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The Tumor Microenvironment and DNA Repair

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Abstract

Genetic instability is one of the hallmarks of cancer cells. As tumors grow, they progressively acquire mutations that ultimately allow them to invade normal tissues and metastasize to distant sites. This increased propensity for mutation also leads to cancers that are resistant to therapeutic intervention. Recent evidence has shown that the tumor microenvironment plays a major role in the etiology of this phenomenon; as tumors are exposed to repeated cycles of hypoxia and reoxygenation, they downregulate a number of DNA repair pathways, thus leading to genetic instability. Understanding the mechanisms involved in this process may provide insights into the development of novel treatment strategies.

Introduction

Historically, tumors have been thought of as homogenous, clonal expansions of a single cell, typically induced by a change, or series of changes, in the genetic make-up of that individual cell. This concept drove much of the research regarding tumor biology and the clinical practice of oncology. Over time, however, we have come to realize that, in the process of expanding from a single cell to a clinically relevant neoplasm, a considerable amount of inhomogeneity arises within a tumor, which impacts both the biology of the cancer as well as treatment of the patient.

One major area of study regarding this intra-tumoral variability involves the different cell populations that arise within a given tumor. Much of this work has focused on the cancer stem cell, a cell whose hallmark is the ability to generate identical daughter stem cells as well as more differentiated tumor cells (reviewed in (1)). These studies have broadened our concept of the tumor from that of a cluster of identical, ever-dividing cells to a much more complex tissue, with distinct cellular subtypes that have different abilities to divide and to metastasize, and potentially differential sensitivities to therapeutic agents and interventions.

The other major area of study regarding the inhomogeneity within a tumor involves the variability that arises due to the environment in which the tumor exists and develops, commonly referred to as the tumor micro-environment. As a tumor matures, there are many complex interactions that take place between the cancerous cells and the surrounding normal tissues that influence the ways in which a particular tumor grows (reviewed in (2)). Ultimately, a tumor will grow to such a size and/or at such a rate that portions of the tumor are unable to maintain

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an adequate blood supply. This, in turn, leads to regions within the tumor that are nutrient-deficient, have an altered pH, and are hypoxic. The effects of hypoxia, in particular, are varied and profound, and have a significant impact on the effectiveness of many different therapeutic modalities.

One of the classically understood impacts of hypoxia is the effect it has on the ability to treat with ionizing radiation. Most of the effect of clinically utilized ionizing radiation is a result of the indirect action of radiation, which involves the formation of free radicals that then go on to damage DNA. This process requires oxygen for the formation of these radicals and, as such, hypoxic conditions dramatically reduce the DNA damaging effects of radiation, thereby decreasing the ability of radiation to kill cancer cells.

More recent studies have shed light on the important biological changes that take place within a tumor under hypoxic conditions (reviewed in (3)). These include alterations in angiogenesis, cellular metabolism, and genetic stability. The study of angiogenesis, in particular, has been the focus of a large body of cancer research and has lead to the development of novel therapeutics used in clinical practice today, most notably bevacizumab (Avastin, Genentech, South San Francisco, CA; reviewed in (4)).

While the relationship between hypoxia and alterations in angiogenesis is at least somewhat intuitive - hypoxic cancer cells signal for increased blood flow in an attempt to receive more oxygen - the relationship between hypoxia and genetic stability is much less clear. A growing body of scientific work, however, has helped to shed light on this topic and will serve as the focus of this review.

Evidence of a link between hypoxia and genetic instability

Genetic instability is a hallmark of cancer cells and is thought to be responsible for much of the aggressive behavior that tumors show (reviewed in (5)). As cancer cells accumulate more and more genetic mutations, they acquire phenotypes that allow them to grow, invade, and metastasize. The mechanism underlying this mutator phenotype has typically been attributed to the loss of particular genes within cancer cells that protect the genome from mutation, most notably p53 (reviewed in (6), (7)). In fact, it is precisely the functional loss of these genes that has been accepted as one of the fundamental causes of cancer.

Evidence suggests that there are additional mechanisms at work that enhance the underlying genetic instability of cancer cells, including the effect of hypoxia. One of the first pieces of evidence to support this theory came from experiments designed to understand the mechanism of hypoxia-induced methotrexate (MTX) resistance in Chinese Hamster Ovary (CHO) cells (8). It was known that this resistance was due, at least in part, to the amplification of the dihydrofolate reductase gene; however the underlying mechanism was unclear. Rice *et al* demonstrated that the resistance to MTX in response to hypoxia was dose-responsive (increasing exposure to hypoxia led to increased MTX-resistance) and that this correlated with an increasing amount of total DNA per cell under increasingly hypoxic conditions. They showed that this was due to an overreplication of DNA during S-phase and postulated that hypoxia led to a transient inhibition of DNA synthesis, thereby resulting in “an increased capacity of cells for initiation of DNA replication.” In the discussion of their work, the authors eloquently stated the following:

> “We propose that [chromosomal] alterations may be related to a hypoxic state, in particular in those regions of a tumor where the blood supply is partially compromised and from which the progressively malignant cells are derived. Thus, the hypoxic state may well induce overreplication-recombination events leading to spontaneous drug resistance, oncogene amplification events, aneuploidy, and various forms of

> chromosomal rearrangements resulting in increased cellular heterogeneity and malignant progression."

Evidence of altered DNA replication under hypoxic conditions was subsequently confirmed in other model systems including mammalian cancer cell lines (9).

Although it had been postulated that the same sort of hypoxic conditions used in cell culture experiments exist in solid tumors, this was first demonstrated by an experiment in which a tumorigenic mouse cell line was grown in a mouse xenograft model (10). This cell line had been modified with a lambda phage vector that carried a reporter gene used to measure mutation rates. After growing in either standard cell culture conditions or as a mouse xenograft, cells were harvested and comparison of reporter DNA revealed a five-fold increase in the rate of mutation in the cells grown as tumor xenografts.

To confirm that the increased mutation rate was primarily caused by the hypoxic state of the xenografted cells, the same cell line/reporter construct was grown in culture under various hypoxic conditions. This experiment demonstrated that both the rate of mutation and the types of mutations (point mutations, transversions, and deletions) found in the xenografts could be almost entirely explained by the effect of hypoxia. Moreover, this experiment showed that repeated, transient exposure to hypoxic conditions, as is typically found in solid tumors, is more mutagenic than prolonged hypoxia alone.

Taken together, these data provided the basis for the assertion that the environment in which a tumor grows, especially the hypoxic nature of that environment, impairs the genetic stability of the individual cancer cells within that tumor. More recent work has shed light on many of the mechanisms underlying these hypoxia-induced changes.

Mechanisms of Hypoxia-Induced Genetic Instability

Although early work suggested that the mechanism of hypoxia-induced genetic instability was the stalling of DNA replication forks under hypoxic conditions followed by subsequent DNA overreplication, as described above (8), this theory couldn't fully explain the types of mutations seen in the tumor xenograft experiments. Rather, these mutations suggested either that hypoxia causes an increase in spontaneous damage to DNA or that it inhibits DNA repair processes (11). Both of these hypotheses have now been shown to be correct, with evidence demonstrating that reoxygenation of hypoxic cells has directly damaging effects on DNA and that hypoxia induces inhibition of DNA repair.

The initial evidence demonstrating that significant DNA damage occurs during the reoxygenation of transiently hypoxic cells came from a series of experiments designed to study the activity of the tumor suppressor genes ATM and ATR under hypoxic conditions (12). Hypoxia alone is sufficient to induce ATR-mediated phosphorylation of downstream targets, including p53. ATM-mediated phosphorylation, however, does not occur to a significant extent under hypoxic conditions, but rather is initiated once hypoxic cells are reoxygenated. Given that ATM signaling typically occurs in response to DNA damage, this finding suggested that reoxygenation acts as a DNA damaging event.

To test this theory, a human cancer cell line was grown under hypoxic conditions, then harvested in either hypoxic, partially hypoxic, or normoxic conditions, and subsequently analyzed by comet assay, which is used to quantify DNA damage (12). This experiment found that cells grown and harvested under hypoxic conditions showed no difference in the amount of DNA damaged per cell compared with cells grown and harvested under normoxic conditions. In contrast, cells grown under hypoxic conditions and then harvested in the presence of oxygen demonstrated a significant increase in the amount of DNA damaged per cell. The level of

damage seen was comparable to treating the cells with 4-5 Gy of ionizing radiation and the mechanism for this was shown to be directly related to the formation of DNA-damaging reactive oxygen species under reoxygenation conditions. Thus, the cycles of hypoxia and reoxygenation that typically occur in solid tumors were proposed to be directly mutagenic, thereby enhancing a tumor's genetic instability.

The other proposed mechanism of hypoxia-induced genetic instability is that of hypoxia-specific inhibition of DNA repair. There are five major DNA repair pathways, which can be subdivided into those involved in the repair of single-strand damage and those involved in the repair of double-strand breaks (reviewed in (13), (14)). There is now a growing body of evidence demonstrating that pathways involved in single-strand damage, including nucleotide excision repair (NER) and DNA mismatch repair (MMR), as well as those involved in double-strand breaks, including homologous recombination (HR), are inhibited by hypoxia.

The initial experiments which showed that DNA repair pathways are inhibited by hypoxic conditions analyzed the NER capacity of hypoxic cells (11). UV-damaged DNA (which is repaired by the NER pathway) was transfected into cells that were subsequently grown under normoxic or hypoxic conditions. The cells grown under hypoxic conditions demonstrated a significantly reduced ability to repair the UV-induced DNA damage. The key to this experiment was that, by using DNA which was damaged prior to transfection and prior to exposure to hypoxic conditions, the results definitively demonstrated that there was truly a defect in DNA repair, not just an increase in DNA damage under hypoxic conditions, as had previously been suggested.

The MMR pathway has also been shown to be inhibited by hypoxic conditions ((15), (16)). The hallmark of MMR deficiency is the instability of simple, repeated sequences of DNA. To study MMR activity in hypoxic cells, a reporter plasmid that was designed to specifically identify MMR defects was transfected into cells that were subsequently analyzed under normoxic or hypoxic conditions (15). This experiment found that hypoxia induced an increase in MMR-specific genetic instability, the mechanism of which was shown to be a decrease in the activity of the MMR gene *Mlh1*.

In contrast to hypoxia-induced NER pathway inhibition, which is thought to take place at the enzymatic level (as no change in NER gene expression is detectable secondary to hypoxia), changes in the MMR pathway due to hypoxia were shown to take place at the level of transcription (15), as demonstrated by decreased levels of *Mlh1* mRNA under hypoxic conditions. This inhibition was shown to be dependent on the activity of histone deacetylases in a hypoxia-specific manner but, interestingly, was not dependent on the activity of the HIF-1α transcription factor, one of the key genes involved in transcriptional responses to hypoxia.

Other MMR genes, including *Msh2* and *Msh6*, have also been shown to be downregulated by hypoxic conditions (16). As with the *Mlh1* data, hypoxia induced transcriptional downregulation of *Msh2* and *Msh6*, which correlated with an increase in genetic instability. In contrast to the *Mlh1* data, this transcriptional inhibition was proposed to be dependent on HIF-1α. It should be noted, however, that there is also clear evidence for HIF-1α independent downregulation of MMR (17), and thus the exact role of HIF-1α in this context is still in question. Additionally, the work on *Msh2* and *Msh6* found no decrease in transcription of *Mlh1* mRNA in response to hypoxia, whereas the work on *Mlh1* found no decrease in transcription of *Msh2* mRNA in response to hypoxia. The discrepancies in these data sets may be due to differences in cell lines, specific growth conditions, or other yet unidentified factors. Work is ongoing to sort out these differences. However, the overarching conclusion from both

papers is the same: hypoxia leads to the transcriptional inhibition of MMR genes, which in turn leads to genetic instability.

The most recent DNA repair pathway that has been shown to be inhibited by hypoxia is the HR pathway. This was first discovered in a large-scale microarray-based screen to identify gene expression changes in response to prolonged hypoxia (18)). This screen confirmed that expression of NER genes expression is not significantly altered by hypoxia, whereas MMR gene expression is decreased. Additionally, this screen identified *Rad51*, a key HR gene, as being significantly downregulated by hypoxia. Interestingly, this downregulation persisted for up to 48 hours after cells were reoxygenated and was independent of HIF-1α expression. As with the MMR experiments, an HR-specific reporter construct demonstrated that the decrease in Rad51 expression under hypoxic conditions corresponded to a decreased capability for HR. BRCA1, another HR gene, was subsequently shown to also be downregulated by hypoxia (19).

Molecular mechanisms of hypoxia-induced inhibition of DNA repair

The data presented above provided the first insights into the cellular mechanisms underlying the increased genetic instability seen in hypoxic cancer cells. More recent work, however, has focused on elucidating the underlying molecular mechanisms responsible for the changes described. In particular, significant advances have been made in our understanding of the transcriptional regulation involved in both hypoxia-induced MMR inhibition and HR inhibition.

An initial observation that the proliferation-promoting transcription factor *c-Myc* is downregulated by hypoxia (20) led to the hypothesis that *c-Myc* might be involved in the transcriptional down-regulation of DNA repair genes (17). To test this, quantitative chromatin immunoprecipitation experiments (qChIP) were performed using an anti-c-Myc antibody and then analyzed for the binding of various DNA repair gene promoters. These experiments identified the MMR genes *Mlh1* and *Msh2*, which were previously shown to be downregulated by hypoxia (described above), as being regulated by c-Myc in a hypoxia-dependent manner. In brief, hypoxia was shown to transcriptionally downregulate c-Myc expression, which leads to decreased levels of c-Myc bound to the promoters of *Mlh1* and *Msh2*. Additionally, hypoxia leads to increased binding of the repressive transcription factors Mad1 and Mnt to the *Mlh1* and *Msh2* promoters. The net effect of these changes is that hypoxia leads to the transcriptional downregulation of these MMR genes, which, in turn, leads to genetic instability.

Of note, the effects of c-Myc/Mad1/Mnt on the MMR genes were shown to be HIF-1α independent, consistent with the initial work on hypoxia-dependent *Mlh1* inhibition, described above (15). However, a recent study has demonstrated a novel, indirectly HIF-1α-dependent mechanism for hypoxia-induced MMR gene repression (21). In that paper, the authors identified the HIF-1α dependent transcription factors DEC1 and DEC2 as regulators of MLH1 that act in a hypoxia-dependent manner. They showed that this particular effect of DEC1 and DEC2 was, in fact, HIF-1α dependent. As before, there is clearly some controversy as to the exact role of HIF-1α in the hypoxia-induced transcriptional downregulation of MMR genes.

The molecular mechanisms regulating the hypoxia-responsive HR genes are distinct from those regulating the MMR genes. Initial experiments identified a 250-bp region upstream of the *BRCA1* gene that regulates transcription in a hypoxia-responsive manner (19). E2F1, a transcription factor that had previously been shown to regulate *BRCA1* in conditions other than hypoxia ((22), (23), (24)), was found to have multiple, conserved binding sites within this 250-bp region. Subsequent experiments demonstrated that hypoxia induces a qualitative shift in the types of E2Fs bound to the *BRCA1* promoter – from activating E2F1 complexes under normoxic conditions to repressive E2F4 complexes in hypoxia.

E2Fs have also been shown to regulate the HR gene *RAD51* in a hypoxia-specific manner (25). As with *BRCA1*, there is a conserved E2F binding site upstream of *RAD51* and changes from normoxia to hypoxia induce a similar shift in binding from activating E2F1 to repressive E2F4. Mechanistically, this change was shown to be due to a hypoxia-induced increase in the prevalence of hypophosphorylated p130, which forms functionally active repressive complexes with E2F4. The exact mechanism by which hypoxia leads to the dephosphorylation of p130 is unclear, and further work to clarify this is needed.

One additional mechanism of hypoxia-induced HR gene downregulation involves the role of microRNAs (26). miRs are small, noncoding RNAs, which were relatively recently discovered as regulators of gene expression at the mRNA level (reviewed in (27)). Initial experiments demonstrated that certain miRs, including miR-210 and miR-373, are upregulated in a hypoxia-responsive and HIF-1α dependent manner. A bioinformatics approach revealed putative binding sites for these miRs in the 3'-UTR of the HR gene *RAD52* and overexpression experiments confirmed that these miRs can, in fact, inhibit Rad52 expression. Moreover, the hypoxia-induced inhibition of *RAD52* was partially blocked by a specific miR-210 anti-miR molecule. Taken together, these data demonstrate a novel role for miRs in the hypoxia-induced inhibition of HR.

In summary, a number of varied mechanisms have been discovered that are responsible for the inhibition of multiple DNA repair pathways in response to hypoxic conditions. This, in turn, leads to an increase in the genetic instability of tumors. The implications of these findings for clinical practice are just starting to be understood.

Hypoxia and Genetic Instability in Clinical Practice

Taken together, the data presented above paint a picture of the tumor microenvironment as an environment that is hostile to cancer cell DNA and promotes genetic instability within cancer cells. Frequent cycles of hypoxia and reoxygenation lead to the formation of reactive oxygen species that act as DNA damaging agents; periods of hypoxia lead to stalled replication forks which then lead to overreplication of DNA; and hypoxia-induced transcriptional inhibition of multiple DNA repair pathways leads to a durable impairment in the fidelity of DNA replication. The clinical implications of these findings are far reaching and include novel rationales for fractionated radiotherapy, new targets for medical therapies, and a better understanding of how tumors become resistant to treatment.

One of the classically taught rationales for fractionating radiation therapy is that fractionation allows tumor cells that are hypoxic (and therefore radioresistant) the time to become more normoxic (and therefore more radiosensitive) during subsequent treatments. While this rationale still holds true, the data presented above add another layer to our understanding of this mechanism. Under conditions of hypoxia, tumor cells begin to downregulate DNA repair genes, including those involved in double-strand break (DSB) repair. Because these changes occur at the level of transcription, they are durable, often requiring 48 hours or more after the reintroduction of normoxic conditions for cells to regain the ability to properly repair DNA damage. Thus, cells that are transiently hypoxic, as are found in solid tumors, are sensitized to DNA damaging agents (e.g. ionizing radiation) in the immediate post-hypoxic period (28).

The study of hypoxic conditions is also relevant to a number of novel therapeutics coming to market that act specifically to inhibit DNA repair processes. One of the most promising classes of these new drugs is the Poly-ADP Ribose Polymerase (PARP) inhibitors (29). PARP serves a critical role in the process of base excision repair, which is important for the repair of single-strand DNA damage (reviewed in (30)). In the presence of PARP inhibitors, single-strand damage is not properly repaired and goes on to form DSBs. Under otherwise normal conditions, these DSBs are repaired by the HR pathway. However, under circumstances in which the HR

machinery is impaired, PARP inhibitors lead to cell lethality. This concept of selective synthetic lethality, in which two non-lethal events are induced to occur together in specific target cells, thereby killing those cells, is becoming an increasingly attractive technique for designing targeted therapies that have minimal side effects.

PARP inhibitors are currently being studied in patients whose tumors have genetic defects in HR, typically BRCA1/2 mutant breast cancers. However, the data presented in this review suggest a rationale for expanding the use of PARP inhibitors. Since hypoxia leads to the down regulation of HR genes, tumor cells in the hypoxic and post-hypoxic periods would be expected to be sensitized to PARP inhibition. We have confirmed this hypothesis in unpublished data (Figure 1; Denise Hegan, unpublished). Thus, any tumor that experiences significant periods of hypoxia, including most solid tumors, might be expected to at least partially respond to PARP inhibitors.

Another area where the study of the tumor microenvironment is likely to yield clinical benefit is that of acquired resistance to treatment. Understanding the mechanisms underlying this resistance is an important step in the development of strategies to counter it. Although there are an extraordinary number of strategies employed by cancer cells to evade treatment, many of them can be understood by the data presented in this review. One such mechanism is that of increased resistance gene expression in hypoxic conditions, as described above for MTX resistance (8). Another mechanism comes as a result of the hypoxia-induced decrease in MMR function described above. It has been shown that cells that lack MMR function are resistant to certain DNA damaging agents, including platinum-based chemotherapies (31). The mechanism for this is thought to be a form of damage tolerance, in which cells that are lacking MMR function no longer recognize certain forms of DNA damage as being damage. This likely plays a role in resistance to a number of clinical agents, including platinum-based therapies, drugs such as cytoxan and temozolamide, as well as ionizing radiation. Since hypoxia leads to an inhibition of MMR, hypoxia itself can be seen as indirectly promoting this resistance.

Thus, the data presented herein provide not only explanations for clinically observed phenomena, but also opportunities for future clinical research. Since hypoxia occurs preferentially in malignant, rather than normal cells, therapies that take advantage of hypoxic cell biology are likely to provide an additional means to enhance the therapeutic index of specific treatments. Hypoxia-activated drugs, for example, may provide a novel means to exploit the DNA repair pathway defects in hypoxic cancer cells in a highly targeted manner ((32), (33)).

Concluding Remarks

Under stressful conditions, particularly those that are damaging to DNA, it might be expected that a cell would act to protect its DNA even more than under normal circumstances. Thus, the fact that the cellular response to hypoxia includes the inhibition of DNA repair pathways is often seen as counterintuitive. There are two explanations for this phenomenon, both of which are likely to be, at least in part, correct.

The first explanation stems from the fact that DNA repair is a metabolically demanding process, requiring significant amounts of ATP to proceed. Thus, under nutrient-deprived conditions, cells might downregulate DNA repair pathways in attempt to conserve energy. The process of autophagy, in which cells consume themselves under stressful conditions (reviewed in (34)), is likely to play a part in this and is an area of active investigation in cancer biology.

The other explanation is rooted in evolutionary biology. Evolutionary studies have demonstrated that under stressful conditions, single-celled organisms like *E. coli* acquire a “mutator phenotype, in which their rate of spontaneous mutations increases (35). This allows

for new mutations to randomly arise that are protective against the stressor, and the cells carrying these mutations are then selected for. The same sort of selective pressures exist on cancer cells, and the benefits of a mutator phenotype can clearly be applied to the cancer cell (even if they are to the detriment of the patient). Thus, by inhibiting DNA repair pathways under hypoxic conditions, cancer cells have an increased likelihood of developing phenotypes that allow them to survive, grow, and even metastasize.

Regardless of the reasons why these changes occur, the data presented in this review demonstrate that they do. By understanding the effects of the tumor microenvironment on cancer cell biology in the lab, we have been able to develop strategies to better treat patients in the clinic. And by exploiting the effects of hypoxia on DNA repair pathways in malignant neoplasms, we expect to see improvements in cancer therapy in the years to come.

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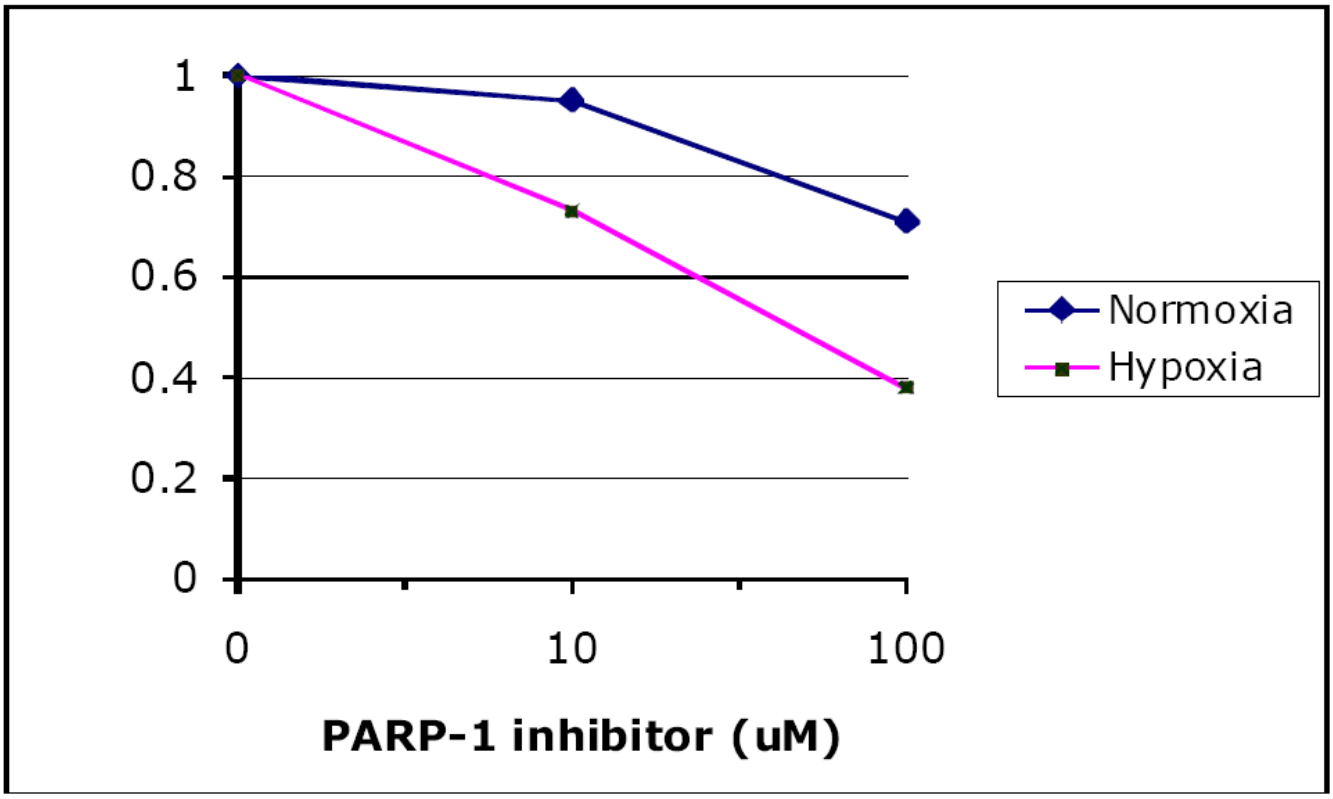

Figure 1.
Hypoxia sensitizes cancer cells to PARP inhibition. MCF7 breast cancer cells were grown under normoxic or hypoxic conditions for 48 hours, as previously described (10). The cells were then exposed to 0, 10, or 100μM of the PARP-inhibitor KU0058684 and assayed for cell survival by colony formation. The fraction of surviving cells is shown.