Clinical Cancer Research

Targeting Hypoxic Cells through the DNA Damage Response

Monica Olcina, Philip S. Lecane, and Ester M. Hammond

Abstract

Exposure to hypoxia-induced replication arrest initiates a DNA damage response that includes both ATR- and ATM-mediated signaling. DNA fiber analysis was used to show that these conditions lead to a replication arrest during both the initiation and elongation phases, and that this correlated with decreased levels of nucleotides. The DNA damage response induced by hypoxia is distinct from the classical pathways induced by damaging agents, primarily due to the lack of detectable DNA damage, but also due to the coincident repression of DNA repair in hypoxic conditions. The principle aims of the hypoxia-induced DNA damage response seem to be the induction of p53-dependent apoptosis or the preservation of replication fork integrity. The latter is of particular importance should reoxygenation occur. Tumor reoxygenation occurs as a result of spontaneous changes in blood flow and also therapy. Cells experiencing hypoxia and/or reoxygenation are, therefore, sensitive to loss or inhibition of components of the DNA damage response, including Chk1, ATM, ATR, and poly(ADP-ribose) polymerase (PARP). In addition, restoration of hypoxia-induced p53-mediated signaling may well be effective in the targeting of hypoxic cells. The DNA damage response is also induced in endothelial cells at moderate levels of hypoxia, which do not induce replication arrest. In this situation, phosphorylation of H2AX has been shown to be required for proliferation and angiogenesis and is, therefore, an attractive potential therapeutic target. *Clin Cancer Res; 16(23); 5624–9. ©2010 AACR.*

Background

Most solid tumors develop in an environment of below optimal oxygen concentration (hypoxia). This low oxygen level occurs as a result of inefficient tumor vasculature and the high metabolic demand for oxygen; essentially an issue of low supply and high demand. Many elegant studies have shown that this situation is therapeutically significant, as hypoxic cells are more resistant to both chemo- and radiotherapy (1, 2, 3). Hypoxia has also been shown to increase both invasion and metastasis, therefore, contributing to more aggressive disease (4–6). For these reasons, the ability to image hypoxic areas and target these cells has become an area of intense scrutiny. The ability of cancer cells to survive and thrive in these conditions results from their ability to hijack pathways necessary for embryonic development in hypoxic conditions. The principle mediators of the hypoxic response are the hypoxia-inducible factor (HIF) transcription factors, which are composed of an oxygen-labile α subunit (HIF-1α, HIF-2α, HIF-3α, and HIF-4α) and a shared constitutively expressed protein (HIF-1β/ARNT; ref. 7). In *in vivo* settings, hypoxia occurs as a gradient of oxygen tensions ranging between normal levels (6%), mild hypoxia (0.5 to 3%), and anoxia (0%; ref. 8). The HIF proteins are responsive to a wide range of oxygen tensions. HIF-1α and HIF-2α possess structurally similar domains, and their stability is regulated through two oxygen-dependent degradation domains (NODDD and CODDD), which allow their proteolytic degradation (9). However, expression of HIF-1α and HIF-2α has been shown to differ between hypoxic tissues, indicating they may have different roles (10). For example, HIF-1α has been shown to be involved in causing cell cycle arrest following moderate hypoxia by inhibition of c-Myc, whereas HIF-2α may enhance cell cycle progression by promoting the activation of c-Myc and some of its target genes (11).

In contrast, severe levels of hypoxia ($<0.1\%$ O_2) have been shown to induce a specific hypoxic response not observed at milder hypoxia levels. This response includes the unfolded protein response, cell death, and the DNA damage response (DDR), which are induced at severe levels of hypoxia (12–15). The DDR involves a complex collaboration between signaling pathways activated as a result of different types of DNA-damaging stresses. In brief, signals such as a double strand break are detected by a group of proteins known collectively as sensors, including the MRN complex (Mre11-Rad50-NBS1). This initial detection of DNA damage leads to activation of the PI3-kinase, ATM, and subsequently ATR. This response is amplified by a group of mediator proteins, including MDC1 and 53BP1

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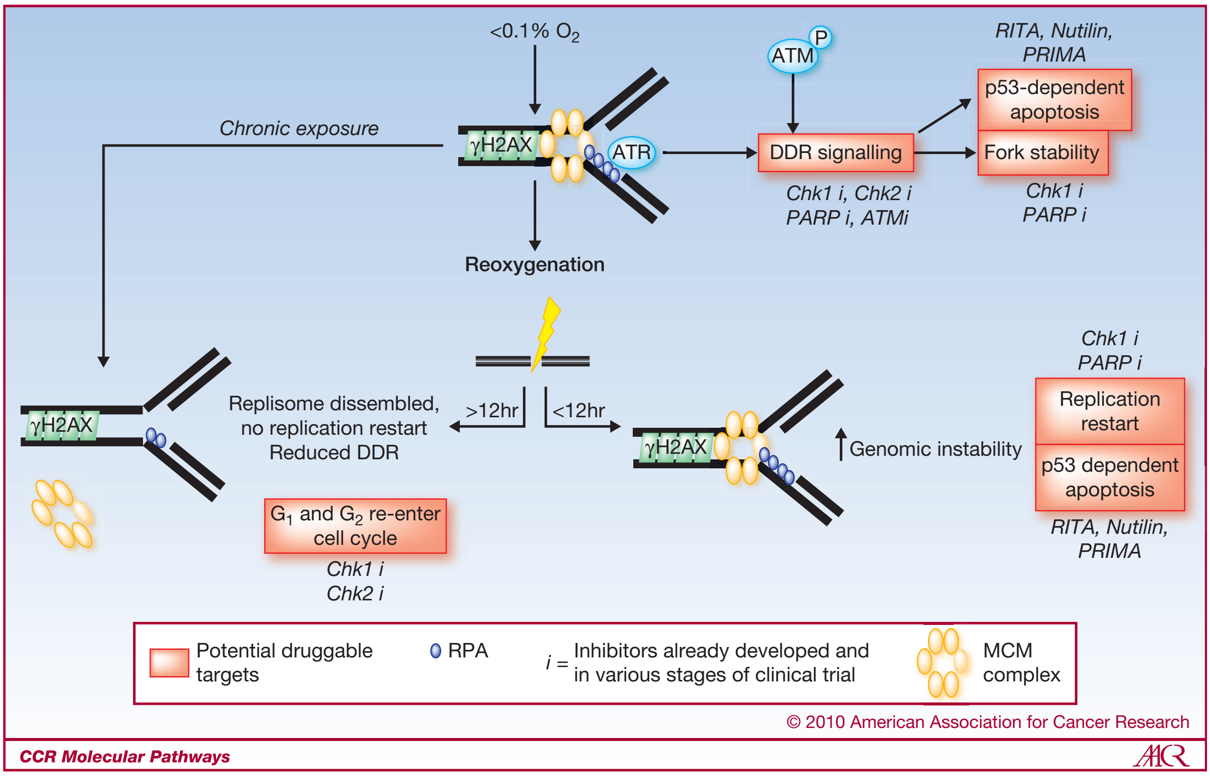

Fig. 1. Hypoxia-induced replication arrest triggers a DDR. Chronic exposure to levels of oxygen below 0.1% lead to replisome disassembly, therefore, preventing any possible replication restart during reoxygenation. In contrast, hypoxic cells arrested for only short periods of time do undergo replication restart, but do so in the presence of reoxygenation-induced DNA damage and hypoxia-repressed DNA repair. Highlighted in red are the potential therapeutic targets for targeting cells cycling through hypoxia and/or reoxygenation or angiogenesis discussed in the text. Specific targets that have inhibitors in clinical trials are indicated in italics.

(16). Ultimately, these pathways are involved in mediating DNA-repair cell-cycle checkpoint activation and/or apoptosis, in order to maintain genomic stability following such insults (17).

The DDR activated at severe levels of hypoxia (<0.1% O_2) involves an induction of rapid replication arrest. The enzyme responsible for nucleotide production is ribonucleotide reductase, which is dependent on cellular oxygen for its function and is, therefore, likely to be severely compromised in hypoxic conditions (18). In support of this theory, we recently measured nucleotide levels in hypoxic cells *in vitro* and found a rapid and significant decrease in levels in response to hypoxia (19). Regions of single-stranded DNA (ssDNA) accumulate at stalled replication forks in hypoxic conditions and, in turn, become coated with replication protein A (RPA; ref. 20). This sequence of events is believed to be the signal for the hypoxic induction of the DDR, which includes the ATR-dependent phosphorylation of, for example, p53, H2AX, and Chk1 (Fig. 1; refs. 21, 22). Interestingly, these phosphorylations occur in the apparent absence of DNA damage, unless factors essential to replication fork stability are also inhibited and/or depleted. Despite this finding, the ATM kinase is also active in hypoxia as shown by increased autophosphorylation and an ability to phosphorylate Chk2 (23–25). ATM has previously been shown to be active in the absence of DNA damage. although, hypoxia is one of the few physiologically relevant stresses to do this (26, 27). ATM-dependent Chk2 phosphorylation under hypoxic conditions has been shown to lead to phosphorylation of p53 at serine 20 and BRCA1 at serine 988 (28). The trigger that initiates ATM-mediated signaling is currently unclear. However, it seems likely that replication stress-induced ATR in hypoxic conditions contributes to the signaling (29). Hypoxia-induced replication arrest is reversible if oxygen levels are restored within an acute time frame (up to approximately 8 to 12 hours). After longer more chronic exposures, a disassembly of the replisome is observed, as well as a failure to restart DNA synthesis, even in the presence of available nucleotides. Specifically, in response to chronic hypoxia exposure, the MCM complex is transcriptionally repressed and becomes detached from the chromatin (Fig. 1; ref. 19).

Although hypoxia does not lead to an accumulation of DNA damage as detected by either comet or 53BP1 foci formation assay, reoxygenation induces significant levels of DNA damage through the action of reactive oxygen species (30). This damage, in turn, leads to an ATM-Chk2–mediated G_2 arrest to allow repair. Tumor cells lacking

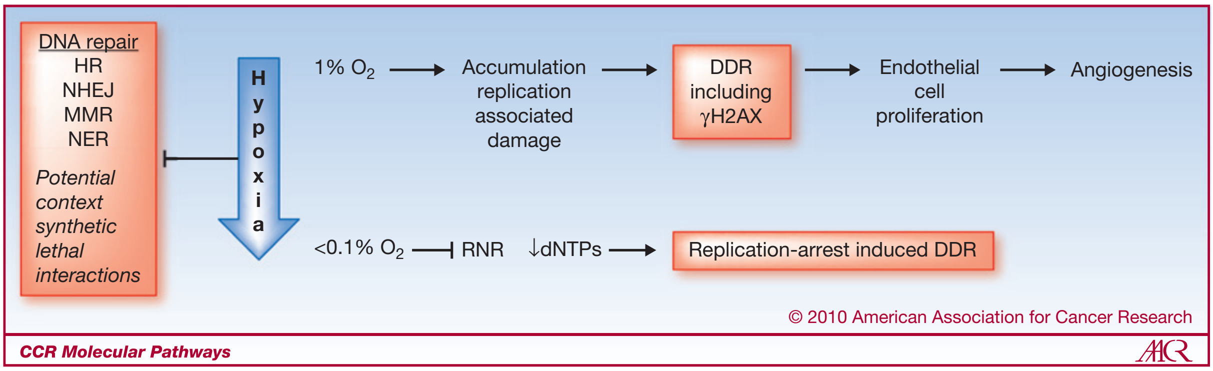

Fig. 2. Schematic of the DDR to hypoxia. The DDR to hypoxia is both oxygen and cell-type specific, for example endothelial cells have been shown to be reliant on γH2AX for proliferation and angiogenesis. At severe levels of hypoxia, nucleotide levels decrease, leading to stalled replication and a DDR. Highlighted in red are the potential therapeutic targets for targeting cells cycling through hypoxia and/or reoxygenation or angiogenesis discussed in the text. HR, homologous recombination; NHEJ, nonhomologous end joining; MMR, mismatch repair, NER, nucleotide excision repair; RNR, ribonucleotide reductase; dNTP, deoxyribonucleoside triphosphates.

Chk2 show reduced reoxygenation-induced arrest and increased apoptosis (23, 25, 31).

More recently, a hypoxia-mediated induction of a DDR has been observed in conditions that do not cause replication arrest (Fig. 2; ref. 32). This work showed that, in response to hypoxia (1% O_2), γH2AX was induced in proliferating endothelial cells and that, even more surprisingly, this was required to maintain proliferation and hypoxia-induced neovascularization in these conditions (32; reviewed in refs. 33, 34). Intriguingly, there was no apparent role for γH2AX in developmental angiogenesis, as loss of γH2AX only reduced hypoxia-induced neovascularization in pathologic settings, for example, hind leg ischemia, retinopathy, and tumor angiogenesis. The induction of a DDR in these conditions was attributed to the accumulation of the low level of DNA damage, which occurs during normal replication. This DNA damage may be potentially more prevalent in hypoxic conditions, as many essential components of the DNA repair pathways have been shown to be repressed in hypoxic conditions (for a recent review, see ref. 35). Homologous recombination, mismatch repair, and nonhomologous end joining have all been shown to be less effective in hypoxic conditions, suggesting that a general response to hypoxia is repression of DNA repair. The mechanisms of repression are varied and include roles for HIF and micro RNAs (miR; refs. 36, 37). For example, components of the mismatch repair pathway, MLH1 and MLH2, have been shown to be repressed under hypoxic conditions. MLH1 repression seems to correlate with increased levels of di- and trimethylations on H3K9, owing to an increase in histone methyltransferase G9a (38). Key members of the homologous recombination pathway, RAD51 and BRCA1, have also been shown to be downregulated in hypoxia. A proposed mechanism for RAD51 and BRACA1 downregulation is the formation of a repressive E2F4/p130 complex at the E2F site on the promoter of these genes (39).

Why a cell actively represses these pathways is unclear, although it may simply be an energy saving measure. Importantly, the hypoxia-mediated repression of DNA repair seems to occur at a variety of oxygen tensions; that is, it does not just occur in regions of severe hypoxia (<0.1% O_2), which occur at the border of necrotic areas. This finding is highlighted by the involvement of HIF, which, as previously mentioned, is stabilized in relatively moderate hypoxic conditions. Our own *in vitro* data show that, although the kinetics of repression of BRCA1 or RAD51 may differ between exposure to 0.02% and 0.2% oxygen, for example, expression levels do decrease in both cases. The implications of these results are that larger proportions of tumors will have repressed DNA repair. Repression of genes involved in DNA repair has been proposed to have a substantial role in increasing genomic instability in tumor cells, which may contribute to the aggressiveness of hypoxic tumors (35). Interestingly, the hypoxia-induced DDR also seems to be repressed after chronic hypoxia exposure; for example, Chk1 is rapidly and robustly phosphorylated during the acute time frame but then decreases (19). The reason behind this observation is not clear, although it was also noted that the number of RPA foci in hypoxia-arrested cells also decreases with increasing exposure to hypoxia. This finding would suggest that the hypoxia-induced signal leading to ATR activation decreases with exposure time. It is possible that this decrease is due to residual polymerase activity, although this remains to be shown conclusively.

Clinical-Translational Advances

Targeting the DDR has become a popular strategy for the development of novel therapeutics, with many now reaching clinical trials and showing promise (40). Both ATM and Chk1 inhibitors have been developed. Unfortunately, toxicity was observed with some of the early versions of these compounds (41). Second generation Chk1 inhibitors such as AZD7762, however, are proving to have some encouraging effects (42). For example, it was recently shown *in vitro* that AZD7762 in combination with the nucleoside analog gemcitabine showed enhanced lethality and that AZD7762 acts a radiation sensitizer both *in vitro* and in *in vivo* xenograft experiments (43, 44). Increasing evidence suggests that DDR inhibitors may be able to effectively target hypoxic cells, because loss or inhibition of several key players in the DDR such as ATR and ATM have been shown to sensitize cells to hypoxia and/or reoxygenation. Cells experiencing hypoxic conditions severe enough to induce a replication arrest are reliant on factors such as ATR and Chk1 to preserve replication fork integrity and prevent DNA breaks (20). Reoxygenation of cells in this state induces DNA damage and a checkpoint response. Indeed, in *in vitro* studies cells exposed to hypoxia and/or reoxygenation are sensitive to loss or inhibition of Chk1 or Chk2, therefore, suggesting that the inhibitors of these kinases currently in clinical trials may show increased toxicity to hypoxic cells (20, 31, 45). Sensitization of tumor cells to hypoxia and/or reoxygenation by inhibition of members of the damage response pathway may be of particular therapeutic importance, because it is those cells that are cycling through hypoxia and/or reoxygenation that are responsible for the worst prognosis (45).

Unfortunately, when considering the targeting of hypoxic cells *in vivo*, the problem of drug delivery arises. Hypoxic regions occur in tumors because of a limited blood supply resulting from an inefficient and chaotic vasculature. This reduced blood supply leads to the limited delivery of chemotherapeutic agents to hypoxic regions. For this reason, the value of Chk inhibitors to target hypoxic regions will probably be in combination with agents known to induce either reoxygenation or vessel normalization (46). For example, it has been proposed that the addition of anti-angiogenic therapies such as vascular endothelial growth factor receptor (VEGFR) antagonists to conventional chemotherapy may lead to a transient increase in vessel normalization, resulting in a more efficient delivery of drugs and an increase in tumor oxygen levels (47). Furthermore, reoxygenation as well as an increase in blood flow and tumor shrinkage occur following fractionated radiotherapy, which can again improve the efficiency of subsequent radiotherapy and chemotherapy (48). Some studies have also suggested that chemo- and radiotherapy may target tumor and circulating endothelial cells, as well as endothelial progenitor cells, and hence have a direct anti-angiogenic effect (49). A further complexity arises from the need to quantitatively measure hypoxia *in vivo* in order to evaluate novel therapy combinations. As mentioned, imaging and measuring tumor hypoxia have been areas of intense scrutiny. Options include the further development and/or validation of biomarkers amenable to measurement in bodily fluids, the imaging of hypoxic regions in tumors using, for example, nitroimidazole derivatives, or measurement of tumor oxygenation directly using an Eppendorf electrode (50–52).

The repression of DNA repair pathways in hypoxia also renders cells sensitive to the loss (or inhibition) of alternative pathways, resulting in context synthetic lethality. This term has been adopted to describe the synthetic lethal interaction between the loss of pathway A through therapeutic intervention and the loss of pathway B through its repression by the cellular context. Inhibitors of poly (ADP-ribose) polymerase (PARP) are now in phase II clinical trials and showing some promise for the treatment of breast cancers with BRCA1 mutations. Given the repression of BRCA1 and other factors essential to homologous recombination in hypoxia, we and others have proposed that hypoxic cells may be sensitive to PARP inhibitors. The PARP inhibitor ABT-888 has already been shown to radiosensitize tumor cell lines in hypoxic conditions (53). The clinical implications of this finding are that a wider range of tumor types might be sensitive to PARP inhibitors, that is, solid tumors with hypoxic fractions rather than just those showing BRCA loss or BRCAness (54).[1]

The combination of Chk1 inhibitors with other therapies capable of inducing damage, such as radiotherapy, inhibitors of DNA replication, or topoisomerase inhibitors, has also been studied. As previously mentioned, the use of the second generation Chk1 inhibitor AZD7762 and the nucleoside analog gemcitabine has been shown to have some synergistic effects, attributed to activation of origin firing, destabilization of stalled replication forks, and entry of cells with unrepaired DNA damage into mitosis (44). These effects may be further potentiated in hypoxic cells that, as mentioned above, show an increased sensitivity to Chk1 inhibition and harbor defects in DNA repair. Importantly, checkpoint and homologous recombination defects have also been proposed to have a major contribution to the radiosensitization observed by the combination of AZD7762 with radiation (55).

The pharmacologic reactivation of p53 may be an effective way of targeting hypoxic tumors because loss of p53 has been shown to select for a loss of the apoptotic response in hypoxia (56). p53 reactivation and induction of massive apoptosis (PRIMA), Nutilin, and reactivation of p53 and induction of tumor cell apoptosis (RITA) are among some of the compounds that are currently under investigation (57). RITA is a small molecule activator of p53. RITA has been shown to inhibit growth and induce p53 dependent apoptosis *in vivo* (58). Furthermore, RITA has been found to induce a DDR that could lead to increased p53 and H2AX phosphorylation. A block in HIF-1α and a downregulation of HIF-1α target proteins, such as VEGF, may also be mediated by RITA. These results suggest that reactivation of p53 in the hypoxic tumor could prove to be an important strategy for targeting the death of cells by reactivating p53-dependent apoptosis and potentially decreasing aberrant angiogenesis (58). Many of the chemotherapy drugs in current use are also reliant on p53-dependent apoptosis for their effects, so RITA and other small molecule reactivators of p53 may also have an important role to play in combination with conventional cancer treatments (59).

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[1] R. G. Bristow, E. M. Hammond, unpublished observations.

Concluding Remarks

The hypoxic fraction of a tumor represents the most therapy resistant, likely to metastasize, and aggressive tumor cells. It has been suggested that this fraction also potentially contains the highest numbers of cancer stem cells (60). For these reasons, any advance in the eradication of hypoxic cells during therapy is likely to have a positive effect on disease progression and patient survival. Although DDR inhibitors as single agents are unlikely to be effective against hypoxic cells, they may well have significant effects used in combination. The design of clinical trials will be critical in determining these potential benefits, that is, the scheduling of DDR inhibitors with, for example irradiation or anti-angiogenic therapies. The development of accurate biomarkers, able to provide reliable predictive and prognostic information will also be of great aid when choosing those patients that will benefit the most from therapies targeting the DDR.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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