Review article:

AUTOPHAGY AND RADIOSENSITIZATION IN CANCER

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ABSTRACT

Autophagy is a natural self-degradative process by which cells eliminate misfolded proteins and damaged organelles. Autophagy has been shown to have multiple functions in tumor cells that may be dependent on the tumor type and the treatment conditions. Autophagy can have a cytoprotective role and be thought of as a survival mechanism or be cytotoxic in nature and mediate cell death. Radiation, one of the primary treatments for many different types of cancer, almost uniformly promotes autophagy in tumor cells. While autophagy produced in response to radiation is often considered to be cytoprotective, radiation-induced autophagy has also been shown to mediate susceptibility to radiation. This review addresses the complexity of autophagy in response to radiation treatment in three different cancer models, specifically lung cancer, breast cancer and glioblastoma. A deeper understanding of the different roles played by autophagy in response to radiation should facilitate the development of approaches for enhancing the therapeutic utility of radiation by providing strategies for combination treatment with unique radiosensitizers as well as preventing the initiation of strategies which are likely to attenuate the effectiveness of radiation therapy.

Keywords: Autophagy, ATM, DNA damage response, lung cancer, glioblastomas, breast cancer, radiosensitization

OVERVIEW

Autophagy is an evolutionarily conserved process, which morphologically involves the formation of double-membrane bound autophagic vacuoles or autophagosomes (Levine and Klionsky, 2004; Mizushima and Levine, 2010), that degrade and recycle proteins and cellular organelles by fusion with lysosomes (Levine and Klionsky, 2004; Mizushima and Levine, 2010). Autophagy can play a critical role in cancer cell survival by allowing cells to survive under conditions of metabolic and genotoxic stress (Wilson et al., 2011; Michallet et al., 2011; Qiang et al., 2013; Fung et al., 2008). On the other hand, excessive autophagy or self-eating may para-

doxically lead to a type of programmed cell death (PCD), or type II PCD, a distinct cellular suicide pathway (Ouyang et al., 2012). In the case where autophagy induction serves a prosurvival function in response to genotoxic stress, inhibiting autophagy may chemosensitize and radiosensitize tumor cells, thus increasing the efficacy of treatment (Janku et al., 2011; Palumbo and Comincini, 2013). Conversely, inducing autophagic cell death within transformed cells resistant to apoptosis may be a viable therapeutic strategy for these types of cancers.

Ionizing radiation, which is effective in the treatment of multiple types of malignancies, has been found to promote autophagy in virtually every experimental tumor model system. The autophagy induced by radiation is generally thought to provide a cytoprotective function, which can be suppressed to enhance the response to radiation (Paglin et al., 2001). However, there are also examples where the autophagy induced by either radiation alone or radiation in combination with another treatment modality results in cell death. This review will provide examples of the functional roles of autophagy induction by radiation in three experimental tumor cell models, specifically breast cancer, lung cancer, and glioblastoma.

LUNG CANCER

Incidence and current treatments

Lung cancer is one of the leading causes of cancer-related death in the United States. Lung cancer has a relatively poor prognosis with a very low median survival rate. Lung cancer can be either primary, originating in the lung itself, or secondary, originating elsewhere in the body and metastasizing to the lungs (lungcancer.org). Primary lung cancer can be either non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC) (lungcancer.org). NSCLC is more common and less aggressive, accounting for 80 % of lung cancers, but can be difficult to diagnose since it is asymptomatic in

its early stages. SCLC accounts for 10-20 % of disease incidence and is frequently associated with smoking (lungcancer.org; Pesch et al., 2012). However, this section of the review will be limited to a discussion of studies relating to non-small cell lung cancer.

The standard treatment regime for NSCLC involves surgery but the majority of NSCLC patients are not eligible for surgical resection; as such, radiation is one the most commonly used and efficacious strategies for treatment of lung cancer (Peng et al., 2008; Shin et al., 2012; Sharieff et al., 2013). Patients are generally treated with cumulative doses of ~ 60 Gy divided into 1.8-2 Gy fractions, often in combination with the chemotherapeutic agents, cisplatin or paclitaxel. Unfortunately, current therapies such as radiation and chemotherapy are usually only palliative (Saintigny and Burger, 2012; Siegel et al., 2013); consequently there is a compelling need for the development of alternative therapies, which might be more effective and selective.

Manipulating autophagy as therapeutic strategy

Cytoprotective autophagy and radiation resistance

Recent studies by Kroemer's group (Ko et al., 2013) have supported the cytoprotective function of radiation-induced autophagy by demonstrating that inhibition of autophagy induced by radiation increased the effectiveness of the radiation treatment. Knockdown of the autophagy related genes ATG5 and Beclin-1 resulted in radiosensitization, indicative of the fact that autophagy can prevent radiation induced cell death in H460 and A549 NSCLC cell lines. Studies in immunodeficient tumor bearing mice further showed that tumors with defective autophagy were more sensitive to radiation.

Studies by Apel et al. (2008) demonstrated an increase in autophagic vesicle formation in response to radiation in A549 non-small cell lung cancer cells. Furthermore, autophagy was shown to be cytopro-

tective in nature since the clonogenic survival fraction was increased when autophagy related genes such as Atg3 and Atg12 were inhibited. Interestingly, this effect was more pronounced in cells with mutant p53. As defective p53 could be related to prevention of apoptosis, these studies suggest that survival in response to blocked autophagy is likely to be more pronounced in situations where apoptosis is compromised. In this context, although p53 is well established to have a central role in the promotion of apoptosis, its involvement in autophagy is less well defined (Rikiishi, 2012; Su et al., 2013).

Studies by Cheng et al. (2013) in H1299 non-small cell lung cancer cells with induced p53 found that inhibition of autophagy by 3-methyl adenine led to a significant reduction in cell viability after radiation, supporting its cytoprotective function. Furthermore, inhibition of apoptosis by using Z-VAD increased tumor cell survival rate. Taken together, these studies support the pro-survival/protective role of autophagy induced by radiation in non-small cell lung cancer.

Cytotoxic or cytostatic autophagy

Given the relative radioresistance of NSCLC, a number of studies has been designed to identify compounds which might sensitize resistant NSCLC cancer cells to radiation treatment. In all the studies indicated below, radiosensitization was associated with an increase in autophagy.

Kim et al. (2011) reported that the zinc ionophore, PCI-5002, could increase the radiosensitive fraction of PCI-500 NSCLC cells, an effect that was accompanied by increased autophagy and decreased caspase-3 cleavage. PCI-5002 treatment also reduced the growth rate of xenografted tumor cells, indicating that this compound has the capacity to sensitize lung tumor cells to radiation both *in vitro* and *in vivo*. Although the data support the premise that radiation sensitization was occurring through the promotion of autophagy, these studies did not directly assess whether inhibition of au-

tophagy could reverse the observed radiosensitization.

Berberine, a major component of herbal medicine used to treat inflammation in Europe and Asia, has been shown to enhance radiosensitization of NSCLC cells both *in vitro* and *invivo* (Peng et al., 2008). Autophagy induction was detected by acridine orange staining of autophagic vesicles, electron microscopy, and immunoblotting of LC3 while there was only a minor increase in the extent of apoptosis. Of critical importance, the autophagy inhibitor, 3-methyl adenine, was shown to reverse radiosensitization by berberine while radiosensitization was not reversed by the broad spectrum apoptosis inhibitor, Z-VAD. Berberine used in combination with radiation produced a substantial reduction in tumor volume compared to the tumor growth delay by either radiation or berberine alone.

Shin et al. (2012) utilized non-invasive aerosol gene delivery in an effort to improve the effectiveness of radiation therapy in NSCLC. Overexpression of Beclin-1 via inhalation in combination with radiation led to a significant decrease in tumor progression via prolonged activation of autophagic cell death in a k-ras^{LA1} lung cancer mouse model. Increased autophagy was detected by TEM analysis and immunoblotting to assess levels of autophagy related genes. The combination treatment also resulted in a decrease in phospho-Akt, which further led to mTORC1 and mTORC2 downregulation. This observation is significant in that phosphorylation of Akt and mTOR has been known to correlate with tumor progression and tumorigenesis (Choe et al., 2003).

While apoptosis has long been thought to be the primary form of cell death in response to radiation, it actually accounts for a minor portion of cell death in irradiated solid tumors (Verheij and Bartelink, 2000). In this context, Kim et al. (2006) showed that knockdown of the pro-apoptotic proteins, Bak and Bax, increased lung tumor radiosensitivity *in vitro*, decreased mTOR-

Akt signaling and increased expression of pro-autophagic genes such as the Atg-5-Atg12 complex. These investigators further demonstrated that autophagy was the cell death mechanism that contributed to this radiosensitization by reversing the sensitization effects using both the pharmacological inhibitor, 3-MA and by genetically silencing the autophagy related genes, Atg5 and Beclin-1. To further explore the potential of apoptosis inhibition to sensitize lung cancer for therapy, M867, a novel chemical caspase-3 inhibitor, was utilized in combination with radiation *in vivo* in a mouse hind limb xenograft tumor model, where it was shown that M867 in combination with radiation produced a prolonged tumor growth delay (Kim et al., 2008a). The use of caspase double knockout cells (caspase 3/7 deficient) provided additional support for the conclusion that autophagy was the mechanism responsible for increased radiosensitivity. Irradiated DKO cells were shown to have elevated levels of autophagy; while knockdown of autophagic genes such as ATG5 and Beclin-1 resulted in decreased sensitization while overexpression of these autophagy associated genes increased radiosensitivity.

In a study that addressed the potential utility of inhibiting both apoptosis and mTOR signaling to improve the efficacy of radiation therapy, Kim et al. (2008b) also showed that the H460 NSCLC cell line is more sensitive to radiation in the presence of a caspase inhibitor, Z-DEVD. Sensitivity was further increased when used in combination with RAD001, which caused an increase in autophagy via the inhibition of mTOR. *In vivo* studies using H460 NSCLC cells as xenografts confirmed the outcome of the *in vitro* studies in that the combination of either Z-DEVD and radiation or RAD001 and radiation induced a significant delay in tumor growth compared to radiation alone. These studies suggest that sensitization can occur even if levels of apoptosis are significantly reduced. Autophagy induction (based on GFP-LC3 and LC3II

protein levels) was significantly increased by the combination treatment of radiation, Z-DEVD and RAD001 both *in vivo* and *in vitro*. Of particular importance for supporting the role of autophagy induction in radiosensitization, knock down of the autophagy related genes ATG5 and Beclin-1 reversed radiosensitization. Studies by Moretti et al. (2009) using H460 cells support these observations in that the broad-spectrum caspase inhibitor Z-VAD radiosensitized H460 cells both in cell culture and in tumor-bearing animals.

Clinical NSCLC specimens have been reported to have mutations in multiple oncogenes and tumor suppressors including EGFR, K-RAS and p53 (Scagliotti et al., 2004; Choi et al., 2010). The epidermal growth factor receptor (EGFR) appears to play a central role in cell proliferation and growth as activation of EGFR leads to collateral activation of several pro-survival signaling pathways (Scagliotti et al., 2004); therefore, inhibition of EGFR signaling pathways represents a promising cancer treatment strategy. Pre-clinical and clinical studies have provided evidence supporting the potential value of targeting EGFR signaling to enhance the anti-tumor effects of radiation (Steiner et al., 2007). However, therapeutic resistance from a number of factors such as downstream or alternative signaling pathways remains an issue. Choi et al. (2010) have shown that simultaneous inhibition of both EGFR and K-RAS using RNA interference increased radio-sensitivity in A549 and H460 NSCLC cell lines. This group had also previously reported that targeting Akt using siRNA increased radiosensitivity in EGFR or Ras activated cell lines (Kim et al., 2005). These investigators further showed that inhibiting Akt interferes with DNA double strand break repair as measured by γ-H2AX foci induction and decline. Both autophagy and apoptosis were demonstrated to be the modes of cell death in this study, as inhibition of either response rescued the cells from radiosensitization. This study makes it clear that

targeting more than one component of a tumor specific signaling pathway would likely be necessary to overcome radiation resistance (Kim et al., 2005)

Recently, studies conducted in our laboratory have shown that combination treatment of radiation with vitamin D and its analog, EB 1089, led to an increase in radiosensitivity in NSCLC. We demonstrated that this sensitization appeared to be a consequence of the switch from cytoprotective autophagy induced by radiation alone to a novel 'cytostatic' form of autophagy by the combination of 1,25-D3 or EB 1089 with radiation. Furthermore, we showed that both pharmacological inhibition, employing inhibitors such as chloroquine and bafilomycin, and genetic suppression by silencing autophagy related genes such as Atg5 and Beclin-1 sensitized NSCLC cells to radiation alone, but inhibition of the cytostatic form of autophagy induced by the combination treatment reversed sensitization (manuscript in preparation).

Conclusions

In summary, while it is clear that radiation-induced autophagy can be cytoprotective in non-small cell lung cancer, sensitization to radiation using various novel compounds and strategies is often associated with promotion of autophagy. Given the double (or triple) edged nature of autophagy, an improved understanding of molecular interactions that underline the mechanism(s) by which autophagy functions could ultimately lead to the identification of new targets for both diagnostic and therapeutic approaches in non-small cell lung cancer.

Breast cancer

Current standard treatment

Breast cancer is the second leading cause of cancer-related deaths among women in the United States, with over 39,000 deaths estimated in 2013 (Siegel et al., 2013). Along with surgery and chemotherapy, radiation is a widely used component of cancer therapy and is a fundamental tool in the treatment of breast cancer. Although radiation is an effective modality in the treatment of breast cancer, there is always the likelihood of disease reoccurrence from the dysregulation of cell signaling pathways that promote tumor cell survival (Dent et al., 2003). For instance, chemotherapeutic agents that kill tumor cells primarily through the induction of apoptosis may become less effective as the cell develops strategies to evade apoptosis (Fesick, 2005). Therefore, the possibility of activating alternative cell death pathways holds promise. In recent years, studies have reported the activation of autophagy in tumor cells following anticancer therapies such as radiation and chemotherapy (Paglin et al., 2001; Wilson et al., 2011; Debnath, 2011) as well as nonconventional therapies (Gorka et al., 2005).

Manipulating autophagy as therapeutic strategy

Cytoprotective autophagy and radiation resistance

Recent studies have demonstrated that breast tumor resistance to anticancer therapies, including chemotherapy and radiation, can be enhanced through the upregulation of autophagy (Wilson et al., 2011; Ouyang et al., 2012; Bristol et al., 2012). Increasing evidence suggests that autophagy inhibition augments cytotoxicity in combination with varying anticancer therapies including radiation. A study by Chaachouay et al., (2011) on the role of autophagy in resistance of breast tumor cells to ionizing radiation found that radiation treatment of MDA-MB-231 cells markedly increased the level of LC3-II and thus the process of autophagy as also demonstrated by acridine orange staining of autophagosomes and Western immunoblotting (Chaachouay et al., (2011). Moreover, pretreatment of radioresistant MDA-MB-231 cells with the early stage autophagy inhibitor, 3-methyl adenine or the late stage autophagy inhibitor, chloroquine, significantly reduced clonogenic

survival of irradiated cells (Chaachouay et al., 2011). Similarly, studies in our laboratory found that the autophagy induced by radiation alone was cytoprotective in that pre-treatment with chloroquine or genetic inhibition of Atg5 or Atg7 increased sensitivity to radiation of both MCF-7 and ZR-75-1 breast tumor cells (Wilson et al., 2011; Bristol et al., 2012).

It is well known that autophagy is a multi-step process that appears to be regulated by various signaling pathways (Cao et al., 2006; Botti et al., 2006). Specifically, the ER stress and mTOR signaling pathways have been shown to be activated in response to radiation in various cellular systems (Qin et al., 2010; Hoyer-Hansen and Jaattela, 2007). With regard to radiation induced autophagy in breast cancer, studies by Nagelkerke et al. (2013) suggested that knockdown of the PERK/ATF4/LAMP3-arm of the unfolded protein response (UPR) system as well as chemical inhibition of PERK radiosensitized MDA-MD-231 cells. An analysis of DNA damage repair proteins found that knockdown of LAMP3 attenuated the DNA damage response after radiation. Confocal microscopy found that LAMP3 knockdown resulted in a marked decrease in γ-H2AX foci positive cells post radiation, indicating that the DNA damage repair signaling response is decreased. Although the exact mechanism involving LAMP3 mediated radioresistance is not completely clear, LAMP3 is involved in autophagy while ATF4 is essential for hypoxia-induced autophagy (Rouschop et al., 2010; Rzymski et al., 2010). Also, autophagy has been shown to be necessary for DNA repair as induced DNA damage has been found to trigger autophagy and regulate the removal of DNA repair factors from the cells (Rodriguez-Rocha et al., 2011; Robert et al., 2011). These studies support the premise that interference with autophagy signaling can potentially overcome radioresistance, in part by compromising DNA repair capacity.

Cytotoxic autophagy

While these as well as other studies have found radiation-induced autophagy to be cytoprotective (Ito et al., 2005; Lomonaco et al., 2009), our laboratory has demonstrated that autophagy can likewise have a cytotoxic function in the radiosensitization of breast tumor cells (Wilson et al., 2011; Bristol et al., 2012). Specifically, the hormonally active form of vitamin D in combination with radiation was found to eradicate p53 wild-type, estrogen receptor-positive ZR-75-1 breast tumor cells (Wilson et al., 2011) and MCF-7 breast tumor cells (Bristol et al., 2012) through the promotion of autophagy. In contrast to sensitization that occurs with pharmacological or genetic inhibition of the cytoprotective autophagy induced by radiation alone, inhibition of the autophagy induced by vitamin D + radiation protected the breast tumor cells from the treatment combination (Wilson et al., 2011; Bristol et al., 2012).

Studies by Moretti et al. (2009) also demonstrated that promotion of autophagy can enhance sensitivity to radiation in breast tumor cells. In this report, the Pan-caspase inhibitor, Z-VAD, was shown to sensitize MDA-MB231 cells to radiation both in cell culture and in a tumor xenograft model. Taken together, these studies demonstrate the dual and opposing functions of autophagy in relation to breast tumor cell sensitivity to radiation.

Conclusions

Both cytoprotective and cytotoxic functions of autophagy have been identified in response to radiation in breast cancer, although the cytotoxic functions required the involvement of vitamin D/vitamin D analogs or a blockade to apoptosis. However, the specific signaling pathways mediating these dual functions of radiation induced autophagy have not been resolved. Furthermore, the basis for autophagy-mediated radioresistance in breast tumor cells is not entirely clear, although potential effects on DNA repair capacity are intriguing and may

lead to the development of novel therapeutic approaches for enhancing the efficacy of radiation therapy.

Glioblastoma multiforme

Current standard treatment

The most common primary brain tumor arises from glial cells (termed "gliomas"), with glioblastoma multiforme (GBM) being the most prevalent and aggressive malignant primary brain tumor (Furnari et al., 2007). The median age of GBM diagnosis is approximately 64 years old, with the disease appearing more commonly in men than women and in Caucasians than African-Americans (Ostrom et al., 2013). Approximately 50,000 new cases were diagnosed between 2006-2010 in the United States alone (Ostrom et al., 2013).

GBMs are technically a subtype of astrocytoma, the most common form of glioma (accounting for roughly 75 % of diagnosed cases) (Ostrom et al., 2013). The World Health Organization (WHO) classes astrocytomas into four prognostic grades (I-IV). As the grade increases, so does the aggressiveness of the neoplasm. GBMs, classed as grade IV, are significantly more proliferative and invasive than their grade I-III counterparts (Kleihues and Cavanee, 2000). As such, the current median survival time of GBM patients is a mere 12-15 months, even with aggressive therapy (Stupp et al., 2005).

The first step in GBM treatment involves neurosurgical resection followed by adjuvant chemo- and radiotherapy (Omuro and DeAngelis, 2013). Radiotherapy is typically given as a 60 Gy dose divided into 30 fractions, and the DNA alkylating agent temozolomide is orally administered alongside the radiation treatment (Stupp et al., 2005). Additionally, the FDA has approved another alkylating agent, carmustine, which can be implanted as an impregnated wafer following surgical resection of the tumor (Omuro and DeAngelis, 2013). Recently, bevacizumab (a VEGF monoclonal antibody that works to prevent angiogenesis) has come under investigation as a first-line agent to be used in combination with radiation and temozolomide, but the efficacy of this treatment remains to be demonstrated (Gilbert et al., 2013; Wick et al., 2013). As it stands, there is a clear need for new therapies and strategies exploiting untapped and largely uncharacterized mechanisms in order to better control and ultimately cure this devastating disease.

Autophagy in glioblastoma multiforme

Few studies have been conducted investigating the "natural" function of autophagy in GBM. Several early reports provided evidence that spontaneous autophagy was reduced in GBM (Ito et al., 2005; Kanzawa et al., 2004; Paglin et al., 2001). The first extensive study involving the expression of a key autophagy-regulating protein, Beclin-1, in the context of gliomas was published in 2007 (Miracco et al., 2007). Examining over 200 primary brain tumor samples, Miracco and coworkers found decreasing expression of Beclin-1 with increasing tumor grade. Importantly, Beclin-1 mRNA expression was significantly lower in GBM samples when compared to normal brain tissue extracted from the same patient.

In a follow-up report, they examined cytoplasmic Beclin 1 expression as a prognostic indicator for high-grade gliomas. They found that a high cytoplasmic Beclin-1 score positively correlated with patient survival and improved performance status (Pirtoli et al., 2009). Conversely, a decrease in cytoplasmic Beclin-1 indicated tumors with a higher cell proliferation rate and a decreased incidence of apoptosis. A similar line of investigation found that another marker of autophagy, LC3, was also a prognostic indicator for GBM. High expression of LC3 was likewise predictive of improved survival of GBM patients (with a poor functional performance score) (Aoki et al., 2008). These studies reinforce the observation that autophagy is downregulated in the most advanced forms of gliomas.

Research such as this, and related work done in other cancer types, have given support to the idea that targeting autophagy might open up novel strategies for tumor treatment, especially for diseases like the highly aggressive and lethal GBM (see (Lefranc and Kiss, 2006), for perspective). Several studies have investigated the role current therapies have on the autophagic process in GBM, as well as novel treatments specifically targeting autophagy as their primary mechanism of action. Many laboratories have spent much time searching for unique pathways and targets to radiosensitize high-grade gliomas and thus extend the life expectancy of patients afflicted with GBM. Efforts over the past decade have clearly made a case for the modulation of autophagy as one way in which to enhance current chemo-radiation treatment.

Manipulating autophagy as therapeutic strategy

While the literature examining autophagic markers as prognostic indicators for GBM all seem to agree that a decrease in autophagy implies a more aggressive disease, the work done on targeting autophagy for radiosensitization paints a much murkier picture. GBM cells are known to be particularly radioresistant and the resulting effects of autophagy on these cells after irradiation (IR) is conflicting. Some studies have shown that activation of autophagy after IR leads to cell death, while others have demonstrated that autophagy actually has a cytoprotective role and its activation leads to enhanced cell survival (Zhuang et al., 2009). Specifically, some of the earliest work found that after IR, glioma cells underwent cell cycle arrest and autophagy without subsequent apoptosis (Ito et al., 2005). Treatment with the potent autophagy inhibitors 3-methyladenine (3MA) and bafilomycin A1 resulted in enhanced radiosensitivity. This radiosensitivity was presumably mediated by impaired DNA repair, as more pronounced and prolonged γ-H2AX foci, a marker for DNA double strand breaks, was also noted. These results suggest that, at least in a subset of malignant glioma cells, the inhibition of autophagy could be a viable option for radiosensitization.

A more comprehensive study released in 2012 provides conflicting results (Palumbo et al., 2012). Two different glioma cell lines with known differences in radiosensitivity were used. The more radiosensitive cells, as expected, were susceptible to low- and intermediate-doses of radiation and associated with autophagy activation. siRNA-mediated knockdown of Beclin-1 and ATG-7 abrogated radiosensitivity, either alone or in combination with temozolomide (Palumbo et al., 2012). However, this result was not observed with the more radioresistant glioma cells, in contrast to the earlier report which showed no such radiation dose bias upon pharmacological inhibition of autophagy (Ito et al., 2005). Interestingly, rapamycin-induced autophagy activation not only increased the radiosensitivity of the more radiosensitive glioma cells, as expected, the more radioresistant cells were equally radiosensitized (Palumbo et al., 2012). While this observation could be caused by some sort of off-target effect affecting these two cell lines differently, it supports the notion that autophagy modulation could increase the radiosensitivity of glioma cells. A more likely explanation is that the two glioma cell lines differing in radiosensitivity chosen for this study have other genetic differences that might contribute.

In line with these results, work performed combining high-linear energy transfer (high-LET) radiation with oxaliplatin-induced autophagy and significantly decreased xenografted GBM tumor growth when compared to radiation alone (Benzina et al., 2008). *In vitro*, human GBM cells experienced significantly more DNA DSBs when the combination treatment was delivered. Interestingly, it was shown that exposure to both oxaliplatin and high-LET ra-

diation induced autophagy in a dose-dependent manner. When the glioma cells were transplanted into the flanks of nude mice, the combined treatment with oxaliplatin and radiation significantly decreased tumor growth with no overt signs of toxicity (Benzina et al., 2008). These findings support the idea that autophagy and enhanced radiosensitivity go hand-in-hand.

It is well known that autophagy is chiefly regulated by the mammalian target of rapamycin (mTOR) pathway (Meijer and Codogno, 2004), a pathway which is also known to play a role in radioresistance (Kim et al., 2006). Therefore, it was hypothesized that inhibition of AKT/mTOR signaling could have an anticancer/radiosensitizing effect. To this end, two versions of the same glioma cells were treated with an AKT inhibitor (Fujiwara et al., 2007). Using human malignant glioma cells, as well as a matched radioresistant glioma variant expressing the constitutively active epidermal growth factor receptor variant III (EGFRvIII) resulting in increased AKT signaling, it was demonstrated that AKT inhibition reduced cell viability and activated autophagy, but not apoptosis, in both cell lines. Thus, it was concluded that AKT inhibition radiosensitized both glioma cell lines by enhancing autophagy.

This observation was recently partially corroborated in a study using the dual phosphatidylinositol 3-kinase (PI3K)/mTOR inhibitor NVP-BEZ235, a drug which is currently being evaluated in a phase 1 clinical trial (Cerniglia et al., 2012). Exposure of glioma cells to NVP-BEZ235 significantly increased radiosensitivity. However, knockdown of AKT1, p110α, or mTOR, while still demonstrating increased radiosensitivity, was not able to show the same degree of response seen with the drug-treated cells. Additional experiments suggested that NVP-BEZ235 also interfered with DNA repair, providing a possible mechanism for the underlying difference. Interestingly, while NVP-BEZ235 treatment induced autophagy and radiosensitized cells, these effects occur by distinct mechanisms. When autophagy inhibitors were added to the NVP-BEZ235 and radiation combination, significantly more radiosensitization/cell killing was recorded, suggesting that, at least with this pharmacological agent, the induction of autophagy was actually cytoprotective (Cerniglia et al., 2012). It should be noted, however, that these experiments were conducted only in head and neck squamous cell carcinoma cells (the other line used in the study), so the effect of NVP-BEZ235 on glioma cells could be different and thus remains unclear. Furthermore, off-target effects on NVP-BEZ235 cannot be ruled out, and equally possible, less than 100 % knockdown of these key players could also affect the interpretation of these findings.

An expanding body of literature indicates that glioma stem cells (GSCs) identified as the CD133+ subpopulation of cells within the GBM tumor mass is actually responsible for the initiation and growth of the malignancy. As these GSCs have been implicated as the underlying cause of radioresistance (Bao et al., 2006; Beier et al., 2007), it would be of great interest to target this type of cells to enhance the efficacy of radiotherapy. One study reported that CD133+ GSCs induced autophagy after radiation and these cells could be radiosensitized by inhibiting autophagy (Lomonaco et al., 2009). It was noted that CD133+ GSCs expressed higher levels of the autophagy-associated proteins ATG-5, ATG-12, and LC3. The use of bafilomycin A1 or shRNA-mediated knockdown of ATG-5 and Beclin-1 resulted in significant radiosensitization of the CD133+ population. Therefore, it was concluded that the inhibition of autophagy could be an effective strategy to induce radiosensitivity in GSCs.

However, this finding is also contested. A subsequent study found that transient exposure of GSCs to rapamycin induced cellular differentiation and significantly increased radiosensitivity by activating autophagy (Zhuang et al., 2011). *In vitro*, the

combination of radiation and rapamycin treatment prevented the formation of intracranial GBMs after GSCs were inoculated into nude mice. Additional *in vivo* experiments demonstrated that the rapamycin and radiation regimen reduced tumor growth and increased the survival of mice. Finally, isolated GSCs exposed to rapamycin and radiation showed reduced viability and clonogenicity. These effects were all abolished when GSCs were triple-treated with rapamycin, radiation, and 3MA, indicating that the rapamycin effect was indeed due to autophagy induction (Zhuang et al., 2011). This work suggests that autophagy plays a key role in the regulation of the differentiation and radiosensitivity of GSCs, though in contrast to previous work, it infers that the induction of autophagy, not the inhibition, mediates these outcomes.

Conclusions

GBM is a highly aggressive and fatal disease. While astounding strides have been made for other cancer types in terms of improved survival and quality of life, glioma patients still lag significantly behind. Novel therapeutic strategies desperately need to be brought to the clinic to combat this devastating malignancy.

The role of autophagy in neoplastic progression, and the response to various anticancer agents and treatments, remains multifaceted and unclear. On the one hand, autophagy provides a pathway by which cancer cells can "clean out" and recycle damaged and possibly deleterious organelles and proteins. On the other hand, the induction of autophagy opens up an avenue by which cell death can occur in the face of defective apoptosis. In some patients, in certain contexts, the induction or inhibition of autophagy could therefore be used to enhance existing treatments, especially radiotherapy. The work reviewed in this section illustrates this point – for seemingly every study which suggests that inhibition of autophagy radiosensitizes glioma cells, another group releases a similar report with a conflicting result. It is clear that these types of experiments need to be carried out under explicitly defined conditions using isogenic cells differing in only one gene and fundamental characteristic.

The contextual importance of GBM anti-neoplastic therapy is not limited to autophagy. For example, recent work has demonstrated that the *in vivo* inhibition of ataxia-telangiectasia mutated (ATM), a kinase critically involved in the DNA damage response (Valerie and Povirk, 2003), was most successful in radiosensitizing human glioma cells with a mutant p53 background (Biddlestone-Thorpe et al., 2013). It is well established that classic autophagy is p53-mediated (Green and Kroemer, 2009; Kenzelmann Broz et al., 2013; Tasdemir et al., 2008). Therefore, if gliomas with mutant p53 undergo radiosensitization and apoptosis more readily than wild type cells it is likely that, under these circumstances, p53 acts to preserve cellular integrity and survival through an autophagic process. As p53 wild type cells are, by some accounts, more prone to undergo autophagy, it can be interpreted that the induction of autophagy in gliomas is functioning in a cytoprotective manner. Regardless, the differential p53 response to ATM inhibition opens up an excellent therapeutic opportunity for targeting gliomas in a cancer-specific manner while protecting normal brain.

A growing body of literature has made it clear that there is no "one size fits all" strategy by which we can hope to effectively treat all cases of GBM. Rather, it will be important to develop patient-specific treatment plans which involve manipulating autophagy or other cellular processes to maximize therapeutic efficacy and patient survival. Though much work remains to be done, it is evident that autophagy modulation offers a promising, novel approach to GBM treatment.

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