Subject Review

DNA Repair and Resistance of Gliomas to Chemotherapy and Radiotherapy

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Abstract

The importance of DNA repair as a resistance mechanism in gliomas, the most aggressive form of brain tumor, is a clinically relevant topic. Recent studies show that not all cells are equally malignant in gliomas. Certain subpopulations are particularly prone to drive tumor progression and resist chemo- and radiotherapy. Those cells have been variably named cancer stem cells or cancer-initiating cells or tumor-propagating cells, owing to their possible (but still uncertain) origin from normal stem cells. Although DNA repair reduces the efficacy of chemotherapeutics and ionizing radiation toward bulk gliomas, its contribution to resistance of the rare glioma stem cell subpopulations is less clear. Mechanisms other than DNA repair (in particular low proliferation and activation of the DNA damage checkpoint response) are likely main players of resistance in glioma stem cells and their targeting might yield significant therapeutic gains. (Mol Cancer Res 2009;7(7):989–999)

Gliomas: Luckily Rare...

Primary brain tumors can be classified in terms of what type of cell the tumor began as (reviewed in refs. 1, 2). Gliomas are the most common form of primary brain tumors. Among gliomas, there are astrocytomas [World Health Organization (WHO) grade I and II], which derive from star-shaped astrocyte-like cells. High-grade astrocytomas are called anaplastic astrocytomas (grade III) and multiform glioblastomas ([GBM] grade IV). Compared with lung and breast cancer, which have rates of about 60 per 100,000 in Western countries, the rate of gliomas is ∼tenfold lower. Nevertheless these cancers have serious effects on the health-care system in general, and especially on patients and their families. Not only do glioma patients have to cope with the diagnosis of incurable disease, they and their families are usually also confronted with the patient’s decrease in cognitive and emotional function as a result of cerebrodental disease. Despite advances in surgery and radiotherapy, the prognosis for malignant gliomas remains poor, the median survival of GBM patients being 5 to 8 months, depending on the patient's age (2). Alkylation agent-based chemotherapy has been shown to modestly increase response rates and survival times of high-grade glioma patients when used as an adjuvant to surgery and radiation (3, 4). Some more efficacy can be observed in lower-grade gliomas such as oligodendroglioma (5), but serious side effects such as cumulative lymphopenia and consequent high incidence of infections occur after prolonged treatments (6). Although, the most frequently used alkylation agent for glioma treatment, temozolomide (TMZ), is well-tolerated by most patients and has favorable pharmacodynamic and pharmacokinetic properties (3). The inefficacy of currently available chemotherapeutic and radiotherapeutic agents depends on a number of resistance mechanisms among which DNA repair may play a role (7-10).

DNA Repair and Resistance of Bulk Gliomas

O6 MethylGua DNA Methyltransferase

Many studies have indicated O6 methylGua DNA methyltransferase (MGMT) as a possible resistance factor to alkylating chemotherapy in brain tumors (3, 11), although negative results have been also reported (Table 1; ref. 12). Two major cell-killing and apoptotic alkylated lesions arising from drugs used in brain cancer chemotherapy are O6-methylguanine (O6-meGua) and O6-chloroethylguanine (O6-chloroethylGua). These lesions are removed by the specific action of MGMT thus counteracting alkylating agents’ killing effects. Levels of MGMT expression may in some cases predict TMZ sensitivity in adult human glioma patients (13-16). A stronger correlation with overall survival, regardless of treatment, is observed in pediatric glioma patients (17). MGMT promoter methylation actually represents part of a genetic signature of less-aggressive gliomas, apart from treatment (Fig. 1A; refs. 15, 18, 19). In MGMT-negative glioblastomas TMZ further enhances radiation responses by increasing the degree of radiation-induced double-strand DNA damage (20). However, the role of MGMT may have been overestimated by the relatively simple assay procedures for its promoter methylation status and enzymatic activity, and should not be generalized (21). For instance, no correlation between MGMT promoter methylation and MGMT protein expression was recently observed in serial stereotactic specimens from anaplastic astrocytomas and glioblastomas (22). One advantage of MGMT targeting is that local intracerebral administration of MGMT drug inhibitors that may sensitize tumor cells to TMZ, is feasible and without important side effects.
effects (23, 24). Viral inhibitors may further be envisaged, on the basis of the fact that some viral proteins directly target and inactivate some DNA repair genes (25). For instance, a combination of oncolytic adenovirus Delta-24-RGD and TMZ induces a therapeutic synergy in glioma cells and significantly improves the survival of glioma-bearing mice by silencing the MGMT gene (26).

Mismatch Repair

The relatively low incidence of microsatellite instability in pediatric and adult high-grade gliomas would suggest that the involvement of mismatch repair (MMR) defects in alkylating agent resistance is limited (27, 28). However, inactivating somatic mutations of the MMR gene MSH6 confer resistance to alkylating agents in glioma in vivo and concurrently unleash accelerated mutagenesis in resistant clones as a consequence of continued exposure to alkylating agents. Hence, when MSH6 is inactivated in gliomas, alkylating agents may convert from inducers of tumor cell death to promoters of neoplastic progression (Table 1; ref. 29). Loss of MSH6 has been recently found in a subset of ionizing radiation (IR) + TMZ-treated glioblastoma recurrences (30). The association with tumor progression during TMZ treatment was significant, mirroring the alkylator resistance conferred by MSH6 inactivation in vitro. MSH6 deficiency may therefore contribute to the emergence of recurrent glioblastomas during TMZ treatment (30). These conclusions have been yet recently questioned by Maxwell and coworkers (31) who could not find any effect of MMR deficiency on resistance of adult malignant glioma to TMZ. Promoter hypermethylations but not structural alterations in the MLH1 gene have been observed in low grade gliomas, associated with their development and progression (32). MLH1 promoter hypermethylation may be an early event in the development and progression or the clonal evolution of gliomas, this gene inactivation proving stable even on tumor recurrence (32). Conversely, proficient MMR is required for repair of interstrand crosslinks via stabilization of RAD51-containing repair intermediates and may thus contribute to resistance to crosslinking agents (33).

Base Excision Repair

Albeit resistance to TMZ has been ascribed to elevated levels of MGMT and or reduced MMR, >80% of the DNA lesions induced by TMZ are N-methylated bases that are recognized by DNA glycosylases and not by MGMT (34, 35). Studies with isogenic cells deficient or overexpressing the DNA base excision repair (BER) enzymes pol beta and alkyladenine DNA glycosylase (AAG) have shown that the BER pathway is a major contributor to cellular resistance to TMZ and its efficacy depends on specific BER gene expression and activity (35, 36). APE1/Ref-1, the main AP endonuclease of mammalian cells, significantly increases during DNA-PK influences the response to cisplatin as well (50).

Fanconi Anemia Pathway

The Fanconi anemia (FA) pathway may contribute to resistance of glioma tumors to alkylating agents (51). A glioma cell line deficient in the FA repair pathway (HT16) exhibited increased sensitivity to TMZ and BCNU relative to FA-proficient glioma cells. Moreover, inhibition of FA pathway by curcumin, a polyphenol present in the curry spice turmeric, or by small interfering RNA (siRNA) suppression caused increased sensitivity to TMZ/BCNU in the wild-type U87 glioma cell line. The BCNU sensitizing effect of FA inhibition seemed additive to that of MGMT inhibition. Yet, curcumin has many effects beyond targeting the FA pathway, being e.g., a nuclear factor kappa-B (NF-KB) inhibitor and a reactive oxygen species (ROS) scavenger (52, 53), and curcumin sensitization of glioma cells to several clinically used chemotherapeutic agents (cisplatin, etoposide, camptothecin, and doxorubicin) and IR also correlates with reduced expression...
of bcl-2 family members as well as DNA repair enzymes that do not take part in the FA pathway (MGMT, DNA-PK, Ku70, Ku80, and ERCC-1; ref. 54). Leaving aside the underlying mechanism, a role for curcumin as an adjunct to traditional chemotherapy and radiation in the treatment of brain cancer could be worth exploring (54).

**Other Resistance Mechanisms in Bulk Gliomas**

Abrogation of p53 wild-type function strongly attenuates TMZ cytotoxicity. Conversely, agents designed to stabilize the wild-type conformation of p53 sensitize glioma cells to TMZ cytotoxicity. Thus, determination of p53 status may help in identification of glioma patients who will or will not respond to TMZ (13). Contrary to what can be observed for TMZ, chloroethylating drugs are more toxic for p53-mutated glioma cells in which both apoptosis and necrosis are induced (55). Inactivation of p53 by pifithrin-alpha or siRNA downregulation sensitize p53 wild-type but not p53-mutated glioma cells to 3-[(4-amino-2-methyl-5-pyrimidinyl) methyl]-1-(2-chloroethyl)-1-nitrosourea (ACNU) and BCNU. ACNU and BCNU provoke the formation of DNA double strand breaks (DSBs) in glioma cells that precede the onset of apoptosis and necrosis. These DSBs are repaired in p53 wild-type cells but accumulate in p53-mutated cells. Therefore, functional p53 may have opposing effects in gliomas treated with methylating or chloroethylating agents and, therefore, the p53 status should be taken into account when deciding which therapeutic drug to use (55). After IR, expression of dominant-negative p53 selectively sensitizes glioma cells (56) and p53-mutated glioma cell lines repair DSBs more slowly and less effectively than p53-wild-type glioma cell lines or normal human astrocytes (46).

Expression of apoptotic proteins correlates in part with resistance of gliomas to chemotherapeutic agents (57, 58). Astrocytes per se have been found to express relatively high levels of antiapoptotic Bcl-2 and Bcl-XL proteins and low levels of proapoptotic Bad and Bid proteins suggesting that astrocytes may be less vulnerable to proapoptotic stimuli compared with other cell types. Hypermethylation of the proapoptotic gene caspase-8 is also a common hallmark of relapsing gliomas (59).
Microarray analyses showed that 78 out of 17,000 genes were differentially expressed between parental cells and TMZ-resistant variants isolated in the human glioma cell line SNB-19 (60). None were implicated in DNA repair whereas several genes involved in differentiation were downregulated. Thus, in this model, the acquisition of resistance to TMZ may result from selection of less differentiated preexistent resistant cells in the parental tumor (60).

**DNA Repair and Resistance of Glioma Stem Cells**

**Glioma Stem Cells**

More than 25 years ago, Rosenblum and coworkers semi-nally proposed that chemoo- and radio sensitivity of human malignant brain tumors could be dependent on inherent differences in the sensitivity of clonogenic stem cells to radiation and or chemotherapeutic agents (61). As for other cancers, the existence of glioma cells endowed with features of primitive progenitor cells and tumor-initiating function has been shown (62-65). Most gliomas contain considerable portions of cells expressing stem cell markers such as CD133, Nestin, Sox-2, and Musashi-1 (66, 67), and expression of some of them has been shown to be prognostic of decreased overall survival (68-70). In particular, expression of the CD133 stem cell antigen negatively correlates with glioma patient survival in several studies (71-75). CD133+ is often expressed on the surface of human glioma stem cells and importantly, CD133+ cells are preserved during orthotopic transplantations of human gliomas in the mouse (76). However, significant subpopulations of CD133− stem cells may also exist in gliomas (77). For instance, Beier and coworkers (78) have observed that 11 out of 15 primary glioblastomas contained a significant CD133+ subpopulation that displayed asymmetrical cell divisions yielding cells expressing markers characteristic for all three neural lineages. However, the remaining four cell lines were driven by CD133− tumor cells that also fulfilled stem cell criteria (e.g., pluripotency and tumorigenicity in nude mice

**in vivo**). Consistently, analysis of the expression profiles of nine glioma cell lines established under neural stem cell conditions yielded two distinct clusters (79). Four cell lines were characterized by the expression of neurodevelopmental genes, a multipotent differentiation profile, expression of CD133, and formation of highly invasive tumors in vivo. The other five cell lines shared expression signatures enriched for extracellular matrix-related genes, had a more restricted differentiation capacity, contained no or fewer CD133+ cells, and displayed reduced tumorigenicity and invasion in vivo. Hence, CD133+ stem cells may maintain only a subset of primary glioblastomas, the remainder deriving from CD133− tumor stem cells with distinct phenotypical features (78-82). Additional cell surface markers have been investigated to hallmark glioma stem cells including the aforementioned Nestin, Sox-2, and Musashi-1 as well as CD117, CD71, CD45, and A2B5 (83-85), but at the moment, CD133 remains prominent. Whether the currently identified glioma stem cells may be the true cell-of-origin for tumor initiation and progression or the results of such processes is still an open question (86, 87). Further, gliomas produce factors [e.g., urokinase plasminogen activator (uPA) and its receptor (uPAR)] that selectively attract neural stem cells (88-93) and this has originated the question about whether stem cells may be attracted by, rather than originate, the tumor (66, 94-96). Notwithstanding, gliomas do not clash with the general cancer-stem-cell-related concept that if only a rare subset of tumor stem cells, whatever their origin, drives tumor formation, then it is important to identify this population and develop therapies that target it (97-103).

Some established glioma cell lines that have been maintained over years in culture contain a subpopulation of stem cells (104, 105). For instance, in the absence of serum a combination of basic fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) maintains in the C6 glioma cell line a population enriched in stem cells (referred to as “side population”) with the ability to efflux the fluorescent dye Hoechst 33342. Moreover, C6 side population cells, but not
nonside population cells, can generate both side population and nonside population cells in culture and are largely responsible for the in vivo malignancy of that cell line. Importantly, C6 side population cells can produce both neurons and glial cells in vitro and in vivo. Thus, some cancer cell lines may contain a minor subpopulation of stem cells that can be maintained indefinitely in culture, and which is crucial for their malignancy (104-108). This cannot be generalized yet: controversial data exist about the presence and properties of cancer stem cells in the established and tumorigenic glioma cell lines D54, TJ905, U87, and U251 (109-111).

Altered Pathways in Glioma Stem Cells

It is difficult to find normal pathways in glioma stem cells. p53 regulates the proliferation of stem and progenitor cells in the brain (112). In p53-mutant neural stem cells, transcriptome analyses identified the dysregulation of several cell cycle regulators, in particular a pronounced downregulation of p21 expression, implicating p53 as a growth suppressor of glioma-initiating stem cells (113). Deregulation of additional processes that control cell cycle progression, such as the p16-CDK4-RB pathway and other cyclin-dependent kinase pathways may also promote the generation of brain cancer stem-like cells (114, 115). Subventricular zone stem cells often express the PDGF receptor, perhaps contributing to their peculiar ability to respond to PDGF (116). Activation of signaling pathways like this, which is often accompanied by Ras inactivation (117), has been implicated in neural stem cell transformation (116). Within the vertebrate central nervous system (CNS), the Sonic Hedgehog (Shh) pathway acts as a morphogen or mitogen that regulates the patterning, proliferation, and survival of neural stem cells (118, 119). Gli1 is a primary mediator of Shh signaling (120) that likely serves as a protective mechanism against premature mitosis in nonmalignant stem cells. Dereguation of Gli1 has been observed in brain tumor-derived stem cells (120). Aberrant expression of additional developmental pathways, such as WNT, Notch, and transforming growth factor-beta/BMP has been also...
Resistance Mechanisms in Glioma Stem Cells

Gliomas frequently display hyperactivation of the phosphatidylinositol-3-kinase (PI3K)-Akt pathway, a protumorigenic signaling cascade that contributes to the pathogenesis of several human cancers (130-134). The PI3K-Akt pathway can be activated in tumors through different mechanisms, including inactivation of the inhibitory effects of the phosphatase and tensin homolog (PTEN) tumor suppressor (130-134). Hyperactive Akt signaling promotes tumorigenic cell behaviors by increasing cell survival, proliferation, invasion, and angiogenesis. Treatment with an Akt inhibitor potently reduces the numbers of viable glioma stem cells relative to matched nonstem cells (135). Further, delayed resolution of γ-H2AX foci after IR and radiosensitization have been observed in glioma cells after induction of PTEN and concomitant decrease of activated Akt (131).

These results together hint at a link between Akt activation and radioresistance in glioma stem cells. Bao and coworkers (136) have shown that glioma stem cells may resist radiation through preferential activation of the DNA damage checkpoint response. The fraction of tumor cells expressing CD133 was enriched after radiation in gliomas. In both cell culture and the brains of immunocompromised mice, CD133+ glioma cells survived IR in increased proportions relative to most tumor cells, which lack CD133. CD133+ tumor cells isolated from both human glioma xenografts and primary patient glioblastoma specimens preferentially activated the DNA damage checkpoint in response to radiation and repaired IR-induced-DNA damage more effectively than CD133− tumor cells (136, 137). In addition, the radioresistance of CD133+ glioma stem cells could be reversed with a specific inhibitor of the Chk1 and Chk2 checkpoint kinases. In the mouse, G2 and G1-arrested neural stem cells rapidly increase after IR and this correlates with phosphorylation of cdc2 and p53, respectively, and inactivation of the Notch signal (138). Resistance of glioma stem cells to chemotherapeutic drugs has been further reported (139, 140). An expression signature dominated by HOX genes, which comprises CD133, emerged as a predictor for poor survival in glioma patients treated with concomitant chemo-and radiotherapy (141). Taken together, these results suggest that CD133+ tumor cells represent the cellular population that confers glioma resistance to chemo- and radiotherapy and that activation of the DNA damage checkpoint response may be a major mechanism in this regard. One further important resistance mechanism could be multidrug resistance. As aforementioned (see “Glioma Stem Cells”), a peculiarity of many stem cell populations (side populations) is their relatively high expression of ATP-binding cassette (ABC) drug transporters, which confer them the ability to efflux the fluorescent dye Hoechst 33342 and protection from cytotoxic agents (142). Side-population cells have been isolated in the CNS, and similar to hematological malignancies, expression of multidrug resistance-associated protein genes has been found elevated in cancer stem-like cells isolated from glioblastoma and astrocytoma (143-145). Finally, elevated expression of antiapoptic
proteins [e.g., the myeloid cell leukemia-1 (Mcl-1) protein] can be found in glioma stem cells (144, 146) and reduced expression of major histocompatibility complex genes may also contribute to their resistance to therapies (147, 148).

The relative importance of DNA repair as a resistance mechanism in glioma stem cells still is an open question. We have recently examined DNA repair in five stem and nonstem glioma cell lines (Fig. 2; ref. 111). The stem lines fulfilled the defining criteria proposed by Vescovi and coworkers (142). The population doubling time was significantly increased in stem as compared with nonstem cells and enhanced activation of Chk1 and Chk2 kinases was observed in untreated CD133+ as compared with CD133− cells (Fig. 3). In this regard, cell-cycle restriction limits DNA damage and maintains self-renewal of leukemia stem cells as well (149). In our laboratory, neither DNA base excision, nor single strand break repair, nor resolution of pH2AX nuclear foci were increased in CD133+ as compared with CD133− glioma cells (Fig. 4). Hence, reduced proliferation may be a major mechanism in glioma stem cells to resist chemotherapeutic-induced killing, whereas if enhanced DNA repair (per time unit) is a common resistance mechanism in glioma stem cells still requires further studies.

Targeting Cell Cycle Restriction in Glioma Stem Cells

The ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related) kinases are transducers that recognize DNA damage and coordinate the initiation, amplification, and activation of the checkpoint through phosphorylation of many different targets (Fig. 5) (reviewed in refs. 150, 151). Checkpoint kinase-1 (Chk1) and checkpoint kinase-2 (Chk2) are the checkpoint transducers kinases that function downstream of ATR and ATM in the DNA-damage checkpoint signaling pathway. Although structurally dissimilar, Chk1 and Chk2 are serine-threonine kinases that serve as functional analogs. ATM phosphorylates Chk2 at various sites including serine 19 and threonine 68, whereas ATR phosphorylates Chk1 at serines 280, 296, 317, and 345. Significant crosstalk exists between the ATM/Chk2 and ATR/Chk1 pathways (152). Together, the proximal transducers ATM and ATR and the distal transducers Chk1 and Chk2 phosphorylate a variety of effector molecules, such as p53 and CDC25 phosphatases, culminating in cell cycle arrest. In particular, the three CDC25 isoforms, A, B, and C, are active in different phases of the cell cycle. CDC25 phosphatases remove inhibitory phosphate groups from cyclin-cyclin-dependent kinase (CDK) complexes, promoting cell cycle progression. In response to DNA damage, the checkpoint kinases phosphorylate CDC25 phosphatases, resulting in CDC25 inactivation through either ubiquitin-mediated degradation or cytoplasmic sequestration. In this manner, the checkpoint kinases serve as negative regulators of the CDC25 phosphatases.

Treatment with ATM and Chk1 and Chk2 inhibitors used alone or in combination may selectively sensitize glioma stem cells to IR and temozolomide (reviewed in refs. 153, 154). In this regard, two interesting novel drugs have been recently developed. The preclinical profile of AZD7762, a potent ATP-competitive checkpoint kinase inhibitor in clinical trials was described by Zabludoff and coworkers (155). AZD7762 was profiled extensively in vitro and in vivo in combination with DNA-damaging agents and was shown to potentiate response in several different settings in which inhibition of checkpoint kinase resulted in the abrogation of DNA damage-induced cell cycle arrest. Dose-dependent potentiation of antitumor activity, when AZD7762 was administered in combination with DNA-damaging agents, was observed in multiple xenograft models with several DNA-damaging agents, further supporting the potential of checkpoint kinase inhibitors to enhance the efficacy of both conventional chemotherapy and radiotherapy and increase patient response rates.

Rainey and coworkers (156) screened a targeted compound library for inhibitors of the ATM kinase, and CP466722 was identified. The compound was nontoxic and did not inhibit PI3K or PI3K-like protein kinase family members in cells. CP466722 inhibited cellular ATM-dependent phosphorylation events and disruption of ATM function resulted in characteristic cell cycle checkpoint defects. Inhibition of cellular ATM kinase activity was rapidly and completely reversed by removing CP466722. Clonogenic survival assays done on HeLa or AT GM02052 cells showed that transient inhibition of

**FIGURE 5.** Cell cycle checkpoint pathways, possible targets in glioma stem cells. Once DNA damage is identified with the aid of sensors, the checkpoint transducers ATM and ATR undergo conformational change and or localization, resulting in their activation. ATM and ATR activate a series of downstream molecules, including the checkpoint kinases 1 (A, Chk1) and 2 (B, Chk2). Chk1 and Chk2 inactivate CDC25 phosphatases, culminating in cell cycle arrest. AZD7762 (AstraZeneca) is a specific inhibitor of Chk1 and Chk2 kinases. CP466722 (Pfizer) is a specific inhibitor of ATM.
ATM was sufficient to sensitize cells to IR and suggested that therapeutic radiosensitization may only require ATM inhibition for short periods of time. The ability of AZD7762 and CP466722 to rapidly and reversibly regulate Chk1, Chk2, and ATM activities thus provides new tools to inhibit constitutive activation of cell cycle checkpoints in CD133+ glioma stem cells, reverse their cell cycle block, and sensitize them to IR and chemotherapy.

Conclusions

In 31 out of 40 (77%) studies on bulk high-grade gliomas, an inverse correlation between DNA repair capacity and therapeutically efficacious has been observed, indicating that nonstem cells in these tumors may frequently adopt DNA repair as a resistance mechanism (Table 1). However, currently available therapies for gliomas seldom are curative, possibly for their inability to eradicate a small subpopulation of highly resistant cells that almost invariably cause tumor relapse (called with some imprecision “glioma stem cells”). Low proliferation rate and constitutive activation of the DNA damage checkpoint response may be major mechanisms of resistance in these cells, conferring increased time for lesion removal or bypass. These features may be common to other tumors as well (149). Drugs targeting cell cycle restriction in glioma stem cells could be of help for complete eradication of the tumor and several novel agents of this kind are under development. In particular ATM and Chk1 and Chk2 kinase inhibitors may effectively sensitize glioma stem cells to IR and alkylating agents by reversing their cell cycle delay. Treating checkpoint activation, rather than DNA repair, might be a fruitful research direction in improving glioma therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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