Radiation Therapy for Glioma Stem Cells

Anthony E. Rizzo and Jennifer S. Yu

Abstract Radiation therapy is the most effective adjuvant treatment modality for virtually all patients with high-grade glioma. Its ability to improve patient survival has been recognized for decades. Cancer stem cells provide new insights into how tumor biology is affected by radiation and the role that this cell population can play in disease recurrence. Glioma stem cells possess a variety of intracellular mechanisms to resist and even flourish in spite of radiation, and their proliferation and maintenance appear tied to supportive stimuli from the tumor microenvironment. This chapter reviews the basis for our current use of radiation to treat high-grade gliomas, and addresses this model in the context of therapeutically resistant stem cells. We discuss the available evidence highlighting current clinical efforts to improve radiosensitivity, and newer targets worthy of further development.

Keywords Glioma • Stem cell • Initiating cell • Glioblastoma • Radiation • Radioresistance • Microenvironment • Hypoxia • DNA damage repair

Radiation therapy is a cornerstone in the treatment of a variety of primary brain tumors. The identification and study of cancer stem cells in a number of primary CNS neoplasms provides new insights into how tumor biology is affected by radiation and the role that cancer stem cells can play in therapeutic resistance and recurrence of some devastating diseases. While the WHO classification system lists numerous CNS tumors, studies identifying and characterizing cancer stem cells are present only in a limited number of them. This chapter focuses on the evidence

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gathered in the study of cancer stem cells related to gliomas. These tumors represent the most common malignant cancers of the CNS and thus a disproportionate amount of investigation is focused on this disease burden. Lessons from glioma may serve as a model for other CNS tumors for which stem cell populations have been identified.

Comprising four different tumor grades, astrocytoma are the most common primary brain tumors [1]. The most common high-grade astrocytoma is glioblastoma (GBM, WHO grade IV astrocytoma) which accounts for 45 % of all primary malignant CNS tumors and carries the gravest prognosis [2]. Despite decades of significant research efforts, the median survival for patients with newly diagnosed GBM receiving the standard of care is between 12 and 15 months, with a 5-year survival rate of approximately 3 % [3]. Optimal therapy for this highly invasive and therapeutically resistant disease includes maximal safe surgical resection [4], radiation therapy [5, 6], and chemotherapy [3]. While surgical resection has been found to provide a survival benefit, less than half of the patients with newly diagnosed disease are candidates for the optimal procedure, gross total resection, and virtually all GBMs will recur following surgery [4, 7]. Radiation therapy is the most effective adjuvant treatment modality for virtually all GBM patients and its ability to improve patient overall survival has been recognized for some time [8, 9].

GBM has long been regarded as a relatively radioresistant cancer, in part due to the persistently high failure rates (up to 90 %) in patients treated with radiation doses up to 80 Gy [10]. Though recurrence may only be delayed with current treatments, the efficacy of adjuvant radiation therapy to improve survival has been supported by level I clinical evidence from randomized controlled trials since the 1970s [8, 9, 11]. The current standard practice of radiation therapy used to treat GBM is based on clinical studies done over the past 40 years. Prospective randomized trials established that whole-brain irradiation did not yield improvements in overall survival or changes in recurrence pattern compared to partial brain irradiation [12, 13]. The practice of delivering 60 Gy to the gross tumor volume is based on a pooled analysis of three successive randomized trials in which a progressive increase in median overall survival was observed among doses ranging from less than 45 to 60 Gy [5]. A dose-effect relationship was suggested for doses above 50 Gy by this study, and following studies comparing a dose of 70–60 Gy demonstrated no survival or local control advantage, which established the dose of 60 Gy [14, 15].

The practice of treating the gross tumor volume and a margin of 2 cm added to the FLAIR and T1-enhancing regions is based on studies of recurrence patterns and microinvasive disease. Early studies established that almost 90 % of recurrences occur within 2 cm of the primary tumor site when assessed by computed tomography (CT) [16, 17]. Magnetic resonance imaging (MRI), specifically the gadolinium-enhanced T1 imaging sequence, is more sensitive than CT in defining tumor extension [18–20]. However, biopsy and autopsy studies further demonstrated that microinvasive disease can be present within the 1–4 cm margins of the gross tumor volume defined by T1-contrast enhancement [21]. More recent retrospective analyses, which evaluate patients that received the current standard of chemoradiotherapy, continue to show a large proportion of recurrence centrally and within the radiation treatment field [22].

The brain tumor stem cell theory as a model for GBM tumorigenesis, therapeutic resistance, and recurrence is still under relatively contentious debate [23]. Despite its continued development and testing, this model has important prognostic and therapeutic implications [24, 25]. For the purposes of this chapter, we use the term GBM-initiating cell (GIC) to refer to a population of cells that are alternately described in the literature as "GBM stem cell" or "brain tumor stem-like cell." These terms are united by a functional definition that is in accordance with the cancer stem cell hypothesis [26]. GICs are a relatively small subpopulation of cells, isolated from bulk tumor specimens, that are experimentally defined by the capacities for self-renewal, differentiation, and maintenance of proliferation [27]. The term GIC is not meant to suggest that this cell population is necessarily the origin of gliomas. Several groups have described GICs using validated functional assays such as serial tumorsphere assay and tumor propagation by in vivo intracranial limiting dilution assay [28]. While a number of cell surface markers have been used to aid in the characterization and isolation of GICs there is no clear universal marker for GICs [29]. This may be due in part to inherent differences between genotypes in human tumors or proposed plasticity of the GIC phenotype [30–33]. Most likely, several different populations of GICs exist within a tumor and each may express different combinations of cell surface markers. Most significant for the assessment of radiation therapy in the treatment of GBM is that GICs display much greater tumorigenic potential than matched non-GIC tumor cells when xenotransplanted into the brains of immunocompromised rodents [34]. Thus, treatments targeting GICs are attractive goals for reducing the recurrence of GBM.

Reflecting on the high propensity for local recurrence of GBM in the face of currently clinically optimized therapeutic practice yields some general questions for further discussion in the context of GICs. First, is radiation an effective treatment for GICs? It would appear clear from prior data that radiation is effective at reducing the tumor bulk, but what about its efficacy on GICs? Second, how do GICs respond to radiation? Clearly there is some population of tumor cells that are not eradicated by current practice; if they are GICs as we currently understand them, can any differences be exploited therapeutically? And finally, is radiation targeting these cells effectively? Can radiation shift the balance between GICs and non-GICs or cause non-GICs to adopt the GIC phenotype?

Ionizing radiation causes cell damage in a variety of ways; however the mechanism believed to be most responsible is the generation of reactive oxygen species leading to DNA damage in the form of double-strand breaks (DSB) [35]. The presence of DSBs, either induced exogenously or the result of endogenous forces during the cell cycle, represents potentially fatal obstacles for cells undergoing replication and division [36]. Unsurprisingly, the presence of DSB stimulates the activation of an array of proteins referred to as the DNA damage response (DDR), which is essential for cells to recover from DSBs [37]. The DDR encompasses a diverse but interconnected set of cellular processes including the damage sensors which initiate and transduce its signal and effectors that modulate cell cycle progression, DNA repair, autophagy, mitotic catastrophe, necrosis, senescence, and apoptosis [37]. The primary function of this cascade is the prevention of DSBs from being transmitted

to later generations of cells as mutations or chromosomal aberrations [36]. This prevention can take the form of cell cycle arrest to allow for repair or organized cell destruction in the event of severe damage. It is important to note that it is thus not the actual insult of ionizing radiation that initiates destruction of cells, but rather the ensuing challenges to DNA replication and transcription. Accordingly, a variety of mechanisms ranging from the reduction of DSB generation, increased capacity for DNA repair, or overcoming cell cycle arrest or organized destruction are implicated in resistance to radiation therapy.

Consistent with its reputation as a radioresistant cancer, cell lines derived from gross specimens of GBM have been found to possess aberrant constitutive activation of a range of DDR proteins [38]. This would suggest that bulk tumor cells may be responsible for radioresistance and recurrence following radiation therapy. However, in response to ionizing radiation GICs are found to preferentially activate a number of critical components of the DDR (specifically ATM, Rad17, Chk2, and Chk1) and more efficiently repair DNA damage in comparison to matched non-GICs [39]. Irradiated GICs also have a lower percentage of apoptotic cells than matched non-GICs [39, 40]. Apoptosis, programmed cell death, is one protective mechanism activated by the DDR cascade in response to extensive DNA damage [35]. The ability to preferentially overcome cell cycle arrest and repair DNA damage compared to non-GIC bulk tumor cell places GICs in a position to expand their population and repopulate the tumor. Evidence in support of GICs as a source of therapeutic resistance can be found by studying tumor composition following radiation therapy. Expansion of the GIC population within recurrent tumors has been confirmed by histological analysis of GBM samples collected at the time of salvage surgery, after initial chemoradiation [41]. In addition, many clinical trials (though not all) have failed to show a benefit to radiation dose escalation [42], radiosurgery boost [43, 44], or brachytherapy boost [45, 46]. These data suggest that while radiation may be effective at reducing the tumor bulk by targeting non-GICs, it exerts a selective pressure favoring the outgrowth of an aggressive recurrent tumor through the expansion of the GIC population. Efficient repair of DSBs is one possible mechanism for the superior response to ionizing radiation seen in GICs, and while the exact cellular mechanisms responsible for this superior response are still being elucidated, we will endeavor to outline the current understanding.

It is important to distinguish that thus far, the study of radioresistance in GICs has not revealed an overexpression of proteins directly involved in the DDR cascade. GICs commonly have a basal activation or preferential activation of these proteins in response to ionizing radiation or possess mechanisms to overcome cell cycle arrest checkpoints [39, 47, 48]. Interestingly, the specific proteins that are preferentially activated and the degree to which they are activated vary between patient-derived GIC specimens, suggesting that there is not a single mechanism for radioresistance [49]. The cellular processes that support preferential DDR activation in response to radiation therapy could be indirectly stimulated by other aberrations that are specific or predominant in the maintenance and proliferation of a GIC population. One could further hypothesize that the resultant radioresistance of GICs is a corollary of signaling that supports the GIC phenotype.

A better understanding of the molecular response to radiation therapy can identify targets for therapies aimed at the specific cellular components. Realizing this potential involves dissecting the DDR cascade. This signaling cascade includes multiple sensor, transducer, and effector proteins. An important sensor of DNA damage is the MRE11-RAD50-NBS1 (MRN) complex [50, 51]. This complex rapidly binds to and assembles at foci of DNA damage and is an important activator of transducing proteins that continue the cascade [52]. Two important transducing proteins are the serine/threonine protein kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) [53]. These two proteins are members of the phosphatidylinositol 3-kinase (PI3K) family and are key regulators of DSB repair. ATM and ATR each activates checkpoint effector proteins CHK2 and CHK1, respectively [53]. Each of these components may play a role in distinguishing the GIC response to ionizing radiation compared to non-GIC.

The involvement of the MRN complex as a potential starting point for preferential activation of DDR proteins is significant for GICs. An upstream regulator of the NBS1 protein in this complex is preferentially expressed in GICs. The cellular surface marker L1CAM is preferentially expressed on GICs to maintain cell survival and tumor growth [54]. Subsequent study of this marker identified a new function of L1CAM in promoting DDR checkpoint activation and radioresistance of GICs through regulation of NBS1 [55]. The L1CAM intracellular domain (L1-ICD) translocates to the nucleus in response to radiation, and it was found that knockdown of L1CAM reduced levels of the transcription factor c-Myc [55]. c-Myc is known to directly regulate NBS1 and is required for the ATM-dependent CHK2 activation, the downstream effector of the MRN complex in DDR [56, 57]. Taken together, the preferential expression of L1CAM provides a basis for the preferential activation of DDR proteins by GICs in response to ionizing radiation.

The transcription factor c-Myc is an important regulator of stem cell biology in both normal and cancer cells, and its transcriptional targets regulate proliferation, apoptosis, and malignant transformation [58]. The oncogenic potential of c-Myc was recognized in the 1980s, and further studies have demonstrated that overexpression of it correlates with poor prognosis in a variety of human tumors [59]. This transcription factor is found to not only be required for GIC proliferation, growth, and survival, but it is also expressed at higher levels in GICs compared to matched non-GICs [60]. Knockdown of c-Myc significantly reduced GIC growth and proliferation, and altered the expression of numerous cell cycle regulators downstream of c-Myc. This effect was seen preferentially in GICs compared to matched non-GICs, which were minimally disturbed [60]. The connection between L1CAM, c-Myc, and the DDR machinery illustrates one of the ways that radiation resistance appears tightly linked to the biology that defines GICs. Thus as a regulator of both stem cell maintenance and radioresistance L1CAM signaling represents an attractive target for modulation of radiation therapy.

DSBs are sensed by the MRN complex and ATM, and these sensors are interdependent for the recognition and signaling of DSBs. When both ATM and the MRN complex are recruited to the foci of DNA damage the MRN complex accelerates phosphorylation of inactive ATM dimers leading to their dissociation [51, 53, 61, 62].

Each phosphorylated ATM monomer further activates itself by auto-phosphorylation in a feed-forward mechanism to activate effector proteins including CHK2 kinase [63]. The CHK2 protein is a molecular switch which directly activates various targets. These include proteins involved in cell cycle progression, DNA repair, and stimulation of apoptosis. The second transducing protein, ATR, functions similarly to ATM, but predominantly in response to endogenous DNA damage. ATR may also be activated in response to DSBs induced by ionizing radiation, though to a lesser extent than ATM [64]. The signaling cascade downstream of ATR begins with activation of CHK1 [64]. CHK1 and CHK2 demonstrate over-lapping, but nonredundant, roles in their effects on cell cycle progression, DNA repair, and apoptosis [65]. One general distinction is the implication of ATM-CHK2 in the G1 checkpoint, with ATR-CHK1 having a more significant role in modulating the S- and G2-phase checkpoints [66].

While the direct contributions of the ATM-CHK2 and ATR-CHK1 remain unclear, several findings support continued investigation of inhibitors of these proteins as therapeutic targets. First, ATM-CHK2 is preferentially activated in GICs and inhibition of the CHK1/2 proteins improves GIC sensitivity to ionizing radiation [39]. Second, ATM expression correlates with radioresistance in bulk GBM cells [67], while its inhibitors have been found to increase the radiosensitivity of bulk GBM cells and GICs treated with temozolomide and radiation [68, 69]. Other targets for drug development can be found downstream of the CHK1/2 proteins. The ATM-CHK2 cascade activates transcription factors that alter the expression of numerous genes including the receptor tyrosine kinase c-MET, which has specific significance for GICs [70].

c-MET is a receptor tyrosine kinase with downstream targets involved in a variety of cellular signaling pathways including proliferation, motility, migration, and invasion [71]. c-MET is overexpressed in approximately 29 % of GBM patients and directly correlates with poor prognosis [72–77]. While the gene MET is amplified in only 5 % of GBM patients, its function is important in the context of GICs. Subpopulations of GBM cells enriched for elevated c-MET expression from primary GBM possess stem-like characteristics such as in vivo tumor initiation [78]. c-MET is activated after interaction with its ligand, hepatocyte growth factor/scatter factor (HGF/SF), which is secreted in an autocrine fashion by GICs [79]. This autocrine/paracrine loop is important for the maintenance of the GIC phenotype. Irradiation upregulates the expression of c-MET in GICs, highlighting the significance of this receptor and its potential to support recurrence following radiation therapy [78].

In response to ionizing radiation, c-MET expression and activation are increased, as is secretion of HGF, in both bulk GBM and GICs. These effects were linked to the DDR by their abrogation upon treatment with an ATM inhibitor [70]. In addition to supporting the maintenance and proliferation of the GIC phenotype, c-MET is also found to stimulate tumor angiogenesis by induction of vascular endothelial growth factor (VEGF) expression [80]. Furthermore, resistance to bevacizumab, an anti-VEGF monoclonal antibody, can occur through c-MET activation of pro-survival and invasion mechanisms [81]. Given the potential for tumor repopulation

and recurrence afforded by processes stimulated by c-MET, the prospect of blocking ionizing radiation-induced c-MET signaling could have tremendous therapeutic benefit. Both in vitro and in vivo models have been used to test this hypothesis by targeting the c-MET receptor with genetic approaches in combination with ionizing radiation. Combined therapy with ionizing radiation and c-MET inhibition decreased cell proliferation and tumor growth compared to ionizing radiation or c-MET inhibition alone [80, 82], while another approach, targeting the c-MET ligand, HGF, with three neutralizing antibodies, also decreased tumor volume [83]. Most significantly, dual inhibition of the c-MET receptor and HGF-ligand expression combined with ionizing radiation reduced proliferation and tumor volume while increasing apoptosis, DNA fragmentation, and animal survival [84]. These data are strong support for investigating c-MET inhibitors such as cabozantinib (XL-184; Exelixis), in combination with conventional GBM therapy [85].

A number of clinical trials are currently assessing new drugs targeting HGF/c-MET signaling. Several of these drugs have completed studies in other solid tumors such as skin, lung, and thyroid cancers, all of which are often driven by similar molecular mechanisms found in GBM [86]. Cabozantinib, a pan-tyrosine kinase inhibitor with high affinity for c-MET and VEGFR2, is being testing in a phase II clinical trial for recurrent GBM [87, 88]. Most notably, cabozantinib is also currently under investigation in a phase I trial assessing it in combination with concurrent temozolomide and radiation therapy [85]. Another approach to therapy aimed at the HGF/c-MET pathway is ligand sequestration with a biologic drug. A monoclonal antibody against HGF, rilotumumab (AMG-102; Amgen), is currently under investigation in two phase II trials as a single-agent therapy for recurrent GBM and as a combination therapy with bevacizumab [89, 90].

Another component of radioresistance through the DDR may involve the Polycomb group protein BMI1. The Polycomb group proteins act as epigenetic silencers, and repress the expression of a range of proteins involved in the regulation of stem cell function during embryonic development and may be directly involved in tumor initiation [91]. BMI1 is part of the Polycomb repressive complex 1 (PRC1) and it has been found to be essential in the maintenance of the stem cell phenotype in both neural stem cells (NSCs) and GICs [92, 93]. Elevated expression of BMI1 in glioma correlates with poor patient survival [94]. It has been recently described that ionizing radiation stimulates the accumulation of BMI1 in chromatin and in DDR proteins. Knockdown of BMI1 impaired the DDR and increased GIC radiosensitivity [92]. While a mechanism of BMI1 in radioresistance is unclear, current evidence suggests that it represents a promising target for improving radiosensitization of GICs.

Thus far our discussion has focused on radioresistance mechanisms that relate directly to activation of the DDR in response to ionizing radiation, but GIC therapeutic resistance may involve molecular characteristics in addition to the radiation stress response. It is very likely that there are multiple, nonexclusive pathways that contribute to the radioresistance of GICs. It is also possible that pathways involved in radioresistance may overlap and have interplay with signaling involved in the maintenance and proliferation of the GIC phenotype, as evidenced by the previous discussion of the L1CAM surface marker. Two pieces of evidence support

alternative pathways. First, preferential activation of CHK1 and CHK2 in GICs has been described in the absence of ionizing radiation [48, 49]. Second, enhanced GIC survival following ionizing radiation has been reported without differences in DNA repair capacity compared to non-GICs [95]. These findings suggest that elevated DNA repair in response to DDR activation may not be the only method of radiation resistance at work in GICs. A number of other signaling pathways that are important in the maintenance of the stem cell-like phenotype may play a role in the therapeutic resistance of GICs.

The NOTCH signaling pathway is a highly conserved regulator of cell fate in both embryonic and adult tissues. Its effect is largely dependent on the context of its stimulation, but in the majority of tissues it contributes to the maintenance of an undifferentiated state. Unsurprisingly, the NOTCH receptor is over-expressed in a variety of cancer stem cells including GICs [96, 97]. NOTCH is a cell surface receptor with an intracellular domain activated upon ligand binding similarly to L1CAM. Following the binding of its ligand, DELTA/JAGGED, the NOTCH receptor, is activated via proteolytic cleavage by γ-secretase to promote the release and nuclear translocation of the NOTCH intracellular domain (NICD) [98]. The activation of NICD promotes the activation of the PI3K/AKT pathway and expression of NOTCH-regulated genes. These genes include c-Myc, Hes1, and Hey1, which are responsible for promoting self-renewal and GIC maintenance [96, 99–101].

NOTCH is important for GICs in the absence of ionizing radiation. There is evidence that treatment with high concentrations of γ-secretase inhibitors decreases tumorsphere formation, proliferation, and xenograft growth, as well as increases differentiation. Ionizing radiation induces NOTCH activation in GICs, resulting in the expansion of the GIC population [100]. Inhibition of NOTCH similarly improves the radiosensitivity of GICs [102, 103]. γ-Secretase inhibitors also enhanced the radiation-induced cell death and impaired the clonogenic survival of GICs in comparison to non-GICs. Furthermore, knockdown of NOTCH sensitized GICs to radiation and impaired xenograft tumor growth. Exogenous expression of constitutively active NICD protected GICs from radiation and the effect of γ-secretase inhibitors was attenuated [100]. Importantly, the inhibition of NOTCH signaling did not demonstrate changes in the DDR of the GICs, but reduced the activity through the PI3K/AKT pathway in response to radiation therapy. Taken together this evidence supports the synergistic effect that γ-secretase inhibitors can have with radiation therapy in GBM treatment.

There are currently several clinical trials evaluating γ-secretase inhibitors in the treatment of patients with GBM [104]. One promising γ-secretase inhibitor is RO4929097 which has been studied in a phase I trial in combination with chemoradiotherapy for newly diagnosed glioma [105]. Investigation of this compound has also moved into phase II studies as a single agent in patients with recurrent GBM [106, 107]. RO4929097 is also being studied in phase II trials as combination therapy with the tyrosine kinase inhibitor cediranib (AZD2171/AstraZeneca) in multiple solid tumors, and with bevacizumab in patients with recurrent high-grade gliomas [108, 109].

The downstream mechanism of radiosensitization through NOTCH inhibition highlights an intracellular signaling axis that has long been studied in GBM biology, the PI3K/AKT axis [110]. This pathway is a mediator of cell survival and invasion signaling pathways and is commonly dysregulated in GBM [111, 112]. Similarly, upstream regulators of PI3K/AKT are found commonly mutated in GBM, increasing signaling through this axis [113–115]. This axis is known to be important for GIC maintenance, as direct inhibition of AKT alone preferentially increased apoptosis, and reduced neurosphere formation, migration, and invasion in GICs compared to non-GICs [116, 117]. In established GBM cell lines radiation therapy is found to stimulate AKT activation and increase both survival and invasion signaling [118–120]. Given the importance of PI3K/AKT in GIC biology it is reasonable to conclude that radiation therapy has similar effects on GICs, and a number of laboratories have found that inhibition of AKT improves radiosensitivity of both GICs and established GBM cell lines in vitro and in vivo [100, 121–124]. While different mechanisms of AKT-mediated radioresistance have been suggested, including effects on DNA repair capacity or the ability to overcome cell cycle arrest, the bottom line is that this pathway represents an integral target for therapeutic radiosensitization [125]. Further elucidation of the downstream effectors of PI3K/AKT involved in radioresistance will be important, but the number and variety of inhibitors of this pathway that are currently in clinical trials are promising.

Another cellular response pathway, downstream of PI3K/AKT, that is induced by radiation and may contribute to the radiation resistance of GICs is autophagy [126]. Autophagy is an intracellular degradation system that cells can use to break down and recycle their contents to provide an alternate source of energy in response to metabolic stress or starvation. This is an important homeostatic process which can contribute to therapeutic resistance in many cancers, or when unchecked can lead to cell death [126]. Ionizing radiation induces autophagy preferentially in GICs compared to non-GICs and GICs are found to express higher levels of autophagy-related proteins (LC3, ATG5, and ATG12) [127]. In further support for autophagy as a target for radiosensitization is evidence that autophagy inhibitors and gene silencing that targets autophagy genes reduce GIC survival and their ability to form neurospheres following radiation [127].

Unfortunately, the benefit of inhibiting autophagy in combination with radiation is not completely clear as other studies demonstrated that activation of autophagy instead of its inhibition can have a radiosensitizing effect. The mammalian target of rapamycin (mTOR) acts as a major checkpoint in the regulation of autophagy signaling, integrating stimulation via PI3K/AKT and the cell's nutrient sensing apparatus [128]. One approach to radiosensitization activated autophagy using inhibitors of the mTOR signaling pathway combined with radiation and observed an increase in radiosensitivity, neural differentiation, and a reduction in the self-renewal and proliferative capacities of GICs [129, 130]. Another approach, which used a combination of cilengitide, an α_v integrin inhibitor that is currently in clinical trials, and radiation to induce autophagy found that it enhanced cytotoxicity and decreased cell survival in GICs [131]. Taken together, the evidence supports a role for autophagy in the GIC response to ionizing radiation, but does not indicate a clear direction for therapeutic intervention.

The in vitro study of GICs has provided an excellent picture of intracellular mechanisms of GBM radioresistance, but some groups have questioned whether it is a sufficient model for studying the radioresistance that GBM displays in situ [132]. The relative difficulty in studying radiation survival curves (considered by many to be the gold standard in assessment of radiosensitivity) in GIC compared to non-GIC populations and conflicting evidence regarding the precise mechanisms of radioresistance (specifically preferential elevations in DSB repair capacity between GICs and non-GICs) suggest that in vitro analysis may be subject to cell line-dependent variability, and by itself is not optimal for therapeutic testing [47, 49]. Another approach, that has accumulated a strong body of evidence, is to assess the role of the tumor microenvironment in the radioresistance of GICs. This model is supported by evidence that stimuli found in the tumor microenvironment are integral in the maintenance and proliferation of GICs in vitro [133–136]. The microenvironment can contribute to differences in DNA repair capacity seen among different GIC lineages, as sections of irradiated tumors generated from GIC versus non-GIC cell populations display differences in DSB repair capacity while when the cell populations were irradiated in vitro the DSB repair capacities were similar [132]. This suggests that there are signaling cues found in vivo that drive the radioresistance of GICs.

The field of radiation biology has recognized for nearly 100 years that a cell's microenvironment can have protective or sensitizing effects on the DNA-damaging properties of ionizing radiation [137]. These effects can be physical, such as the availability of oxygen for the generation of DNA-damaging free radicals, or biological, such as molecular signaling that promotes DNA repair and cell proliferation. In GBM, the therapeutic implications of the microenvironment are becoming apparent as we gain insight into how GICs exist in the context of their tumoral location. The current understanding of GBM tumors identifies specific anatomical and functional locations within the tumor, termed "stem cell niches," where signaling cues and nutrient availability promote the survival and proliferation of GICs [136, 138]. GICs tend to cluster in niches characterized as perivascular and hypoxic, though there may be other general types.

The concept of a niche to support the stem cell phenotype is parallel to the microenvironments identified in the support and maintenance of neural stem cells (NSCs). The NSC niche, well characterized in murine models, is understood to be an interactive structural unit, concentrated around blood vessels, where the NSCs have access to signaling molecules, nutrition, and use of vasculature for migration [138]. GICs have similarly been found to be regulated by relationships to endothelial cells for their maintenance and self-renewal [139].

Paracrine signaling from endothelial cells can support GIC renewal and proliferation [140]. In an interesting in vitro model, GICs were cocultured with or without tumor microvascular endothelial cells (tMVEC) isolated from the same tumor specimen and exposed to radiation therapy and/or chemotherapy with temozolomide. GICs cultured with tMVECs not only recovered from the therapeutic insult more quickly, but the cultures were enriched for the GIC phenotype, as seen in recurrent GBM following chemoradiation [141]. This suggests that a combination of soluble and membrane-bound factors from endothelial cells can contribute to

GIC maintenance and radioresistance. Endothelial cell expression of the NOTCH ligand is found to drive GIC self-renewal and proliferation, in addition to the previously discussed role of NOTCH signaling in radioresistance [142, 143]. Furthermore, endothelial cell production of nitric oxide is found to stimulate transcription of NESTIN, a protein highly expressed in GICs, and Hes1, a target of NOTCH signaling. It was also found that the production of nitric oxide by endothelial cells stimulated the development of the stem cell phenotype in cultured GBM cell lines and the expansion of GICs [144].

GICs are not only passive receivers of this paracrine stimulus from endothelial cells. Lineage tracing studies of GICs in 21 GBM xenografts demonstrated that they gave rise to pericytes in vivo [145]. GICs are capable of recruiting endothelial cells and stimulating tube formation, supporting their active role in remodeling the microenvironment [146, 147]. GICs are key players in this dynamic process, giving rise to tumors with greater vascularity, necrosis, and hemorrhage compared to tumors generated from non-GICs [146]. The model for development of these qualities suggests that rapid growth of the tumor cells surpasses the supportive capacity of the available blood supply, creating hypoxic zones that stimulate angiogenic signals and give rise to disorganized tumor vasculature. Given that necrosis and angiogenesis are both characteristics of GBM, and that extensive necrosis is a negative prognostic factor in GBM patients, a number of therapies have attempted to target the molecular components that drive the development of these microenvironments [4]. One of the most promising of these therapies has been the monoclonal antibody against the cytokine vascular endothelial growth factor (VEGF), bevacizumab [148, 149].

GICs produce much higher levels of VEGF, upregulated 10–20-fold, compared to non-GICs, under both normoxia and hypoxia [146]. Both in vitro and in vivo evaluation of bevacizumab has shown that it is capable of abrogating the angiogenic signaling of GICs and reducing tumor vasculature [146]. Unfortunately, the use of bevacizumab clinically is presenting a more complicated picture, with recent evidence showing no survival benefit in the treatment of newly diagnosed GBM, but there may still be efficacy in progressive or recurrent disease [150]. VEGF is not the only important player in the signaling of the perivascular niche, and the relationship between GICs and endothelial cells has a number of implications for the use of ionizing radiation because interactive signaling with endothelial cells promotes GIC survival [139, 141].

GICs are recruited to endothelial cells via chemotactic signals with SDF-1/CXCR4 and were stimulated to differentiate into pericytes due in part to TGFβ secreted by endothelial cells. The cytokine SDF-1 plays a well-established role in the invasive behavior of GICs, in addition to exerting proliferative and antiapoptotic stimulus on a variety of glioma cell lines in vitro [151–153]. This axis may have a particularly detrimental role in GBM because its expression is induced by hypoxia and it can then support radioresistance and recurrence following ionizing radiation.

TGFβ is an intriguing cytokine because it is found to stimulate either tumor suppression or disease progression in different cell types and tumors [154]. In normal brain tissue TGFβ has an antiproliferative effect, whereas GBM tumors are known to express this cytokine abundantly and to proliferate in response to it [155]. Decades

of research suggests that GBM tumors have overcome the antiproliferative effects, and instead the abundance of TGFβ produced by tumors may exert suppressive effects on the host antitumoral immune response [134, 156]. In GICs, TGFβ improves the tumorigenicity of injected cells in xenograft models and it stimulates transcription factors that play a role in stem cell maintenance [157–159].

Ionizing radiation induces the expression of TGFβ, likely through a mechanism that involves reactive oxygen species, and TGFβ has been directly linked to the DDR and radiosensitivity. In studies of a TGFβ inhibitor combined with ionizing radiation the neurosphere-forming capacity and repair of DNA damage were reduced in GICs and in bulk tumor specimens. There was a corresponding induction of self-renewal signals through NOTCH and CXCR4 when TGFβ inhibition and radiation therapy were combined suggesting a possible escape mechanism for radioresistance [160]. Taken together, this evidence supports a role for anti-TGFβ therapy in targeting GIC radioresistance.

Despite abundant angiogenic signaling in GBM, the rapid growth of the tumor cells will outstrip their ability to stimulate sufficient vessel growth. This phenomena is evident in the highly disorganized vessels and variable oxygen tension across GBM tumors [161]. Most solid tumors, including GBM, contain regions of irregular blood flow creating fluctuating and abnormal levels of oxygen tension [162]. Analysis of normal brain and glioma revealed that the physiological concentration of oxygen in healthy brain tissue ranges from 12.5 to 2.5 % ($pO_2 = 100–20$ mmHg). However in GBM masses there is mild to moderate/severe hypoxia with oxygen concentrations ranging between 2.5 and 0.5 % ($pO_2 = 20–4$ mmHg) for mild and 0.5–0.1 % ($pO_2 = 4–0.75$ mmHg) for severe hypoxia [163, 164]. The result of this loss of oxygen and nutrients is necrosis, a characteristic of GBM. Tumor hypoxia is a negative prognostic factor in GBM patients and is associated with tumor aggression. These correlations may be linked to GIC biology. The hypoxic niche paradoxically represents another supportive microenvironment for GICs as hypoxia increases the expression of some markers of GICs in glioma cells [165–167].

The necrotic cores of GBM have elevated expression of cellular markers of hypoxia and the GIC phenotype [168, 169]. Reduced oxygen levels are found to promote the formation of neurospheres in both GICs and non-GICs, and the stem cell genes *Sox2* and *Oct4* are upregulated in glioma cells under moderate hypoxia [170]. In both normal cells and tumors the response to hypoxia is mediated through induction of the hypoxia-inducible factors (HIFs) [162]. The HIF proteins are heterodimeric and exist as a beta subunit which is constitutively present in the nucleus, and alpha subunits which are typically cytosolic and degraded rapidly in the presence of oxygen. The HIF alpha subunits are analogous to an on/off switch, and when regulated by prolyl hydroxylase, which promotes their ubiquitination and degradation by the proteasome in well-oxygenated environments, they are off. In poorly oxygenated environments the function of prolyl hydroxylase is impaired, allowing the stabilization of the HIFα subunits, their translocation to the nucleus, binding of the beta subunit, and subsequent transcriptional activation of a number of target genes [171]. The alpha subunits HIF1α and HIF2α have well-characterized function, and while there is some overlap they differ in their activity at different levels of hypoxia and in their transcriptional targets [172, 173]. Both HIF1α and

HIF2α are critical for GIC function, with knockdown of either one individually reducing neurosphere formation of GICs in vitro, and in vivo their knockdown correlated with increased survival in mice bearing intracranial xenografts.

While both HIF1α and HIF2α are critical for GICs, HIF2α represents a more attractive therapeutic target. First, both neuronal stem cells and normal endothelial cells rely on HIF1α so targeting this protein will have a limited therapeutic index [174]. Second, HIF2α is preferentially expressed in GICs and upregulates the specific stem cell factors Nanog, Oct4, and Sox2 [30]. Uniquely, HIF2α can also promote the GIC phenotype in non-GIC cells [33, 170, 173]. Experimental expression of HIF2α in non-GICs induced the expression of the genes *Oct4*, *Nanog*, and *c-myc* [30]. Expression of a non-degradable HIF2α increased the ratio of GICs to non-GICs, and overexpression of HIF2α in non-GICs increased their tumorigenic potential in mouse xenograft models [173]. HIF proteins are also important mediators of angiogenic signals from GBM as VEGF is a downstream target of HIF as are other pro-angiogenic signals such as angiopoietins [162, 175, 176].

Taken together the data indicate that HIF2α targeting is an attractive approach to GICs and may be useful in combination with ionizing radiation as it could reduce the proportion of these highly resistant cells within a patient's tumor. The potential for broad spectrum drugs, such as the aminoglycoside digoxin, to decrease HIF protein levels in vitro and inhibit tumor growth in xenografts has been demonstrated [177]. However, this therapy would suffer from the therapeutic index limitations due to its targeting of both HIF1α and HIF2α. It is possible to develop HIF2α specific inhibitors, but unfortunately there are no drugs currently under clinical evaluation [178].

Beyond contributing to the maintenance and potential expansion of the GIC population, tissue hypoxia represents a challenge to radiation therapy in a mechanistic way. A major concept in radiobiology, for more than 60 years, is the recognition that the proportion of hypoxic cells in a tissue decreases its radioresponsiveness. In tumors, the hypoxic cells tend to be clustered in the center of the mass, most distant from the vasculature. The challenge posed by the hypoxic cores of most solid tumors is that the cells can still give rise to recurrence. The hypoxic cores of most solid tumors display radioresistance relative to their level of hypoxia, with a dose modifying effect. The dose modifying effect of the hypoxia found commonly in GBM tumors is such that to achieve the equivalent cytotoxic effect desired at normoxia, three-times the radiation dose must be administered. This suggests that an improvement in tumor hypoxia could dramatically improve the effect of ionizing radiation in GBM.

Because radiation therapy is a mainstay in the treatment of GBM its effect on the normal tissue of the brain, in addition to the tumor, are necessary to assess its therapeutic index. It is also important to understand the impact of radiation on the microenvironments and cellular changes induced in the brain of a GBM patient. Historically, this has not been a major focus of research in the GBM field, but as we learn more about extensive changes in gene expression in both normal and cancer cells induced by radiation we need to give more consideration to these effects [179–181]. One population of normal cells that are particularly sensitive to the effects of

radiation are the NSCs. The relationship between GICs and NSCs is an area of intense study, and the striking similarities in phenotypes and overlapping signaling systems that support each population's maintenance are important to recognize. The subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral periventricular region have been found to harbor and maintain a population of NSCs that can differentiate, migrate, and integrate into other functional brain regions [182].

NSCs are exquisitely radiosensitive, and the regenerative capacity of the cells in both the SGZ and SVZ can be impaired by even low to moderate levels (0.5–5 Gy) of radiation, and these changes may persist for as long as 25 months in rodents and humans [183–185]. While there is significant evidence linking damage (either cytotoxic chemotherapy or radiation induced) to the SGZ and hippocampus to neurocognitive decline, there is less evidence linking damage to the SVZ and decline in function [186]. A better understanding of the potential damage that radiation to the SVZ may cause patients will be necessary in the future.

Similarly important is the need to address questions regarding interaction between NSCs and GBM. Given that certain autocrine/paracrine signaling loops are implicated in the maintenance and survival of both NSCs and GICs, it is possible that NSC niches could support GIC development or even provide a pool for their population to arise from. There is currently no direct evidence elucidating the relationship between NSCs and GICs in tumor cells; however, preclinical models support a number of hypotheses that link the SVZ to tumor recurrence. Tumor-suppressor gene deletions in NSCs have shown that they can be a source of tumorigenesis, giving rise to tumors in the brain that resemble the invasive and malignant potential of human gliomas [187–191]. Models in mice and rats that investigated the vascular niche thought to support the NSCs of the SVZ have shown the potential for secreted factors in this microenvironment to induce glioma-like hyperplasias [192], and migration patterns of NSCs have been found to infiltrate gliomas with both supportive and inhibitory effects on glioma progression [193, 194].

While the nature of the relationship between GICs and NSC niches is currently unclear, a number of studies have implicated the SVZ as a target of radiation to sterilize possible microinvasive disease [195]. Clinical evidence similarly suggests a relationship between glioma progression and involvement of the SVZ. Patients that have tumors contacting the SVZ have been found to have a poorer survival compared to those that do not contact the SVZ [196, 197]. Furthermore, retrospective clinical evaluations are interrogating the role of radiation in a possible therapeutic enhancement targeting the SVZ [198, 199].

In conclusion, the study of GICs has provided us with a powerful model to understand the radioresistance of GBM. The heterogeneity inherent in GBM is partially responsible for the difficulty in finding effective treatment. It is clear that GICs possess a variety of intracellular adaptations allowing them to preferentially survive and even proliferate in response to radiation. This model has helped to identify or support a number of potential targets for therapeutic radiosensitization, which are under clinical investigation to improve patient survival. Unfortunately such targeted therapies may fall short if factors present in the microenvironment of

this disease persist or are even recapitulated by our treatments. The possibility that cellular phenotypes within the tumor are dynamically regulated by extrinsic stress and signaling, such as that stimulated by radiation, suggests that modulation of radiation's effectiveness alone may not be enough to improve patient survival. Further investigation of radiation therapy in GBM and other stem cell neoplasms of the central nervous system should focus on disrupting the intracellular mechanisms of resistance and microenvironmental stimuli.

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