compounds interact with the DNA radicals by hydrogen donation. This interaction leads to restitution of the DNA to the innocuous state. As a result, hypoxia severely compro-

mises ionizing radiation in its ability to kill cells [12, 117, 118].

Preclinical Research on Radiotherapy Under Hypoxic Conditions

Several in vitro studies have confirmed that hypoxia affects cellular radiosensitivity by increasing resistance to ionizing radiation. Vordermark et al. [119] analyzed the effect of exposure to hypoxic conditions (5%, 1%, or 0.1% O_2 for 1 hour) on the cellular radiosensitivity of the FaDu and HT1080 tumor cell lines. In both cell lines, the largest increase in radioresistance was noticed between 5% and 1% O_2, with smaller differences between 2% and 5% O_2 and between 1% and 0.1% O_2 [119].

The impact of reduced oxygen tension on radiation-induced apoptosis was investigated by Weinmann et al. [29]. After a preincubation time of 6 hours in an extremely hypoxic atmosphere ($<0.1\%$ O_2), cells were irradiated with a single dose of 10 Gy, while control cells received the same radiation dose under ambient oxygen conditions. It was noticed that, in unirradiated control cells, anoxia itself induced a small increase in the rate of spontaneous apoptosis. Nevertheless, the apoptotic rate was significantly slower after radiation under extremely hypoxic conditions compared with normoxic controls [29].

Current research focuses on the causal factors of hypoxia-induced radioresistance. Several reports indicated that hypoxia-mediated radioprotection might be linked to antiapoptotic mechanisms. For example, it has been reported that chronic hypoxia protects head and neck cancer cells against radiation-induced apoptosis by inducing Bcl-2 overexpression and by inhibiting mitochondrial transformation of Bax [120]. Moreover, hypoxia blocked radiation-induced cleavage of caspase-3 and poly-ADP-ribose polymerase in TK6 human lymphoblastoid cells [121].

The contribution of HIF-1 to the outcome of radiotherapy is still under investigation. Arvold et al. [122] showed hypoxia-induced radioresistance to be independent of HIF-1α in two mouse embryonic fibroblast cell lines. Williams et al. [123] were also unable to observe any difference in the radiation response of Hepa-1 (HIF-1 wild-type) and HIF-1–deficient cells in vitro. Nevertheless, HIF-1 deficiency promoted radioresponsiveness in vivo. In agreement with these data, small interfering RNA targeted to HIF-1α was associated with an enhancement of radiotherapy in vivo [124].

Concerning the duration of hypoxic treatment, Pettersen and Wang [125] reported that cells irradiated under extremely hypoxic conditions (0.0004% O_2), following prolonged hypoxia, were shown to be radioresistant. However, these cells were relatively more radiosensitive than cells irradiated under hypoxic conditions following acute hypoxia [125]. Zölzer and Streffer [28] also noticed greater sensitivity of chronically versus acutely hypoxic cells. Moreover, their results seemed to indicate that oxygen deprivation involves an increase in the radiation sensitivity of p53 mutants, but not cells expressing wild-type p53. Independently of p53 status, energy depletion could cause a reduced ability to repair radiation damage. The general breakdown of the cellular energy metabolism during chronic hypoxia could bring along a delay in DNA replication, making cells more sensitive to radiation damage [28]. Indeed, Ling et al. [126] demonstrated that radiation sensitivity increases when the cellular energy status declines, because the repair of sublethal radiation damage is energy dependent. Consequently, it is conceivable that the greater sensitivity of chronically versus acutely hypoxic cells results from changes in energy metabolism. This would manifest itself more in p53 mutants, because they are less sensitive than p53 wild-type cells to hypoxia as such [28].

Regarding reoxygenation, several groups noticed that transient exposure to hypoxia followed by reoxygenation promoted greater radiation sensitivity of human tumor cell lines. Kwok and Sutherland [127] suggested that radiosensitization by reoxygenation would require metabolic changes during both the hypoxia and the reoxygenation. Consistent with these data, Danielsen et al. [30] showed that the increased radiosensitivity of human melanoma cells after reoxygenation could be a consequence of the extensive energy depletion induced during the hypoxia pretreatment ($<0.001\%$ O_2).

Other experiments indicated that radiosensitization by reoxygenation after chronic hypoxia is not related to inhibition of the repair of potentially lethal radiation damage [128].

Overall, the intricate mechanisms by which hypoxia influences the outcome of radiotherapy are not completely understood so far, and further research is needed to reveal the molecular-biological pathways involved.

Hypoxia and Radiotherapy in Clinical Trials

The prognostic significance of low pretreatment pO_2 values for responsiveness of tumors to radiotherapy has been the subject of numerous clinical studies. The first clinical proof came from Gatenby et al. [129], who measured oxygen distribution in lymph node metastases of head and neck cancer using polarographic electrodes. They showed substantial differences in pO_2 values of complete responders versus nonresponders [129]. Next, several studies have demonstrated the adverse prognostic influence of low pO_2 on local tumor control, disease-free survival, and overall survival

among patients with cancer of the head and neck or cervix and soft tissue sarcomas (reviewed by Harrison and Blackwell [130]).

Moreover, clinical proof to support this from endogenous hypoxia marker studies looks promising. For example, HIF-1 expression has been shown to predict poor prognosis after radiation in patients with oropharynx cancer [88]. Koukourakis et al. [131] concluded that immunohistochemical assessment of the endogenous markers HIF-2α and carbonic anhydrase 9 was strongly correlated with radiotherapy failure in patients with head and neck cancer. In addition, high tumor expression of VEGF identified patients with prostate cancer at high risk for radiotherapy failure [132].

Impact of Hypoxia on Chemotherapy Response

Hypoxia and Chemotherapy Resistance

In addition to the impact of hypoxia on the outcome of radiotherapy, tumor hypoxia causes resistance to a broad range of cytotoxic agents because of several reasons. First, because many anticancer drugs require molecular oxygen to be maximally cytotoxic, hypoxia provides a direct cause of therapeutic resistance [133]. Second, drug resistance can result from hypoxia-induced inhibition of cell proliferation [134]. As most chemotherapeutic agents are mainly effective against rapidly dividing cells, the slowing down of the proliferation by hypoxia leads to a decrease in cell killing.

Furthermore, as hypoxia arises from insufficient and disordered vasculature, the delivery of many cytotoxic drugs to hypoxic regions is restricted [135, 136]. An important implication of the fluctuating blood flow (causing acute hypoxia) is that tumor cells will be resistant to any chemotherapeutic agent that has a shorter half-life in the bloodstream than the time for which the dysfunctional blood vessel remains closed [117]. In addition, the concentration of cytotoxic agents is higher in regions more adjacent to the blood vessels than further away. This is brought about by the fact that drugs have to reach more peripheral tumor cells by diffusion from a central vessel. However, many chemotherapeutic agents are limited in their diffusion through successive cellular layers because of their reactivity and their metabolism [37].

Another factor that contributes to chemoresistance is the induction of alterations in the cellular genome and proteome by hypoxia. Hypoxia-induced changes in the expression of genes may result in drug resistance and may thus impair treatment with chemotherapeutic agents.

Preclinical Research on Chemotherapy Under Hypoxic Conditions

Preclinical Research on Conventional Chemotherapeutic Agents Under Hypoxic Conditions

In cell culture, most cytotoxic agents show a positive correlation between oxygen tension and their efficacy (Table 1).

For example, Rice et al. [137] demonstrated that Chinese hamster ovary cells became highly resistant to cell killing by methotrexate after they were exposed to extremely hypoxic conditions (95% N_2, 5% CO_2) for several hours. They reported that this resistance resulted from amplification of the dihydrofolate reductase gene [137]. Sanna and Rofstad [80] observed that anoxic stress ($<0.001\%$ O_2) induced methotrexate resistance in two of four human melanoma cell lines. They suggested that the increased cellular drug resistance was a result of hypoxia-induced metabolic or genetic alterations in the tumor cells, such as elevated synthesis of oxygen-regulated proteins [80]. Moreover, HIF-1α knockdown was found to increase drug sensitivity to methotrexate under hypoxic conditions in MCF-7 cells [138].

Sakata et al. [139] found that mammary tumor cells exposed to 5-fluorouracil in air, immediately after an extremely hypoxic treatment ($<0.001\%$ O_2), developed resistance to this drug. They suggested that resistance to 5-fluorouracil could result from the depletion of the intracellular pool of nucleotides that is observed in hypoxic cells. Moreover, continuous presence of oxygen is needed to maintain the active, radical-containing form of ribonucleoside diphosphate reductase, an important enzyme in the metabolism of 5-fluorouracil [139].

Luk et al. [140] showed that transient exposure of a human bladder carcinoma cell line to extreme hypoxia ($<0.002\%$ O_2) promoted a small decrease in doxorubicin sensitivity. This would be partly a result of hypoxia-induced alterations in the distribution of cells in the cell cycle [140]. Other investigators have confirmed that hypoxic stress facilitates doxorubicin resistance in several human tumor cell lines [80, 141]. Comerford et al. [141] revealed that the multidrug resistance (*MDR-1*) gene is hypoxia responsive and they proposed the hypoxia-induced expression of P-glycoprotein as a possible pathway for resistance to doxorubicin. However, this was refuted by Song et al. [142], who reported that MDR-1 regulation would not be involved in hypoxia-induced chemoresistance to doxorubicin and cisplatin in NSCLC cells.

For etoposide, hypoxia-mediated drug resistance has been described by several groups. Whether the HIF-1α pathway is involved in the protection from drug-induced

Table 1. In vitro research on conventional chemotherapeutic agents under hypoxic conditions

Chemotherapeutic agent	Study	% O_2	Cell line	Sensitivity under hypoxic conditions	Possible mechanism
Methotrexate	Rice et al. [137]	95% N_2, 5% CO_2	CHO AA8 cell line	Increased resistance	Amplification of dihydrofolate reductase gene during hypoxia
	Sanna and Rofstad [80]	<0.001% O_2	Four human melanoma cell lines (BEX-c, COX-c, SAX-c, WIX-c)	Increased resistance in two of the four cell lines	Hypoxia-induced metabolic or genetic alterations
	Li et al. [138]	1% O_2	Human breast tumor MCF-7 cells	Increased resistance	Contribution of HIF-1α pathway
5-Fluorouracil	Sakata et al. [139]	<0.001% O_2	Murine EMT6/Ro mammary tumor cells	Increased resistance	Hypoxia-induced depletion of intracellular pool of nucleotides
Doxorubicin	Luk et al. [140]	<0.002% O_2	Three human tumor cell lines (MGH-U1, Hey, A549); 2 murine sarcoma cell lines (EMT6/Ro, KHT-LP1)	Human cell lines, increased resistance; EMT6/RO cells, no difference with normoxia; KHT-LP1 cells, increased resistance	Cell-cycle phase-specific effects of doxorubicin
	Sanna and Rofstad [80]	<0.001% O_2	Four human melanoma cell lines (BEX-c, COX-c, SAX-c, WIX-c)	Increased resistance	Hypoxia-induced metabolic or genetic alterations
	Comerford et al. [141]	2.67% O_2	A multicellular spheroid model of human KB cells	Increased resistance	Hypoxia-elicited P-glycoprotein expression
	Song et al. [142]	0.5% O_2	Human lung adenocarcinoma cell lines SPCA1 and A549	Increased resistance	Contribution of HIF-1α pathway, MDR-1 regulation would not be involved
Etoposide	Piret et al. [143]	1% O_2	Human hepatoma cells HepG2	Increased resistance	Activation of AP-1 pathway
	Schnitzer et al. [144]	0.5% O_2	Human lung adenocarcinoma cell line A549	Increased resistance	Contribution of HIF-1α pathway
	Brown et al. [145]	<0.01% O_2	Human tumor cell lines HT1080 and HCT116	Increased resistance	HIF-1-mediated downregulation of the proapoptotic protein Bid
	Lee et al. [146]	1% O_2	Human lung adenocarcinoma and squamous cell lines A549 and NCI-H157	Increased resistance	Hypoxia-induced activation of PI3K/Akt and ERK survival pathways
Gemcitabine	Koch et al. [25]	1% O_2	Three human testicular germ cell tumor cell lines (NTera-2, 2102 EP, NCCIT)	Increased resistance	

(continued)

Table 1. (Continued)

Chemotherapeutic agent	Study	% O_2	Cell line	Sensitivity under hypoxic conditions	Possible mechanism
	Yokoi and Fidler [147]	1% O_2	Human pancreatic cancer cell line L3.6pl	Increased resistance	Hypoxia-induced activation of PI3K/Akt and ERK survival pathways
Actinomycin D	Sakata et al. [139]	<0.001% O_2	Murine EMT6/Ro mammary tumor cells	Increased resistance	
Carboplatin	Koch et al. [25]	1% O_2	Three human testicular germ cell tumor cell lines (NTera-2, 2102 EP, NCCIT)	Increased resistance	
Bleomycin	Koch et al. [25]	1% O_2	Three human testicular germ cell tumor cell lines (NTera-2, 2102 EP, NCCIT)	Increased resistance	
Paclitaxel	Skvortsova et al. [32]	0.2% O_2	A human vulvar squamous cell carcinoma cell line (A431); a human mammary gland carcinoma cell line (MDA-MB-231); a human bronchioalveolar carcinoma cell line (NCI-H358)	A431 and MDA-MB-231, increased resistance; NCI-H358, not significantly different from normoxia	Hypoxia-induced EGFR expression
Cisplatin	Skov et al. [148]	O_2-free N_2	Three CHO cell lines (AA8, UV20, UV40)	Enhanced cytotoxicity	Hypoxia-dependent potentiation of cisplatin-induced DNA–protein crosslinks
	Yamagata et al. [149]	95% N_2, 5% CO_2	Two human hepatoma cell lines (PLC/PRF/5, HuH-7)	No cytotoxic enhancement	
	Koch et al. [25]	1% O_2	Three human testicular germ cell tumor cell lines (NTera-2, 2102 EP, NCCIT)	Increased resistance	
Mitomycin C	Koch et al. [25]	1% O_2	Three human testicular germ cell tumor cell lines (NTera-2, 2102 EP, NCCIT)	Increased resistance	
	Kennedy et al. [150]	95% N_2, 5% CO_2	Murine sarcoma 180 and EMT6 tumor cell lines	Enhanced cytotoxicity	
	Yamagata et al. [149]	95% N_2, 5% CO_2	Two human hepatoma cell lines (PLC/PRF/5, HuH-7)	Enhanced cytotoxicity	

Abbreviations: AP-1, activator protein 1; CHO, Chinese hamster ovary; EGFR, epidermal growth factor receptor; ERK, extracellular signal–regulated kinase; HIF-1, hypoxia inducible factor 1; MDR-1, multidrug resistance 1; PI3K, phosphatidylinositol 3-kinase.

apoptosis is under discussion. Although Piret et al. [143] suggested HIF-1 would not be involved, others have reported conflicting results that show HIF-1–mediated downregulation of the proapoptotic protein Bid under anoxia [144, 145]. Furthermore, hypoxia-induced resistance was shown to be decreased after specific inhibition of the antiapoptotic protein activator protein (AP)-1. Moreover, activation of the survival pathways PI3K/Akt and ERK would be involved [146]. Interestingly, activation of the PI3K/Akt and MAPK/ERK pathways would

also contribute to hypoxia-mediated resistance to gemcitabine [147].

Meanwhile, hypoxia and/or anoxia are associated with resistance to a growing number of cytotoxic agents, such as actinomycin D (<0.001% O_2) [139], carboplatin (1% O_2) [25], bleomycin (1% O_2) [25], paclitaxel (0.2% O_2) [32], and some other chemotherapeutic agents.

However, for some cytotoxic drugs, the observed results of their cytotoxicity under hypoxic conditions are contradictory. For cisplatin, Skov et al. [148] noticed enhanced drug activity under hypoxic conditions, possibly resulting from a potentiation of cisplatin-induced DNA–protein crosslinks. In contrast, Yamagata et al. [149] could not show any cytotoxic enhancement of cisplatin in two human hepatoma cell lines during hypoxia. In addition, Koch et al. [25] demonstrated that cisplatin was less effective under hypoxic conditions (1% O_2) in three human testicular germ cell tumor cell lines. The same was observed for mitomycin C, as Koch et al. [25] found lower toxicity under hypoxia in three cell lines, although several other in vitro studies have described a greater efficacy of mitomycin C during hypoxia [149, 150].

In conclusion, the ability and efficiency of hypoxia to induce drug resistance greatly depends on the cell line and the cytotoxic agents investigated. Nevertheless, despite their different mode of action and potential ways to induce drug resistance, a relative increase in resistance is seen in a broad spectrum of cytotoxic agents during hypoxia. These observations suggest that rather universally active mechanisms or coactivation of several pathways confer resistance under hypoxic conditions. However, further in-depth studies are certainly required to elucidate the underlying mechanisms.

The Development of Bioreductive Drugs

As the presence of hypoxic areas can be a major obstacle for the successful treatment of tumors, efforts have been made to exploit hypoxia as a potential difference between malignant and normal tissues. This has led to the development of bioreductive drugs, which are only activated in the absence of oxygen and thus selectively eradicate hypoxic cells. Stratford et al. [151] suggested that bioreductive drugs can be subdivided into four main classes: (a) the quinones, such as mitomycin C and EO9; (b) the nitroheterocyclics, such as the hypoxic bioreductive marker pimonidazole; (c) the aromatic N-oxides, such as tirapazamine; and (d) aliphatic N-oxides such as AQ4N [151].

Mitomycin C is regarded as the prototype bioreductive drug. Several in vitro experiments have reported that mitomycin C shows greater toxicity toward oxygen-deficient cells than their oxygenated counterparts [152, 153]. Moreover, oxygenation status at the time of exposure to mitomycin C, rather than preincubation conditions, were shown to be the parameter determining cell sensitivity [154].

A second well-explored bioreductive agent is the benzotriazine-di-N-oxide tirapazamine. As with mitomycin C, in vitro studies indicate that tirapazamine is a potent and selective killer of hypoxic cells. In a variety of murine and human cell lines, a 15- to 200-fold differential toxicity toward hypoxic versus aerobic cells has been observed [153, 155]. Moreover, tirapazamine potentiates the activity of several cytotoxic agents such as cyclophosphamide, fluorouracil, doxorubicin, carboplatin, and cisplatin [156].

The third bioreductive prodrug briefly mentioned is AQ4N, an aliphatic di-N-oxide. As with mitomycin C and tirapazamine, AQ4N is the inactive prodrug of a DNA-targeting cytotoxic agent. Moreover, it was shown that AQ4N enhances the activity of cisplatin, cyclophosphamide, and thiotepa [157–159].

Hypoxia and Chemotherapy in Clinical Trials

Whereas resistance of hypoxic cells to conventional chemotherapy is well documented, clear hypoxic thresholds for chemotherapeutic agents are still not available, though they presumably exist for each agent. Thus, additional research is necessary to provide quantitative data on hypoxia-induced chemoresistance, although this information might be difficult to obtain in clinical settings [160].

Current clinical trials try to overcome hypoxia-induced treatment failure by combining conventional chemotherapeutic agents with bioreductive drugs, in particular, tirapazamine. For example, a phase III trial (the Cisplatin and Tirapazamine in Subjects with Advanced Previously Untreated Non-Small Cell Lung Tumors [CATAPULT] I trial) demonstrated a survival benefit for the combination of tirapazamine and cisplatin compared with cisplatin alone, in patients with advanced NSCLC [161]. Unfortunately, a more recent phase III trial with NSCLC patients showed that the addition of tirapazamine to carboplatin and paclitaxel did not result in superior response, time to progression, or survival, but led to significantly greater toxicity than with carboplatin and paclitaxel alone [162]. As other bioreductive drugs such as EO9 have not been impressive in the clinic, further development is required to improve treatment response.

Impact of Hypoxia on Chemoradiation Response

The combination of radiotherapy and chemotherapy is promising because of its independent cell kill effect and the property of some cytotoxic agents to enhance the effect of radiotherapy. Although the mechanism of action of chemotherapeutic agents and radiation under hypoxic conditions

is still a subject of research, attention is already being given to the outcome of chemoradiation protocols during hypoxia. At present, several different approaches in the combination of chemotherapeutic agents and irradiation are being investigated (Table 2).

First, research has focused on the interaction between conventional anticancer drugs and radiation during hypoxia. It was already described at the end of the 1970s that platinum complexes can act as radiosensitizers of hypoxic tumor cells in vitro [163, 164]. This was confirmed by in vivo studies in the murine SCCVII model, which showed that cisplatin is a potent radiosensitizer. Cisplatin/radiation treatment protocols in animals breathing a reduced oxygen atmosphere were most efficacious when drug exposure preceded irradiation. Therefore, it has been suggested that the primary interaction is a cisplatin-induced increase in the oxygenation status of acutely hypoxic tumor cells [165].

Koukourakis et al. [166] suggested that overexpression of HIF-1α in patients with head and neck cancer was related to important resistance to carboplatin chemoradiotherapy [166]. However, a study of carboplatin in different cell lines was unable to demonstrate a significant difference in radiosensitization whether cells were exposed to carboplatin before or during irradiation in air or under hypoxic conditions [167]. Yet, another study suggested that radiation increases the binding of carboplatin to dsDNA under hypoxic, but not under normoxic, conditions [168].

In addition to the studies on platinum complexes, it has been suggested that a major mechanism of tumor radioenhancement of taxanes is possibly the reoxygenation of radioresistant hypoxic cells [169]. Very recently, the influence of irinotecan and its active metabolite SN-38 on the radiation response of WHO3 human esophageal tumor cells was studied under hypoxic conditions. Hereby, it was noticed that irinotecan, and in particular SN-38, markedly radiosensitized the hypoxic cells [170].

A second approach implies the combination of radiotherapy with hypoxic radiosensitizers, that is, drugs that are able to sensitize ionizing radiation under hypoxic conditions. These "oxygen-mimetic" electron-affinic drugs are activated during hypoxia and fix radiation damage in a way similar to oxygen. They are themselves only slightly toxic to hypoxic cells and substantial kill of hypoxic cells is only achieved when they are given concomitantly with irradiation.

The most investigated hypoxic radiosensitizers are the nitroaromatic compounds. Despite convincing in vitro data, clinical trials with the first-generation nitroimidazoles (metronidazole, misonidazole) were generally disappointing [171–174].

Thereafter, second-generation 2-nitroimidazoles like etanidazole and pimonidazole became available. Because of their lower neurotoxicity, these hypoxic cell radiosensitizers could be given at higher doses than misonidazole [175–177]. Unfortunately, clinical results of these second-generation nitroimidazoles were more or less disappointing too [178, 179].

Consequently, newer nitroimidazole derivates like nimorazole and doranidazole have been examined. An in vitro study with several human pancreatic cancer cell lines indicated that doranidazole had no sensitizing effect when combined with aerobic irradiation, but under hypoxic conditions significant radiosensitizing activity was shown [180, 181]. A recent phase III trial of patients with pancreatic cancer confirmed a superior therapeutic response to radiotherapy using doranidazole [182]. Moreover, nimorazole was reported to significantly improve the outcome of radiotherapy in patients with supraglottic or pharyngeal tumors in a large phase III randomized trial (a Danish Head and Neck Cancer [DAHANCA] trial) [183, 184]. So, although hypoxic radiosensitizers generally did not lead to the hoped success, nimorazole is now part of the standard treatment of most patients with head and neck cancer in Denmark.

A third strategy is the combination of bioreductive drugs with radiotherapy. It has been suggested that regimens combining these hypoxia-selective drugs with radiotherapy would target hypoxic and oxygenated tumor cell populations, respectively.

Indeed, combination of mitomycin C with radiation resulted in enhanced antitumor effects in both preclinical and clinical studies [185, 186]. For example, it has been reported that the combination of radiotherapy with mitomycin C is safe and effective for the treatment of squamous cell carcinoma of the head and neck. However, despite improvements noted in local recurrence-free survival and cause-specific survival, no difference in overall survival was observed [186].

For tirapazamine, in vitro results demonstrated that it has the potential to overcome the radioresistance induced by hypoxia. It has been reported that the addition of tirapazamine to hypoxic melanoma cells before irradiation produced a radiosensitivity similar to that of aerobic cells [187]. Recently, Rischin et al. [188] showed strikingly better locoregional control in patients with hypoxic tumors of the head and neck treated with a regimen in which tirapazamine was added to a standard radiation and cisplatin treatment versus a cisplatin, fluorouracil, and radiation regimen.

For AQ4N, in vivo experiments showed a positive interaction with ionizing radiation [158]. Moreover, it has been reported that AQ4N potentiates the action of irradiation in both a drug and radiation dose-dependent way [158, 189].

Finally, prodrugs activated in hypoxic cells by the reducing species generated by ionizing radiation have been described recently. This new type of bioreductive agent is

Table 2. Overview of preclinical chemoradiation experiments under hypoxic conditions

Class of agents combined with radiation	Drug	Study	Type of study	Effect of the combination with radiation under hypoxic conditions
Conventional anticancer drugs	Cisplatin	Douple and Richmond [163]	In vitro (Chinese hamster V-79 cells)	Radiosensitization of hypoxic cells (DEF 1.3–1.4)
		Douple and Richmond [164]	In vivo (C3H/HeJ mice bearing MTG-B)	Radiosensitization of hypoxic cells
		Yan and Durand [165]	In vivo (murine SCCVII model)	Maximal radiosensitization of acutely hypoxic cells when drug exposure preceded irradiation
	Carboplatin	Yang et al. [168]	In vitro (3 CHO cell lines: K1, AA8, UV41)	No significant difference between radiosensitization in air and under hypoxia (DEF 1.3–1.7)
	Paclitaxel	Milas et al. [169]	In vivo (C3Hf/Kam mice bearing MCA-4)	No significant enhancing effect on tumor radioresponse under hypoxia; radiosensitization a result of reoxygenation of radioresistant hypoxic cells
	Irinotecan	Van Rensburg et al. [170]	In vitro (human esophageal carcinoma cell line WHO3)	Dose-dependent re-establishment of radiosensitivity under hypoxic conditions
Hypoxic radiosensitizers	Metronidazole	Rauth and Kaufman [171]	In vivo (KHT transplantable tumor of C3H mice)	Radiosensitization of a chronically hypoxic cell population in air-breathing mice
	Misonidazole	Rockwell [172]	In vitro (murine EMT6 cells)	Radiosensitization of hypoxic cells (DEF 1.6)
	Etanidazole	Brown and Yu [175]	In vivo (RIF-1 tumor implanted in C3H mice)	Radiosensitization of hypoxic cells at low radiation doses
	Pimonidazole	Denekamp et al. [176]	In vivo (albino mice of the WHT inbred strain)	Radiosensitization of acutely hypoxic cells (DEF 1.1–2.15)
	Doranidazole	Shibamoto et al. [180]	In vitro (4 human pancreatic cell lines)	Significant radiosensitization under hypoxic conditions (DEF 1.25–1.55)
		Mizumoto et al. [181]	In vitro (7 human pancreatic cancer cell lines)	Selective radiosensitization of hypoxic cells
Bioreductive drugs	Mitomycin C	Rockwell and Kennedy [185]	In vitro (murine EMT6 cells)	Enhanced antitumor effects

(continued)

Table 2. (Continued)

Class of agents combined with radiation	Drug	Study	Type of study	Effect of the combination with radiation under hypoxic conditions
	Tirapazamine	Zhang and Stevens [187]	In vitro (3 melanoma cell lines: human MM576 and MM96L and murine B16-F10)	Tirapazamine given to hypoxic cells 1 hour before radiation produces the same radiosensitivity as with aerobic cells
	AQ4N	Patterson et al. [158]	In vivo (NMRI mice with MAC16 or MAC26 tumors; C3H mice with RIF-1 or SCCVII tumors; CBA mice with NT tumors)	Enhanced antitumor effects
Prodrugs activated by hypoxic radiation	OFU001 (1-(2′-oxopropyl)-5-fluorouracil)	Shibamoto et al. [192]	In vitro (murine SCCVII cells)	Hypoxic radiation-activated cytotoxicity
		Shibamoto et al. [193]	In vivo (C3H/He mice bearing SCCVII tumors)	OFU001 is efficiently activated during hypoxic irradiation, but no in vivo efficacy could be proven
	OFU106 (5-Fluorodeoxy-uridine prodrug)	Shibamoto et al. [20]	In vitro (murine SCCVII and EMT6 cells); in vivo (Nalb/c mice bearing EMT6)	In vitro, hypoxic radiation-activated cytotoxicity; in vivo, no marked effects observed
	IQ-FdUrd (5-Fluorodeoxy-uridine prodrug)	Tanabe et al. [194]	In vitro (murine EMT6/KU cells)	Hypoxic radiation-activated cytotoxicity
	Nitroarylmethyl quaternary salts	Wilson et al. [191]	Use of anoxic plasma	Hypoxic radiation-activated cytotoxicity

Abbreviations: CHO, Chinese hamster ovary; DEF, dose enhancement factor.

activated in hypoxic regions specifically by irradiation. Interestingly, because these prodrugs do not rely on enzymes for their bioreduction (as is the case for mitomycin C and tirapazamine), activation can potentially occur in all hypoxic regions of a tumor, including necrotic regions [190]. However, this specific chemoradiation strategy places great demands on the design of the prodrugs. Because a clinically relevant dose of radiation (2 Gy) generates only a small amount of reducing equivalents, the prodrugs need to be capable of releasing very potent cytotoxins [191]. As an example, the uracil prodrug OFU001 (1-(2′-oxopropyl)-5-fluorouracil) is activated by hypoxic irradiation to release 5-fluorouracil [192]. However, in vivo evaluation indicated that the effect of this compound was not high enough to warrant further clinical studies [193]. Therefore, a series of radiation-activated prodrugs of 5-fluorodeoxyuridine (which is generally more potent than 5-fluorouracil) is now being investigated [194]. Although these prodrugs are activated as efficiently as the prodrugs of 5-fluorouracil, marked in vivo effects have not been detected so far [20].

Besides the radiation-activated prodrugs of 5-fluorouracil, a series of quaternary ammonium salts of mechlorethamine has been synthesized. These compounds deactivate the cytotoxic amine effector very efficiently and appreciable cytotoxic activation of the prodrugs can be achieved by a therapeutic dose of irradiation in anoxic plasma [191].

In conclusion, the overall concept of chemoradiation under hypoxic conditions seems promising, but more in-

depth research is needed before the unique features of hypoxia can be maximally exploited.

Concluding Remarks

Our knowledge about hypoxia as a key microenvironmental factor contributing to the development of an aggressive phenotype in cancer is growing day by day. Recent studies have provided mechanistic insights into the wide spectrum of molecular pathways, orchestrated by oxygen deficiency. It has been demonstrated that signaling mechanisms interact, support as well as antagonize each other, which mounts to a complex network of cell death, adaptation, and treatment resistance.

The future poses three great challenges to the oncologist. First of all, further study is needed to accurately characterize the molecular phenotype of the hypoxic tumor microenvironment. As discussed in this review, many hypoxia-induced gene products have been identified, but their exact functions within the tumor microenvironment often remain to be determined. We expect that further work will undoubtedly reveal a spectrum of new and exciting areas in hypoxia signaling.

Next, the application of many chemoradiotherapy combinations is in the pipeline. As solid tumors contain hypoxic regions that are intrinsically more resistant to treatment with chemotherapy or radiotherapy, it is important to investigate combination schedules under both normoxic and hypoxic conditions.

In addition, the continued research into the molecular and cellular responses to hypoxia will contribute further to the development of novel antitumor treatments. For example, drugs targeting HIF-1 will be available soon and there is no doubt that they will have important clinical implications. Moreover, hypoxia can be used to activate therapeutic gene delivery to specific areas of tissue. Many elements of the hypoxia response pathway are good candidates for therapeutic targeting, challenging further insight into the intricate nature of the hypoxic tumor microenvironment.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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