Review

Cancer Research

Replication Stress: An Achilles' Heel of Glioma Cancer Stem–like Cells

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Abstract

Glioblastoma (GBM) is a highly aggressive form of cancer that is resistant to standard therapy with concurrent radiation and temozolomide, two agents that work by inducing DNA damage. An underlying cause of this resistance may be a subpopulation of cancer stem–like cells that display a heightened DNA damage response (DDR). Although this DDR represents an attractive therapeutic target for overcoming the resistance of GBMs to radiotherapy, until now, the cause of this DDR upregulation has not been understood. In a previous issue of *Cancer Research*, Carruthers and colleagues investigated DNA replication stress as an underlying mechanism responsible for upregulation of the DDR and hence the radiation resistance of glioma stem–like cells. Furthermore, the authors explore the efficacy of combined ataxia telangiectasia and Rad3-related kinase and PARP inhibitors as a strategy to leverage these mechanisms and overcome radiation resistance. *Cancer Res; 78(24); 6713–6. ©2018 AACR.*

See related article by Carruthers and colleagues, Cancer Res; 78(17); 5060–71.

The cancer stem cell theory states that a small subpopulation of tumor cells possess unique self-renewal properties that are capable of seeding new tumors and are a source of regrowth following therapy (1). Glioma stem–like cells (GSC) are defined as CD133-positive cells that can initiate new tumors in mice (2). This subpopulation of cells was later shown to be notably radioresistant, a property attributed to an intensified DNA damage response (DDR), which could be targeted with an inhibitor of CHK1/2 (3). Subsequently, the Chalmers' group demonstrated that enhanced G_2–M checkpoint activation and DNA repair were functional consequences of an augmented DDR that treatment with ataxia telangiectasia and Rad3-related (ATR) and PARP inhibitors could overcome to prevent radioresistance (4). Key questions prompted by these studies are what properties of GSCs lead to the enhanced basal level of DNA damage signaling and whether these mechanisms can be leveraged therapeutically to overcome the resistance of GSCs to ionizing radiation.

The underlying cause for elevated DDR in GSCs has previously been attributed to heightened levels of reactive oxygen species (ROS) leading to increased levels of PARP activity and single-strand break repair (5); however, Carruthers and colleagues did not find evidence that ROS levels were elevated in $CD133^+$ GSCs versus GSC-depleted cultures (6). Instead, GSCs displayed both elevated basal levels of activated ATR and CHK1, and elevated markers of replication stress (RS) such as foci marked with the single-stranded DNA binding protein, replication protein A, and the DNA damage markers γH2AX and 53BP1. Untreated GSCs also exhibited reduced replication velocities and asymmetric bidirectional DNA replication forks, indicating increased stalling of replication factories compared with non-GSC populations (6). These observations pointed to elevated levels of RS as causative of DDR activation in untreated GSCs, a hypothesis supported by the high levels of RS in glioblastoma (GBM; ref. 7), broad activation of DDR proteins by RS (8), and the overlap between signaling in response to RS and DNA damage (9). But, can elevated RS increase radioresistance in non-GSC cultures? Carruthers and colleagues demonstrate that slowing DNA replication velocity by aphidicolin treatment imparts a radioresistant phenotype (6). Therefore, slowing DNA replication and artificially creating RS can lead to radioresistance. These observations lead to the next question: what is the mechanism behind elevated RS in GSCs?

RS is associated with oncogene expression and is a common feature of cancers (10, 11). The induction of RS by oncogenes is multifactorial and may be due to aberrant expression of genes that regulate DNA synthesis (e.g., cyclin E), increased origin firing, depletion of deoxynucleotide pools, and formation of hard to replicate secondary structures in DNA such as G4-quadruplexes (12, 13). Emerging evidence implicates a role for oncogene-driven transcription as a source of RS (14, 15). One potential consequence of increased origin firing and elevated transcription is collision between the protein machinery for these two processes, which in turn creates abnormal replication fork structures that can be processed into DNA double-stranded breaks (DSB; refs. 16, 17).

Given the recent evidence that replication/transcription conflicts can induce RS, Carruthers and colleagues explored whether GSCs displayed altered transcription profiles compared with the bulk non-GSC populations. No evidence for altered expression of genes associated with DNA replication or genes known to be induced by RS was discovered. Instead, GSCs overexpressed a significant number of "very long genes" (VLG), sequences in excess of 800 kb in length (6). It is interesting to note that several VLGs upregulated in GSCs are known to play important roles in neurologic development, axon guidance, and synapse formation consistent with a neural progenitor phenotype (6). Some VLGs contain difficult-to-replicate sequences that are hotspots for forming chromosomal gaps and breaks, or common fragile sites (CFS),

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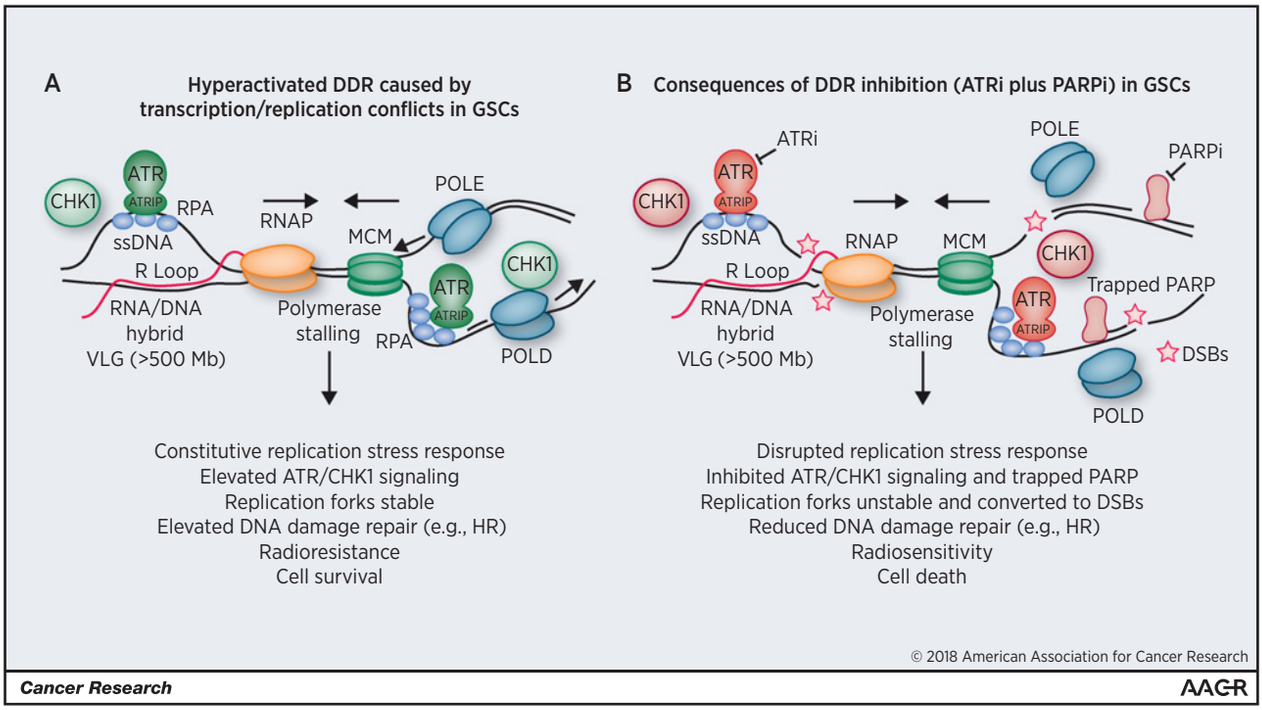

Figure 1.

Targeting RS in GSCs. **A,** Carruthers and colleagues demonstrated that CD133$^+$ GSCs exhibit constitutive RS as shown by elevated ATR and CHK1 kinase signaling (green). ATR is activated by binding to replication protein A (RPA) coating-extended regions of ssDNA through its partner ATR interacting protein (ATRIP). ATR phosphorylates and activates CHK1, thereby initiating a DDR that promotes activation of the intra S and G_2–M phase checkpoints, increases replication fork stability, and regulates DNA repair pathways such as homologous recombination (HR; ref. 35). One potential source of RS in GSCs is the elevated transcription of VLGs by RNA polymerase that may inadvertently collide with late replicating regions of the genome, activating the ATR RS response, which in turn promotes cell survival and radioresistance. **B,** Treatment of GSCs with an ATR inhibitor (red) is selectively toxic due to GSC dependence upon RS response signaling for survival. Inhibition of the RS response leads to increased R loop and replication fork instability that ultimately lead to DSBs, following structure-specific endonuclease processing or DNA breakage. Inhibition of PARP results in base excision repair deficiency and may lead to trapping of the PARP enzyme on ssDNA breaks, creating further dependence upon ATR signaling to promote stability and repair of stalled replication forks. Inhibition of ATR and PARP leads to profound radiosensitization of GSCs. MCM, minichromosome maintenance protein complex helicase; POLE and POLD, DNA polymerase epsilon and delta; RNAP, RNA polymerase II.

that are expressed under conditions of RS, such as through aphidicolin treatment (18–21).

Transcription of VLGs occurs late in the cell cycle and may not be completed until the following cell cycle (22). Therefore, replication factories will inevitably encounter the transcription of a VLG at some point during the cell cycle. Replication stalling at CFS may further increase the incidence of replication/transcription machinery encounters (21, 23). One consequence of replication/transcription collisions is the formation of stable RNA/DNA hybrids (also referred to as R loops), which require enzymes such as RNAse H to resolve (22, 24–27). R loops form when transcribed RNA hybridizes with the complementary DNA strand and displaces the nontemplate strand as single-stranded DNA (ssDNA). R loops have been detected in both bacteria and human cells and are now known to influence chromatin structure, regulation of transcription, immunoglobulin class switch recombination, and, if persistent or collided in a "head on" orientation with the replication machinery, can lead to genome instability when processed into DSBs (28–34).

Slowing and/or stalling of DNA replication, such as that induced by low concentrations of aphidicolin, causes the appearance of RNA/DNA hybrids. Current evidence suggests these hybrids result from inadvertent collision of replication/transcription machinery at VLGs (22). The observation by Carruthers and colleagues that GSCs preferentially overexpress VLGs provided novel mechanistic insight into a source for elevated RS in GSCs. Enhanced transcription of multiple VLGs increases the incidence of replication/transcription conflicts, resulting in DSBs, potentially at CFS (Fig. 1). Indeed, this study provides evidence of DSBs, marked by γH2AX, at sites overlapping replication or transcription, marked by BrdUrd or RNA:DNA hybrids, respectively. Furthermore, this overlap occurred preferentially in CD133$^+$ GSCs compared with bulk GBM cultures (6).

The poor prognosis and relative resistance of GBM to standard therapy underscore the need for more effective therapies. The major question addressed by Carruthers and colleagues is whether the heightened RS in GSCs is therapeutically actionable. The ATR kinase is a master regulator of responses to DNA damage and RS (35). ATR has a direct role in diminishing RS by promoting stabilization and restart of stalled DNA replication forks, as well as preventing aberrant replication origin firing and subsequent nucleotide exhaustion and replication stalling (35). Of relevance to the observation that transcription/replication conflicts may be a source of RS in GSCs (1), ATR activates and promotes the resolution of persistent R loops (33, 36–38). Thus, inhibition of

ATR may present a unique approach to attenuating constitutive DDR signaling exhibited by GSCs and reversing radioresistance.

The PARP1 and PARP2 enzymes bind to ssDNA breaks and are important signal transducers within the DDR pathway. Binding to ssDNA breaks activates PARP1 and PARP2 to posttranslationally modify themselves as well as other proteins by synthesizing negatively charged poly(ADP-ribose) chains. PolyADP-ribosylation recruits proteins involved in ssDNA break repair (e.g., XRCC1) and modifies chromatin structure. Distinct from ATR, PARP also functions during DNA replication and the RS response by regulating fork stabilization and restart, elongation velocity, ligation of lagging strand Okazaki fragments, and homologous recombination repair of stalled DNA replication forks (39–43). Importantly, the therapeutic activity of PARP inhibitors is in part attributed to PARP "trapping," resulting from the loss of autoPARylation that facilitates removal of PARP from DNA. Trapped PARP creates obstacles that impede ongoing DNA replication. The increased abundance of trapped PARP enzymes is hypothesized to be preferentially cytotoxic to cancer cells harboring defects in homologous recombination repair (44, 45).

Consistent with the complementary roles of ATR and PARP in the DDR pathway and the hypothesis that RS is a targetable feature of GSCs, Carruthers and colleagues tested whether the combination of ATR and PARP inhibitors is preferentially cytotoxic and radiosensitizing in GSCs (relative to bulk cells). Although PARP inhibition alone was relatively ineffective, treatment of GSCs with an ATR inhibitor inhibited stem cell–like neurosphere formation *in vitro*, implicating a role for ATR for viability under these conditions. This effect was even more pronounced with the combination of ATR and PARP inhibition. Importantly, the combination treatment enhanced DNA damage in GSCs and diminished the radioresistant phenotype of GSCs. It is likely that ATR and PARP inhibitors synergize by inhibiting multiple points in the DDR. The heightened levels of RS and DDR signaling in GSCs are consistent with the hypothesis that these cells have become dependent upon ATR for viability (46). Trapping PARP through the coadministration of a PARP inhibitor may further strengthen the dependence of GSCs on ATR activity for survival (Fig. 1).

Of particular clinical relevance, Carruthers and colleagues found that although PARP inhibition alone was ineffective in radiosensitizing the GSC models used in this study, the combination of ATR and PARP inhibitors induces profound sensitization of GSCs to radiation, an effect that was significantly greater in $CD133^+$ GSCs than in bulk GBM cells (6). Multiple clinical trials combining PARP inhibitors with radiation or other DDR inhibitors, such as those targeting ATR in both BRCA1/2-mutant and nonmutant cancers, are underway (45, 47). The data presented in this study provide a preclinical rationale for the future clinical development of concurrent ATR and PARP inhibitors with radiation in GBMs, and potentially other cancers with a high RS burden. As an added benefit, inhibition of ATR has recently been shown to inhibit radiation-induced upregulation of programmed death-ligand 1 in tumor cells, diminish radiation-induced $CD8^+$ T-cell exhaustion, and decrease the number of tumor-infiltrating T regulatory cells to achieve a greater antitumor response in a mouse model of Kras-mutant cancer (48). Given the well-characterized immunosuppressive tumor microenvironment associated with GBM, treatment with ATR inhibitors may present two weapons against this disease: targeting the addiction to the DDR pathway and reinvigorating T cells to attack GBM cells following radiotherapy (49, 50).

Disclosure of Potential Conflicts of Interest

M.A. Morgan reports receiving a commercial research grant from, and has received speaker's bureau honoraria from AstraZeneca. No potential conflicts of interest were disclosed by the other author.

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