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Low Dose Ionizing Radiation Responses and Knockdown of ATM Kinase Activity in Glioma Stem Cells

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Genesis of new cells in the mammalian brain has previously been regarded as a negligible event; an assumption that long limited our understanding in the development of neoplasias. The recent discovery of perpetual lineages derived from neural stem cells has resulted in a new approach to studying the cellular behaviour of potential cancer stem cells in the brain. Glioblastoma multiforme (GBM), the most aggressive and lethal brain tumour is derived from a group of cancerous stem cells known as glioma stem cells. GBM cells are impervious to conventional therapies such as surgical resection and ionizing radiation because of their pluripotent and radioresistant properties. Thus in our study, we aim to investigate whether a combination of chemo- and radio- therapies is an effective treatment for glioma stem cells. The study utilizes a specific kinase inhibitor (ATMi) of the ATM (Ataxia-telangiectasia mutated) protein which is an essential protein in DNA-damage responses. In the presence of both low dose radiation and ATMi, glioma stem cells have rapid onset of cell death and reduction in growth. Since DNA damage can be inherited through cell division, accumulated DNA breaks in later generations may also lead to cell death. The limitation of conventional radiation therapy is that administration of fractionated (low) doses to reduce any potential harm to the surrounding healthy cells in the brain outweighs the benefits of high radiation doses to induce actual arrest in the propagation of malignant cells. Our study demonstrates a benefit in using low dose radiation combined with chemotherapy resulting in a reduction in malignancy of glioma stem cells.

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Introduction

As evolution occurred slowly through time, individual cells began to assemble into multi-cellular colonies. The advantages awarded to a community of cells are often greater than those for an individual. Survival and reproduction are part of the communal benefits. Similarly, a parallel can be drawn to single cancer cell in the brain. Its presence as a singular entity in the bustling metropolis of normal neuronal cells may seem harmless and even insignificant at the beginning. But once the cell 'plants its root', a community of cancerous cells arises. The resulting brain tumour is inevitable, in addition, neighbouring healthy cells undergo continuous cellular necrotic insult through hypoxia and nutrient deprivation. Subtle clinical signs and symptoms may begin to manifest as fewer functional neuronal cells are present. Altered personality traits, chronic throbbing headaches and weakness in the extremities are the signs of a brain tumour. However, such clinical features are ambiguous, especially in the early stages of tumour formation and can be mistaken for other neurological disorders. Delay in diagnosis can reduce a patient's median survival time as much as the lack of therapeutic treatment. The current mainstay for brain tumour therapy is surgical resection and radiation (to induce DNA damage and subsequent death of any remaining tumour cells). This combination normally increases survival by an additional 3-9 months.[1] Statistically, the patients will eventually fall victim to the brain tumour. Magnetic resonance imaging (MRI) scans have revealed that poor patient survival is predominantly due to the rapid recurrence of tumour growth at the site of resection rather than additional *de novo* tumour formation in other parts of the brain.[2] This raises the question – How do cancer cells continue to proliferate rapidly despite the removal of an entire tumour (or nearly entire

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tumour) combined with post-operative therapies that induce cell death and should decrease the rate of tumour growth.

Shortcomings of conventional therapies

The reasons for brain tumour recurrence can be outlined in two parts: Firstly, tumours are resistant to radiation therapy, thereby rapidly regaining population size. The progressive accumulation of mutations and epigenetic silencing of key regulatory genes may be a source of radiation-resistance within cancerous brain cells limiting the effects of IR-induced DNA damage responses.[3] Secondly, recent advances have identified a small population of cancer stem cells (CSCs) that has the ability to generate a large number of differentiated progenies, while maintaining its pluripotent form.[4] The failure of traditional therapy to remove CSCs can be attributed to the use of typical cytotoxic drugs that often target the progeny of cancer stem cells which have a higher proliferative index while sparing tumourigenic stem cells with a lower proliferative rate. This demonstrates that the limited understanding of the mechanisms involved in brain tumour initiation, may have led to the unchanged median survival of patients for the past decades as the primary causes of the tumour were unaffected by treatment.[5] Advances in diagnostic tools in recent decades have markedly improved the resolution of computer imaging (eg: MRI, radioisotope imaging, etc) which has lead to better identification of brain tumour diseases. In the United States alone, more than 13,000 deaths are related to brain tumours diseases with ~18,000 new cases being diagnosed annually.[6] Brain tumour related disease is also the second most common childhood cancer.[7,8] The principle hurdle in formulating an effective therapy may lie in targeting these brain tumour stem cells (BTSCs). As a model, we chose the most malignant and common form of brain tumour; Glioblastoma multiforme (GBM) to characterize the molecular properties of glioma stem cells.

Glioblastoma multiforme stem cells

The World Health Organisation (WHO) classification of nervous system tumours has been the gold standard for defining the malignancy of brain tumours by employing a graded system (Table 1).[9] Amongst the various tumours listed, grade IV gliomas or Glioblastoma Multiforme (GBM) accounts for the majority of primary tumours (~77%), and other known (~20%) intracranial tumours cases. Patients that undergo surgical resection have an ~90% chance of recurrent tumour formation. Survival statistics also reflect this with only 40% of patients surviving for the first 6 months post-treatment and only 3.3% surviving after two years.[2] Recent studies support a role of BTSCs in the progression of GBM.[10] This hypothesis postulates that the recurrent tumour growth results from a rare subset of cells that have the capacity to support development and progression of neoplasm. When neural- and glioma-extracted stem cells are compared, only the latter has the capacity to initiate tumour growth when transplanted into an animal model.[11,12] A further feature that supports post-operative stem cell dependent tumour initiation is the determination of the cellular origin of medulloblastoma in a mouse model. When neural stem cells received a deletion in the Patched (Ptc) gene, abnormal stem cells begin to increase their proliferation rate within the ventricular zone (VZ). Later, these Ptc deficient neural cells can be observed migrating towards the cerebellum – the site where medulloblastoma is initiated.[13] Although there is no clear mechanism for the homing of 'medulloblastoma stem cells', this suggests that the source of glioma stem cells may be different from the site of tumour initiation.[14] Potentially, glioma stem cells can also originate in a similar region to 'medulloblastoma stem cells' (eg: subgranular zone) where asymmetrical cell division generates CSC progenies that migrate to other regions and initiate tumour growth.[14] If this hypothesis holds true, then the elimination of glioma stem cells will have a general effect in reducing the recurrence of tumour growth.

Table 1. World Health Organisation (WHO) classification of brain tumours.
Different types of neuroepithelial origin tumours have been classified by WHO according to the cell types and genetic aberrations that predominate in the tumour mass.[9] GBM is the most aggressive form of brain tumour with the lowest survival rate. Abbreviations in table, (OE) Overexpressed, (amp) amplified, (mut) mutated.

Types of astrocytic tumours	General onset of disease	Median survival	Genetic distinction	Pathology
Grade I Pilocytic Astrocytomas	< 40 years of age, common in children and in young adults	8-10 years	No obvious genetic alteration	Begin tumour with loose textured astrocytes
Grade II Diffuse Astrocytomas	< 45 years of age, more common in young to middle-aged adults	5-10 years	p53 mut, PDGF/R OE	Low mitotic index, Infiltrating astrocyte
Grade III Anaplastic Astrocytomas	< 45 years of age	2-3 years	RB mut, CDK4/6 amp, PTEN loss, DMBT1/mxi loss, 19q loss, 11p loss	Increase proliferation, diffuse invasion, angiogensis
Grade IV Glioblastoma Multiforme	> 45 years of age, frequent in fifth decade of life but occassionally occurs in the brain stem of children	6-12 months	PTEN mut/OE, EGER amp/OE/mut, MDM2 OE amp/OE, p53 mut, p16, CyclicD1/3 amp/OE, RB mut, INK4a/ARF loss and 10q loss	Rapid proliferation, diffuse invasion, angiogenesis, necrosis

The role of glioma stem cells in response to radiation therapy

In response to radiation therapy, CSCs show increased proliferation and reduced cell death when compared to differentiated cells. CSC survival may be due to an altered DNA damage response and subsequent cell-cycle regulation. Supporting evidence exists from Bao *et al* who observed that glioma stem cells have significantly increased phosphorylation of Rad17, Chk1 and Chk2 proteins.[15] The latter proteins (Chk1 and Chk2) halt cell-cycle progression to facilitate DNA damage-response proteins relocating to the site of damage. For instance, in the event of a G2/M arrest, sensor protein, Rad17 is able to recruit the DNA-response complex (Rad1, Rad9 and Hus1) to assist in the repair of DNA lesions.[16] Although Rad1, Rad9 and Hus1 proteins are functionally different and activation occurs in defined sections of the cell-cycle, biochemical analysis demonstrates that the activity of these proteins is mainly controlled by a member of the PIKK (Phosphatidylinositol-3 kinase-like) family; the ATM protein.[16-18] The increased phosphorylation of these proteins in CSC may be due to a lack of negative feedback loop to limit the response to damaged DNA, or chronic activation of the initiator kinase (ATM). Consequently, further investigation into the mechanism underlying the dominant activation of signalling in glioma stem cells is required. ATM is essential for the DNA double strand break response and its activation orchestrates several cellular processes such as regulation of apoptosis, cell-cycle checkpoint activation and DNA repair. Modulation of ATM activity may prove to be a starting point to delineate the cause of radioresistance in glioma stem cells. In this study, we focus on the role of ATM in the DNA damage response in GBM and the potential use of ATM inhibitors to increase the GBM response to low dose radiation.

Materials and methods

Established glioma stem cell and lymphoblastoid cell lines

Grade IV GBM neurosphere (NS) line – U-251 was a kind gift provided by Dr Bryan Day. To generate neurospheres, U-251 cells were maintained in NS-media containing NeuroCult® (StemCell Technologies Inc), 20ng/ml recombinant human Epidermal Growth Factor (R&D Systems), 10ng/ml recombinant human Fibroblast Growth Factor (R&D Systems) and 1unit/ml of Heparin (Sigma). For passaging to generate new neurospheres, cells were chemically dissociated using trypsin and replated in a T-25 flask at a concentration of 1 x 10^5 cells per ml. A-T deficient human lymphoblastoid cell line (AT1ABR) and normal human lymphoblastoid cell line (C3ABR) were cultured as suspension cells in RPMI1640 medium supplemented with 6% v/v of fetal calf serum and penicillin/streptomycin.

Ionizing radiation exposure

All cultures were growth to 70%-80% confluency before being subjected to radiation in a GammaCell^{40}Exactor (Best Theratronics Ltd.) with an exposure rate of 960cGy/min. Where indicated ATMi[19] was added to media 2hrs prior to irradiation. Treated cells were harvested at indicated times and trypsinised (only U-251) before fixing with 1% of PFA for 15mins on ice and stored at -20°C with 70% ethanol

Cell proliferation

Growth of neurosphere U-251 cells was observed using a 40x objective microscope (Olympus Ckx41). Photographs of neurosphere cultures with different treatments were taken at indicated time points using an attached digital CCD colour camera (QImaging micropublisher 3.3). An average of 30 neurospheres was photographed from each culture. Neurospheres that resembled clusters of cells rather than true spheres were excluded from analysis.[20] Acquired photographs were then analysed separately on a PC computer for size distribution of spheres using Q-Capture Suite Software (QImaging).

Cell-cycle distribution

All cultures were collected at 16hrs, trypsinised (only U-251), fixed and stored at -20°C as mentioned above. Cells were incubated with Propidum iodide (50ug/ml) (Sigma) and RNAse A (Roche) (100ug/ml) for 30mins before cell-cycle profiles were quantified using a FACScan™ (BD Biosciences). Analysis was performed using ModFit LT™ (BectonDickinson). Histograms are representative of two independent experiments.

RESULTS

Establishing of glioma stem cells culture

The neurosphere culture is based on the fact that stem cells are able to survive in the presence of mitogenic agents but the media does not support the survival of differentiated cells. Tumour was acquired from a GBM diagnosed patient (Figure 1a). Upon removal of the tumour, extensive washes were conducted to remove any excess haemoglobin. The tumour underwent mechanical dissociation to expose the tumour cells to subsequent trypsinization. A heterogeneous population of cells released from the tumour were later separated from tissue debris using a cell strainer. Collected cells were growth in serum-free NS-media containing the appropriate mitogenic agents. After 8-10 weeks of selective growth, only pure stem cells remained.

Proliferation of glioma stem cells after radiation

To date, studies regarding the use of ATM inhibitor to examine radiosensitivity have relied heavily on the use of non-specific PIKK inhibitors such as wortmannin and caffeine to understand the molecular mechanism of the DNA-damage response to radiation.[21] Although such an approach often displayed increase radiosensitivity, data obtained from these studies could raise concern over its specificity as other members of the PIKK family are also inhibited (eg: ATR, DNA-PK, etc.).[21,22] Thus, the use of non-specific PIKK inhibitors is

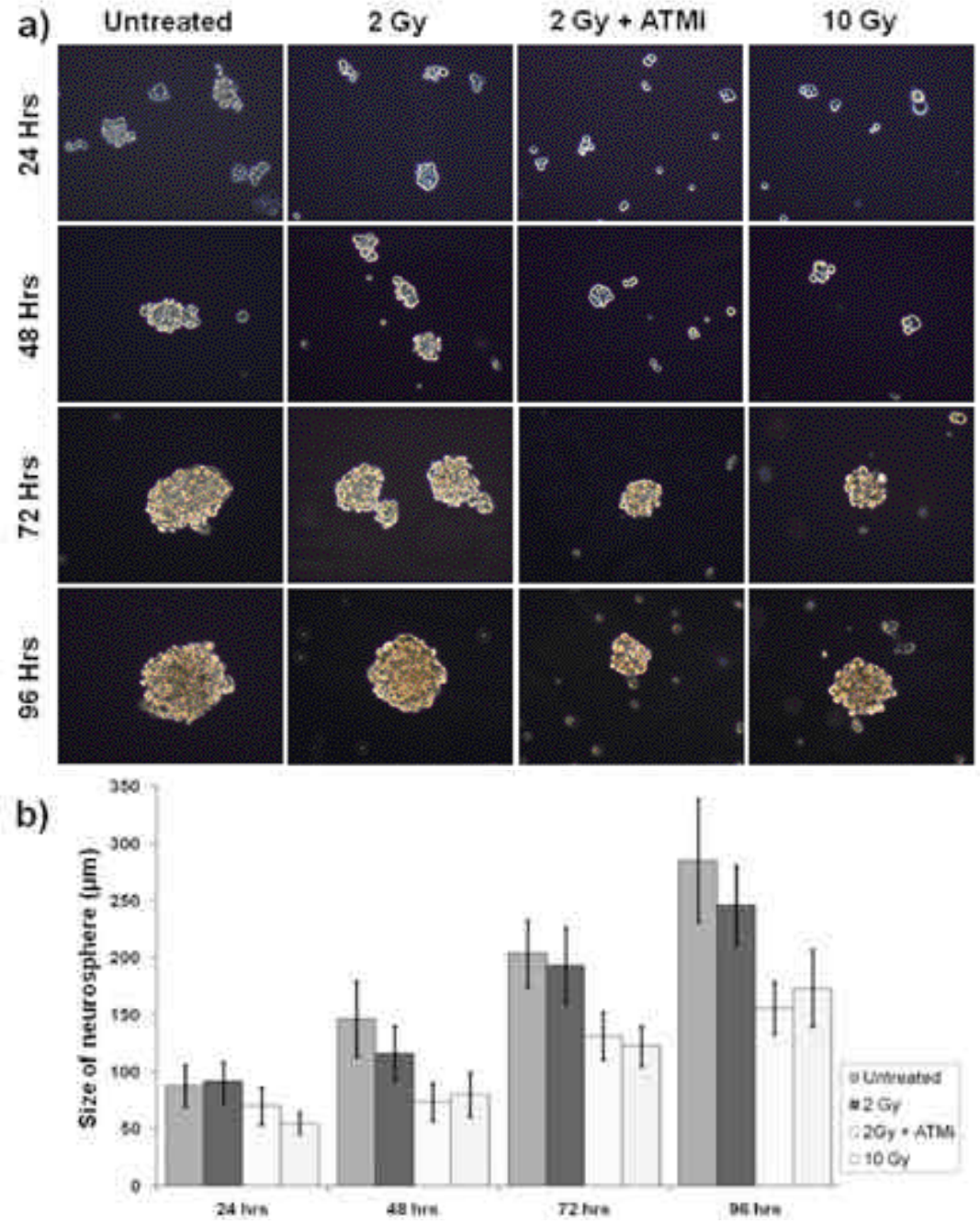

Figure 1. ATMi exacerbates growth retardation of neurospheres in response to low dose IR.
Neurospheres were exposed to the indicated dose of IR. Where indicated cultures were treated with 5µM of ATMi for 2hr prior to IR treatment. **a)** Cultures were photographed at the indicated time points after exposure to IR. The single cells suspension began to produce spheres after 24hrs in all cultures. Differences in the size of spheroids formed between cultures could be identified from 48hrs. **b)** Average neurosphere sizes were determined by Q-Capture Suite. Both untreated and 2Gy treated neurosphere cultures were capable of forming regular spheres whereas 10Gy and 2Gy with ATMi cultures had reduced spheroid formation. Columns show the average size and error bars represent the standard deviation of neurosphere diameter in each culture.

ineffective in identifying novel therapeutic approaches as these studies only partially identified the IR-induced signalling pathways. This lack of molecular tools is one of the reasons why the cause of radioresistance of GBM has been difficult to elucidate.[23] However, with the identification of a specific kinase inhibitor of ATM (ATMi),[19] we can now study the molecular pathways involved in GBM radioresistance in greater detail. In an initial experiment, we examined the growth response of glioma stem cells in the presence of a therapeutically relevant IR dose of 2Gy, followed by the comparison of ATMi in combination with low dose radiation (2Gy) and high dose radiation (10 Gy) alone.

When U-251 neurospheres were exposed to 2Gy of IR, growth was

similar to the untreated neurosphere culture during the first 24hrs. However, a reduction in neurosphere size and number at 48hrs was slightly more obvious in irradiated neurosphere cultures (Figure 1a).[24] The reduction in growth may be due to activation of cell-cycle checkpoints and damage repair pathways. By 96hrs, spheroids formed in the irradiated (2Gy) neurosphere culture (~246um) and untreated (~285um) were a similar size, demonstrating the survival of glioma stem cells in response to low dose IR.

Further comparisons, indicated that neurosphere cultures that were treated with ATMi prior to IR had a significant reduction in spheroid formation. At 48hrs, 2Gy induced IR alone neurospheres had an average diameter of >120μm in size whereas those treated with a combination of ATMi and IR were smaller than ~75μm (Figure 1b). Interestingly, the neurosphere culture that was treated with 10Gy of IR exhibited a similar growth reduction to the culture given the combination of 2Gy IR and ATMi, displaying an average diameter of ~80μm at 48hrs. By 96hrs, spheroid size in both cultures, 10Gy (~173μm) and 2Gy with ATMi (~156μm) were ~1.7 fold smaller than 2Gy IR alone-treated neurosphere cultures (~246μm). This data strongly suggests that radiosensitivity could be induced in glioma stem cells by inhibition of ATM kinase activity. In fact, inhibition of ATM activity allowed a 2Gy dose to appear to be equivalently as lethal to GBM as a 10Gy dose. The data also indicated that ATM may be required for radioresistance in neurospheres.

Effect of ATMi on the cell-cycle distribution of glioma stem cells

One of the hallmarks of A-T deficient cells is the extended delay in G2 phase after irradiation.[25] This delay relies on alternate (ATM-independent) signalling pathways to arrest cells at the G2 cell-cycle checkpoint.[26] Corresponding with reduced neurosphere formation, we attempted to delineate the cell-cycle distribution of glioma stem cells in response to both ATMi and irradiation. Three asynchronous cell types (AT1ABR; A-T deficient lymphoblastoid cell, C3ABR: control lymphoblastoid cell and U-251: glioma neurosphere) were compared following irradiation with or without ATMi pre-treatment. Cultures were treated with 2Gy of IR before harvesting at 16hrs (Figure 2).[19] When exposed to radiation, AT1ABR had a prominent shift to G2 (61.85%) accumulation as compared to G1 phase (13.69%). Conversely, IR treatment of C3ABR cells and U-251 neurospheres resulted in equal numbers of cells in G1 and G2 phase (Figure 2), suggesting normal ATM activation which leads to the phosphorylation of Chk2. However, the inclusion of ATMi prior to IR significantly increased the accumulation of cells in G2 in both C3ABR and U-251 samples.

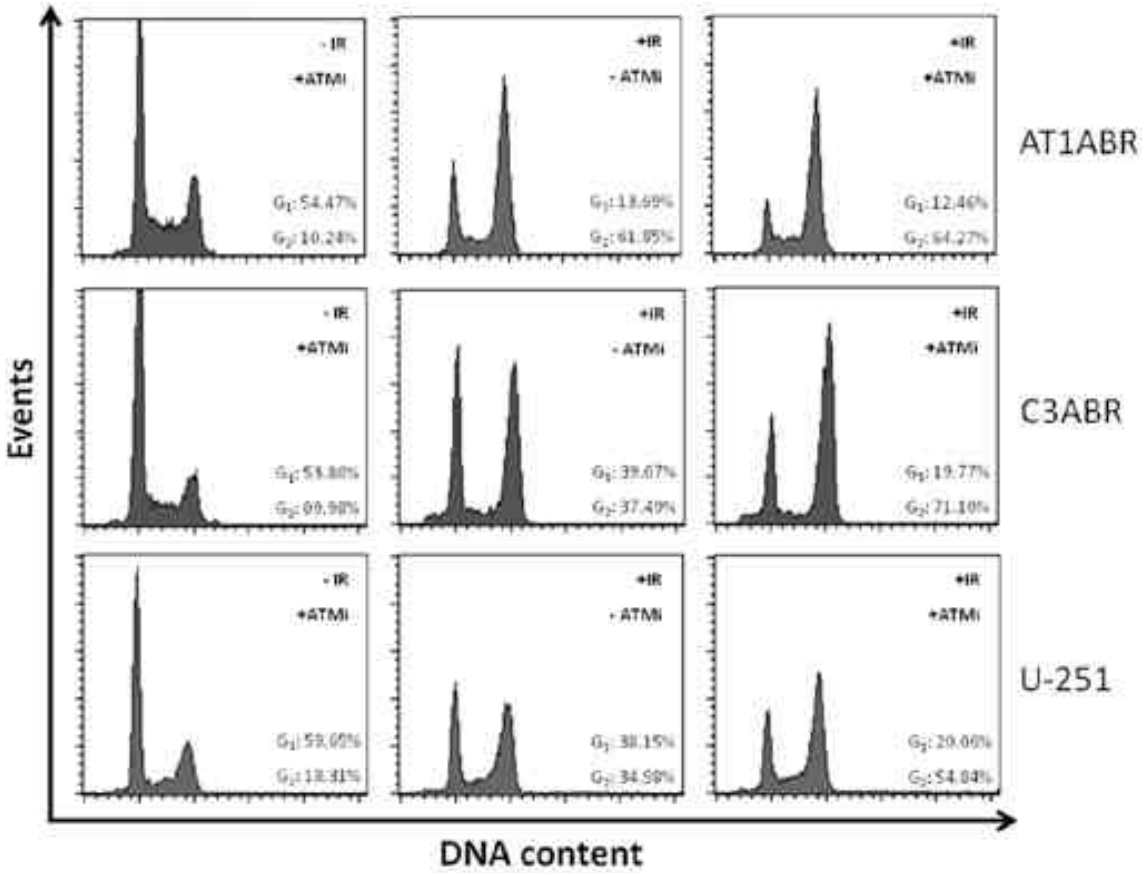

Figure 2. Analysis of cell-cycle.
All cell types; AT1ABR (A-T deficient), C3ABR (normal) and U-251 (glioma) neurospheres were treated with or without 2Gy IR. Where indicated, cells were treated with 5μM of ATMi for 2hr prior to IR treatment. Cells were harvested 16hrs post-irradiation and the cell cycle profile determined by propidium iodide staining and flow cytometry.

The increased in G2 accumulation was consistent with the data from radiosensitive AT1ABR cells. ATMi specificity was confirmed by, 1) the lack of cell-cycle shift in cells that were not exposed to IR, and 2) no change in G2 accumulation in AT1ABR (AT deficient) cells when exposed to IR in the presence or absence of ATMi.

Discussion

To date, the lack of molecular tools for understanding the mechanisms of GBM response to radiotherapy has prevented therapeutic endeavours aimed at improving patient survival.[15,23] In our study, the use of a specific ATM kinase inhibitor provides data showing that ATM activity may be crucial for the survival and radioresistance of glioma stem cells. This is exemplified by the increase in G2 accumulation in response to IR. Absence of ATM kinase activity prevents rapid ATM-dependent phosphorylation of Chk2 therefore arrest of cells at G2 phase requires alternate signalling via the ATR-Chk1 dependent pathway.[26,27] Response through this pathway occurs only at later time points (5-24hrs),[28] once activated, ATR-Chk1 dependent phosphorylation of CDC25 occurs. ^{P}CDC25 then interacts with 14-3-3 protein, translocating to the nucleus and preventing dephosphorylation of CDC2, causing G2 cell-cycle arrest.[26] Compensation for a lack of ATM activation via increased ATR-dependent signalling could manifest as an extended delay in G2 arrest observed in ATMi treated cells. However, the advantage of employing ATM kinase inhibition during IR induction is the lack of early arrest in G2 phase (1-4hr) (Data not shown).[28] Glioma stem cells that have received damage to their DNA in G2 phase can contribute significant chromosome breakage/instability to its next generation of stem cells when they exit the mitotic phase without any G2 arrest.[29-31] However, damaged glioma stem cells that are driven into the G1 phase may allow activation of p53 pathway that can induce cells to undergo either cell death, through the apoptosis pathway, or downstream phosphorylation of p21 resulting in G1 accumulation.[32] In the latter process, DNA can then be repaired by initiation of NHEJ (Non-homologous end joining) prior to DNA synthesis.[26,33] Data obtained from the growth of neurospheres in response to IR alone does suggest that glioma stem cells may be inclined to rapid DNA-damage repair when ATM is present. For example, a comparison of IR treated (2Gy) and untreated neurosphere culture does not show any significant difference in spheroid formation. The importance of ATM activity to glioma stem cell survival is shown in (Figure 1b) where addition of ATMi to cells treated with 2Gy IR could severely limit cell growth compared to 2Gy of IR alone.[34]

GBM radioresistance may be due to altered signalling pathways that have been found during genomic studies of glioma tumours. Out of 206 GBM extracted tumours, ~88% had an altered PI(3)K signalling pathway, which could result in the dysregulation of the downstream activation cascade including the p53, p16 and AKT pathways.[35] ATM can activate more than 900 different phosphorylation sites in 700 different proteins, a large proportion (48%) of which are related to DNA replication, recombination and repair.[36]

To confirm these findings we plan to examine the effect of ATMi pre-treatment in combination with low dose IR on DNA damage responsive signalling pathways, apoptosis, rate of DNA repair and cellular proliferation.

In conclusion, data from the growth response of neurosphere culture treated with ATMi and low dose radiation showed significant reduction in growth, and a delay in G2 cell-cycle arrest providing us with preliminary results that suggest ATM-dependent signalling is important in glioma stem cell survival. The removal of ATM kinase activity and reduction of the resulting DNA damage and repair signalling cascades had a significant impact on the radiation dose response curve of glioma stem cells. In our studies, administration of ATMi to cells treated with 2Gy IR resulted in cellular responses similar to cells treated with 10Gy of IR. Therefore, inhibition of ATM offers a potential platform for formulating effective therapeutic interventions to be used in conjunction with current conventional therapy.

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