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CANCER STEM CELLS AND RADIORESISTANCE

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Abstract

Purpose—Radiation therapy has made significant contributions to cancer therapy. However, despite continuous improvements, tumor recurrence and therapy resistance still occur in a high proportion of patients. One underlying reason for this radioresistance might be attributable to the presence of cancer stem cells (CSCs).

Conclusions—This review discusses CSC-specific mechanisms that confer radiation resistance with a focus on breast cancer and glioblastoma multiforme (GBM), thereby emphasizing the addition of these potential therapeutic targets in order to potentiate radiotherapy efficacy.

INTRODUCTION

Radiation therapy has been in clinical use for over a millennium (DeVita 2008). The era of radiation treatment began at the turn of last century with Roentgen's discovery of X-rays and Pierre and Marie Curie's discovery of radium. The modern era of radiation therapy began in 1950's with the introduction of cobalt teletherapy, which utilized synthetic radium. In 1971, the advent of computed tomography (CT) created a shift from 2-dimentional to 3-dimentional radiation delivery, allowing therapeutic radiologists to deliver beam energy precisely to the tumor. Although still a powerful tool for the control of tumor growth, radiation therapy, like most other anti-tumor modalities, has its weaknesses as tumors develop adaptive response and become more resistant, aggressive, and invasive (Ahmed & Li 2007). A newly emerged plausible explanation for tumor radioresistance is the existence of a subpopulation of cancer stem cells (CSCs) that are intrinsically more resistant to multiple clinical therapies. Moreover, therapeutic treatments may cause the expansion and further genetic mutations and epigenetic alterations of CSCs resulting in acquired therapy resistance. Characterizing the roles of CSCs in both intrinsic and acquired radioresistance and identifying the molecular pathways that maintain CSC stemness are of paramount importance in improving the efficacy of cancer treatments.

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DECLARATION OF INTEREST

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CANCER STEM CELLS AND RADIATION

The CSC hypothesis postulates an intra-tumoral cellular hierarchy generated and maintained by a small population of tumor cells that has the ability to self-renew and to differentiate into the bulk, more mature cancer cells (Reya et al. 2001). A direct implication of the CSC hypothesis is that cell populations with different properties co-exist within the same tumor and CSCs have the ability to create the cellular heterogeneity commonly observed in clinical tumors. This implication is significant, as tumor cell heterogeneity, in the past, had been traditionally explained only by clonal evolution dictated by high genetic instability in tumor cells. The CSC hypothesis emerged after the discovery of a small population of cells in human leukemias that possessed infinite self-renewing activity after they were transferred to immune-deficient mice (Bonnet & Dick 1997). More recently, genetic tracing studies have shown that in mouse tumors, CSCs exist and maintain tumor growth (Chen et al. 2012; Driessens et al. 2012; Schepers et al. 2012). One important aspect of CSCs is their potential resistance to chemotherapeutic agents as well as radiation therapy (Reya et al. 2001; Jordan et al. 2006).

One main mechanism of radioresistance in CSCs compared to non-CSCs appears to be related to their enhanced DNA-repair capacity and reactive oxygen species (ROS) defenses, and their self-renewal potential. Ionizing radiation (IR) and radiomimetic drugs lead to the formation of double-strand breaks (DSBs) in DNA, which normally trigger DNA-damage responses (DDR). When the DDR cannot efficiently repair the DSBs, irradiated cells undergo the so-called mitotic catastrophe, a major cell death mechanism for irradiation-induced DNA damage. Other mechanisms include genomic instability (Morgan & Murnane 1995; Morgan 2003), bystander effects (Morgan & Murnane 1995), and adaptive radioresistance (Ch'ang et al. 2005). Development of tumor radioresistance creates a serious challenge to the current cancer treatments. First described for glioblastoma multiforme (GBM) and for breast cancer, the radioresistance in CSCs seems to be associated with an increased ability to scavenge free radicals formed in response to radiation and with differences in how the DNA DSBs are processed and repaired (Bao et al. 2006; Phillips et al. 2006). One of the common characteristics of normal stem cells (SCs) and CSCs is their better ability to protect DNA from stress-induced damages than the non-stem cells. Indeed, CSCs are shown to be more radioresistant than the non-stem cancer cells and are therefore believed to be responsible for treatment failure and tumor recurrence (Baumann et al. 2008).

Repopulation of recurrent tumors by CSCs has been supported clinically, as the percentages of CSCs are found to increase following cytotoxic chemotherapy in breast cancer patients (Diehn et al. 2009a). In fact, repopulation has long been considered as a cause of treatment failure (Kim & Tannock 2005) although there certainly exist many mechanisms involved in adaptive resistance. It is plausible to assume the presence of different subsets of CSCs with divergent mutations/genomic alterations within tumors, since heterogeneous tumors consist of unstable genomes. Upon chemo- or radiotherapy, the CSC clones with the advantageous genomic alterations to protect against therapy would be selected for and continue to sustain the tumor (Diehn et al. 2009a). Thus, radiation may selectively kill the relatively radiosensitive tumor cell populations leaving the therapy-resistant CSCs alive, thus contributing to adaptive radio-resistance via the selective repopulation from the surviving

CSCs. On the other hand, non-genetic mechanisms may also operate to generate therapy-resistant cancer cells that can repopulate recurrent tumors. For instance, in a recent paper, Lagadec *et al*. showed that IR is capable of reprogramming differentiated breast cancer cells into induced breast CSCs (iBCSCs). The iBCSCs displayed enhanced mammosphere formation and tumorigenicity and also expressed the same stemness-related genes as the BCSCs from non-irradiated samples (Lagadec et al. 2012). Overall, molecular mechanisms underlying radiation resistance in tumors remain poorly understood and clearly these mechanisms are complex and require more extensive characterizations. Below we use breast cancer and glioblastoma as tumor models to discuss the involvement of CSCs in mediating radioresistance and the potential underlying mechanisms (Table 1).

BREAST CANCER

Radiotherapy remains a standard therapeutic modality for breast cancer patients, which has been shown to improve the patients' overall survival (Clarke et al. 2005; Veronesi et al. 2005; Gebski et al. 2006). Nevertheless, tumor resistance to IR has caused a plateau in the survival benefits and one emerging explanation is the existence of CSCs (Gupta et al. 2009; Rosen & Jordan 2009). Indeed, pre-clinical data suggest that breast CSCs (BCSCs) are enriched after radiation and that, vice versa, BCSCs are particularly resistant to radiation (Phillips et al. 2006).

One of the major limitations of radiation therapy is that cells in solid tumors become deficient in oxygen, creating a hypoxic environment. As a potent radiosensitizer, oxygen can increase the effectiveness of a given dose of radiation by forming DNA-damaging ROS and therefore tumor cells in a hypoxic environment can be more resistant to radiation damage than those in a normal oxygen environment. Indeed, data has shown that normal breast SCs and CSCs in some tumors arising in both mice and humans contain lower levels of ROS than their cellular descendants. This indicates that SCs possess and CSCs have kept this attribute perhaps to protect their genomes from endogenous and exogenous ROS-mediated damage. Lower levels of ROS in CSCs are associated with increased expression of free radical scavenging systems (Tothova & Gilliland 2009). Diehn *et al*. (2009b) observed a marked heterogeneity of ROS levels in both normal SC and CSC-enriched populations that may influence the extent to which CSC-enriched populations are resistant to therapies such as IR. They observed that CSCs accumulate fewer DNA single- and double-strand breaks after *in vitro* and *in vivo* irradiation. In addition, the decrease in DNA damage correlates with increased persistence of CSCs, consistent with the model that ROS modulate radioresistance of CSC populations. Furthermore, radioprotection in the CSC-enriched populations is linked to expression of genes involved in glutathione synthesis, including glutamate cysteine ligase (Gclm), glutathione synthetase (Gss), and FoxO1, but not FoxO4, Hif1α, or Epas1. In addition, depletion of glutathione (GSH) in CSCs via treatment with buthionine sulphoximine (BSO) results in decreased colony-forming ability and radiosensitization (Diehn et al. 2009b).

Therapeutic IR causes DNA damage and generates oxidative stress in cells leading to the activation of specific signaling pathways in the irradiated cells (Spitz et al. 2004). Depending on the extent of DNA damage, either pro-apoptotic or pro-survival pathways are

initiated. Phillips *et al*. (2006) were the first to show that the $CD44^{+}CD24^{-/low}$ BCSCs are more radioresistant, supporting a notion that CSCs are more radioresistant than the non-stem cancer cells (Al-Hajj et al. 2003). Upon radiation exposure, breast cancer-initiating cells increased, which was accompanied by radiation-induced Jagged-1 expression and subsequent activation of Notch signaling via the PI3K pathway. Further, treatment with IR caused lower levels of ROS in this population, associated with high intracellular levels of radical scavengers (Phillips et al. 2006). Several drugs to inhibit Notch signaling have been developed and γ-secretase inhibitors are entering Phases I–II clinical trials in breast cancer, offering hope of overcoming BCSC-mediated radioresistance.

Working on a p53 null mammary tumor model, Zhang *et al* (2010) showed that tumor-initiating cells (TICs) exhibit more efficient DNA damage repair than bulk tumor cells when exposed to IR. Also, there was selective activation of the Akt and canonical Wnt signaling pathways in TICs as evidenced by the increased expression of phospho (p)-Akt and increased phosphorylation of β-catenin on serine (Ser) 552 (Zhang et al. 2010). Several groups have also shown that HER-expressing BCSCs are more radioresistant and aggressive compared to their negative counterparts (Cao et al. 2009). For example, it was recently shown that HER2-overexpressing BCSCs are responsible for the radioresistance of $HER2^{-/low}$ breast cancer. Specifically, BCSCs with the phenotype of $HER2^{+}/CD44^{+}/CD24^{-/low}$, compared with the $HER2^{-}/CD44^{+}/CD24^{-/low}$ breast cancer cells, showed an increased aggressiveness, tumorigenesis, and radioresistance thus providing a potential therapeutic target to sensitize breast cancer cells (Duru et al. 2012).

GLIOBLASTOMA MULTIFORME

Glioblastoma multiforme (GBM) is one of the most aggressive human malignancies with rapid growth, high invasiveness and vascularity, treatment resistance, and a poor patient prognosis. IR, either alone or adjuvant after surgery, is part of standard treatment. However, prognosis of patients with glioblastoma still remains poor because of refractory response to radiation and other treatments (Legler et al. 1999; Hegi et al. 2005; Bao et al. 2006; Huang et al. 2007). The exact molecular mechanisms driving resistance in GBMs are incompletely understood but have been in part accredited to glioma stem cells (GSCs), which have been shown to be highly resistant to IR due to more efficient DNA damage repair and prosurvival mechanisms (Bao et al. 2006; Kang et al. 2008). Indeed, identification of a subpopulation of brain tumor cells with potent tumorigenic activity provided earlier supports to the CSC hypothesis in solid tumors (Hemmati et al. 2003; Singh et al. 2003; Galli et al. 2004; Singh et al. 2004). As with BCSCs, GSCs have been reported to be less sensitive to radiation-induced cell killing through preferential activation of DNA damage checkpoint responses and increased capacity for DNA damage repair. There is also evidence for more efficient homologous recombination repair in GCSs contributing to their radioresistance (Lim et al. 2012).

CD133, a marker for normal neural SCs, has been suggested to be a CSC marker in malignant brain tumors as only CD133-positive cells from brain tumor biopsy materials were able to initiate tumor regeneration in xenograft mouse models (Singh et al. 2003; Singh et al. 2004). Also, tumor cells expressing CD133 are enriched after radiation in gliomas,

therefore indicating CD133 expression in treatment failure (Uchida et al. 2000; Hemmati et al. 2003; Singh et al. 2003; Singh et al. 2004). CD133-positive GSCs can survive, change to a proliferative phenotype, and cause recurrence in de novo glioblastomas despite tumor vascular damage after radiotherapy and chemotherapy (Tamura et al. 2013). In a seminal study, Bao *et al*. reported that CD133-positive tumor cells represented the radioresistant cell population and could be the source of tumor recurrence after radiation (Bao et al. 2006). These GSCs contributed to glioma radioresistance via preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity. In both cell culture models and in the brains of immunocompromised mice, CD133-expressing glioma cells survived IR relatively better compared to CD133-negative cells. In addition, CD133-positive glioma cells activated Chk2-dependent checkpoint responses to a greater extent than CD133-negative cells (Bao et al. 2006).

In a follow-up study, the same group shows that L1CAM (CD171) regulates DNA damage checkpoint responses and radiosensitivity of GSCs through nuclear translocation of L1CAM intracellular domain (Cheng et al., 2011). Further, L1CAM, via c-Myc, regulates expression of NBS1, a critical component of the MRE11-RAD50-NBS1 (MRN) complex that activates ataxia telangiectasia mutated (ATM) kinase and early checkpoint response (Cheng et al. 2011). Other groups have also shown that GSCs are more resistant to radiation compared to non-GSCs due to high expression of phosphorylated checkpoint proteins. For example,Squatrito *et al*. (2010) demonstrated that loss of ATM/Chk2/p53 pathway components accelerated tumor development and contributed to radiation resistance in gliomas. Chk2 seemed to be required for glioma response to IR *in vivo* and for DNA-damage checkpoints in the neuronal SCs (Squatrito et al. 2010). Additionally, Facchino *et al*. (2010) reported the requirement of the polycomb group protein BMI1, which is enriched in CD133-positive GBM cells, for the self-renewal of GSCs in an *INK4A/ARF*-independent manner through transcriptional repression of alternate tumor suppressor pathways. BMI1 conferred radioresistance to malignant neural SCs through recruitment of the DNA damage response machinery (Facchino et al. 2010).

Several major prosurvival signaling pathways have been shown to be important for GSCs (Li et al. 2009). Further characterizing and potentially targeting these pathways hold promises to improve the current radiotherapy efficacy in glioma treatment. The epidermal growth factor receptor (EGFR), a member of receptor tyrosine kinases (RTK) family, is shown to play a significant role in the proliferation and neurosphere formation in GSCs. Activation of pro-survival PI3K/Akt pathway, which is downstream of RTKs, has been shown to be more dominant in GSCs compared to non-stem glioma cells. Indeed,Hambardzumyan *et al*. (2008) showed the importance of the activation of PI3K/Akt/mTOR pathway in conferring radioresistance in subpopulations of medulloblastoma cells, a highly malignant brain cancer. Interestingly, three different populations of medulloblastoma cells displayed differential responses upon IR - the main cell population, which was radiosensitive, underwent p53-dependent apoptosis whereas the other two populations were radioresistant, including nestin-expressing SCs and non-proliferating cells (Hambardzumyan et al. 2008).

Another major pro-survival mechanism activated by DNA damage is the NF-κB signaling pathway, which attenuates apoptosis and enhances survival in many cancers (Tang et al. 2001; Danial & Korsmeyer 2004). Accumulating evidence indicates that NF-κB plays an essential function in the response of cells to IR and many studies have investigated the effects of inhibition of NF-κB activity on the radiation response (Ahmed et al. 2009). In a recent study, Bhat *et al* (2013) show that in GBM patients, a mesenchymal gene profile, CD44 expression, and NF-κB activation correlate with poor radiation response and shorter survival suggesting inhibition of NF-κB activity as an attractive therapeutic strategy for GBM (Bhat et al. 2013). They further show that cell types such as macrophages and microglia play an integral role in NF-κB activation, bolstering the importance of influence from the tumor niche (Bhat et al. 2013).

Many studies have directly linked TGF-β to DNA damage responses and radiosensitivity (Kirshner et al. 2006; Wiegman et al. 2007). Hardee *et al* (2013) demonstrated that TGF-β production by GSCs was higher than in bulk glioma cells, which promoted effective DNA damage response and self-renewal, creating microenvironment-mediated resistance. Zhang *et al* (2011) conducted a preclinical study of the antitumor effects of the TGF-β receptor (TGFβR) I kinase inhibitor LY2109761 in combination with radiotherapy. They observed that this inhibitor potentiated radiation responses by coordinately increasing apoptosis in cancer stem-like cells and by blocking DNA damage repair, invasion, mesenchymal transition, and angiogenesis (Zhang et al. 2011).

Yoon *et al*. (2012) recently showed that c-Jun N-terminal kinase (JNK) signaling was crucial for the self-renewal, tumorigenicity, and IR resistance in GSCs. Aberrant Notch activation has been found in various malignant tumors, including breast cancer, leukemia and glioma (Wang et al. 2009). Notch activation through introduction of the notch intracellular domain stimulates growth and sphere formation in glioma cell lines and endows radioresistance to $CD133^+$ GSCs (Zhang et al. 2008; Wang et al. 2010). Findings from Wang *et al* (2010) suggest that JNK upregulates Notch-2 expression, at least via c-Jun, and thereby maintains stemness of GSCs. Their data also suggests that PI3K is an upstream kinase of JNK, independently from Akt signaling, and that PI3K/JNK and PI3K/Akt pathways may have crucial independent roles in the maintenance of glioma stem-like cells and in radioprotective functions (Wang et al. 2010). Finally, Fassl *et al* (2012) have recently shown that Notch1-mediated upregulation of Mcl-1 contributes to the therapy resistance of glioma-initiating cells.

Several other signaling pathways have also been implicated in governing GSC properties. Members of the protein kinase C (PKC) family of serine/threonine kinases are key components of signal transduction pathways that regulate proliferation, cell survival and malignant transformation (Parekh et al. 2000; Steinberg 2004; Griner & Kazanietz 2007).Kim *et al*. (2011) show that PKCδ activation is essential for the expansion and maintenance of glioma stem-like cell populations and acquisition of resistance to cancer treatments including radiation. Lomonaco *et al* (2009) suggested autophagy as an alternative radioprotective mechanism for $CD133^+$ GSCs. In their study, $CD133^+$ cells expressed higher levels of the autophagy-related proteins LC3, ATG5, and ATG12 than $CD133^-$ cells after irradiation and inhibition of autophagy preferentially sensitized $CD133^+$ cells to

radiation and decreased sphere-forming capacity (Lomonaco et al. 2009). Tumor angiogenesis has also been associated with radioresistance. GSCs may promote tumor angiogenesis through elevated expression of vascular endothelial growth factor (VEGF) and the VEGF-neutralizing antibody bevacizumab specifically inhibits GSC angiogenesis. Thus, targeting VEGF in CSCs may improve the clinical efficacy of radiation in solid cancers (Eyler & Rich 2008).

CONCLUSIONS

There is strong experimental and clinical evidence that CSCs have the ability to generate heterogeneous cell populations. They occupy specialized niches and are able to repopulate the tumor after treatments. CSCs may be intimately involved in both intrinsic and acquired tumor resistance to anticancer treatments including radiation therapy. Uncovering the mechanisms that govern the maintenance of CSCs and their resistance to therapy is crucial for developing novel therapeutic strategies to target these cells. Ideally, combining radiation therapy with an agent that radiosensitizes CSCs, targets tumor angiogenesis, and also eliminates differentiated cells would be an effective approach to eradicate primary tumor burden and prevent recurrence.

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Table 1

Radioresistance of CSC in breast cancer and GBM*

Cell Type	CSC Marker	Assays Performed	Proposed Mechanisms
MCF-7, MDA-MB-231	mammosphere; $CD44^{+}/CD24^{-/low}$	ROS measurement; γ-H2AX foci; Jagged-1 expression and Notch-1 activation	Radiation-induced Jagged-1 expression and subsequent activation of Notch-1; lower levels of ROS due to high intracellular levels of radical scavengers in cancer-initiating cells (Phillips et al. 2006)
Mouse mammary tumor cells; human breast cancer cells	$Lin^{-}CD24^{med}$ $CD49f^{hi}$, (mouse); $CD44^{+}$ $CD24^{-/low}$ (human); $Thy1^{+}$ $CD24^{+}$ Lin^{-} (mouse)	Intracellular ROS analysis; alkaline comet assay; γ-H2AX foci; FACS to determine CSC-enriched populations post IR	Lower ROS levels in breast CSC, associated with increased expression of free radical scavenging molecules (Diehn et al. 2009b).
Transplantable p53-null mouse mammary tumors	$Lin^{-}CD29^{hi}CD24^{hi}$	γ-H2AX and 53BP1 foci; MitoSOX and DCF-DA assays; mammosphere formation; FACS to determine CSC-enriched populations post IR	Increased phospho (p)-Akt and β-catenin Ser552 phosphorylation (Zhang et al. 2010).
MDA-MB231, MCF7, radioresistant MCF7/C6, and MCF7 transfected with HER2	$HER2^{+}/CD44^{+}/CD24^{-/low}$	Clonogenic survival assays; Matrigel invasion assays; mammosphere formation; mitochondrial membrane potential ($\Delta\psi_m$) and apoptosis; FACS to determine CSC-enriched populations post IR	HER2-overexpressing breast CSCs responsible for the radioresistance of $HER2^{-/low}$ breast cancer (Duru et al. 2012).
Xenografts derived from primary glioblastoma samples	$CD133^{+}$	CSC-enriched populations post IR; alkaline comet assay; checkpoint responses; γ-H2AX foci	$CD133^{+}$ cancer cells contributing to glioma radioresistance via preferential checkpoint response and DNA repair (Bao et al. 2006).
U87, U373, patient-derived glioma cells	Sphere formation	Sphere-forming assays; quantification of cell death	JNK signaling contributing to resistance of sphere-forming glioma cells to radiation (Yoon et al. 2012).
GSCs from surgical specimens	$CD133^{+}$	Caspase 3/7 cleavage; clonogenic survival assays	Notch regulating GSC radioresistance (Wang et al. 2010).
U87, U373	$CD133^{+}$, sphere-formation	CSC marker expression; clonogenic survival assays; CSC enrichment post IR	PKCδ playing a role in fractionated-radiation-induced expansion of glioma stem-like cells and acquisition of radioresistance (Kim et al. 2011).
GSC from surgical specimens or xenografts	$CD133^{+}$ or $CD15^{+}$	L1CAM expression after IR; phosphorylation of key checkpoint proteins; γ-H2AX foci; induction of DNA damage and comet assays; NBS1 rescue	L1CAM augmenting DNA damage checkpoint activation and radioresistance of GSCs through L1-ICD-mediated NBS1 upregulation and enhanced MRN–ATM–Chk2 signaling (Cheng et al. 2011).
Tumor neurospheres from primary glioblastomas, mouse tumor neurospheres	neurosphere cultures	TUNEL and survival assays	Chk2 required for glioma response to ionizing radiation in vivo and necessary for DNA-damage checkpoints in the neuronal stem cells (Squatrito et al. 2010).
Human GBM samples	Neurosphere cultures; $CD133^{+}$	Colony-forming assays; neutral comet assays; ATM inhibition; γ-H2AX foci; BMI-1 protein levels; ROS concentration	BMI-1 conferring radioresistance in cancerous neural stem cells via recruitment of the DNA damage response machinery (Facchino et al. 2010).
Human GBM samples	Neurosphere cultures; CD133+, $CD15^{+}$, and/or $CD44^{+}$	γ-H2AX foci; apoptosis assays; neurosphere formation; CSC enrichment post IR	Tumor microenvironment cells (macrophages/microglia) playing a role in radioresistance; MES signature, CD44 expression, and NF-κB activation correlating with poor

Cell Type	CSC Marker	Assays Performed	Proposed Mechanisms
			radiation response and shorter survival in GBM patients (Bhat et al. 2013).

*Presented are examples of CSC resistance to irradiation. All radiation assays utilized ^{137}Cs, except in two cases where ^{60}Co was used (Duru et al. 2012) and (Bhat et al. 2013).

Abbreviations:
γ-H2AX: H2A histone family, member X
53BP1: p53-binding protein 1
ATM: ataxia-telangiectasia-mutated kinase
DCF-DA: cellular reactive oxygen species detection assay
GBM: glioblastoma multiforme
GSC: glioblastoma stem cell
IR: irradiation
JNK: c-Jun N-terminal kinases
ROS: reactive oxygen species
MES: mesenchymal
MRN: MRE11-RAD50-NBS1
PKCδ: protein kinase C
TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling