Dexamethasone interferes with Autophagy in Irradiated Malignant Glioma Cells

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ABSTRACT

Dexamethasone interferes with Autophagy in Irradiated Malignant Glioma Cells

Autophagy is either cyto-cidal or cyto-protective to many tumors and study has shown that radiation induces autophagy in malignant glioma cells (Gewirtz AD, 2013, 2014)

Dexamethasone is frequently used to reduce tumor associated brain edema especially during radiation therapy. However, neither the effect of dexamethasone on autophagy or intervening molecular pathways in radiated malignant glioma cells is studied. The purpose of the study was to determine whether and how dexamethasone affects autophagy in irradiated malignant glioma cells

We prepared U373 and LN229 glioma cell lines, of which have different PTEN mutational status and made U373 stable transfected cells expressing GFP_LC3 protein. After performing cell survival assay after irradiation, IC_{50} radiation dose was determined. Dexamethasone dose (10 μM) was determined from the literature, and added to the glioma cells 24 hours before the irradiation. Difference of cell survival by adding-on dexamethasone was evaluated by both cell survival assay and cell cycle analysis. Measurement of

autophagy was visualized by western blot of LC3-Ib/LC3-IIb activation and quantified by both GFP_LC3 punctuated pattern of fluorescence microscopy and acridine orange staining for acidic vesicle organelles by flow cytometry

Dexamethasone was protective and reduced cell death after irradiation hence resulted to increased cell viability in both U373 and LN229. It interfered with autophagy in both U373 and LN229 depending on the PTEN mutational status as the autophagy is decreased in U373 (PTEN mutated) but increased in LN229 (PTEN wild type). Inhibition of autophagy by selective phospho-Akt inhibitor, Ly294002 repressed the action of dexamethasone significantly in PTEN-mt cells leading to increased autophagy and cell death, while in LN229 there was no significant change in both cell death and autophagy level. After autophagy knockdown, dexamethasone decreased cell death in both MG cells. In conclusion, the action of dexamethasone interfered with autophagy and increased cell viability in irradiated malignant glioma cells. This action could be mediated through dysregulation of PI3K/akt/mTOR signaling pathway especially in PTEN-mt, and use of Ly294002 inhibitor could improve treatment in patients with PTEN mutation

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1. Introduction

Glioblastoma multiforme (GBM) is an aggressive primary brain tumors and presents with continuous local tumor growth as well as a tendency for spreading through brain parenchyma. Currently, the standard therapy for GBM includes maximal surgical resection followed by concomitant chemo-radiotherapy with temozolomide (Stupp R, 2005, Davis ME, 2016). As stated, radiotherapy is one of the three (3) main treatment modalities for malignant glioma (MG) treatment used to shrink the tumor or kill residual cancer cells after surgical resection (Baskar R, 2014). Almost all MGs recur in a more aggressive way and there is no standard of care for recurrent GBM (Kewal KJ, 2018). Resistance of the glioma cells to radiation has been one of the limitations to the success of treatment and dose related adverse events preclude to re-radiation in case of recurrent GBM.

A glucocorticoid, dexamethasone has been used for decades in the treatment of GBM to reduce tumor associated cerebral edema especially during radiation therapy (Shields LB, 2015). It is frequently used before and after radiotherpay in order to alleviate the side effects such as nausea and vomiting (Das A, 2004). However, the effect of dexamethasone in cancer cell growth and patient's survival remains contentious, as many

studies have reported that it protects cancer cells from radiation and chemotherapy (Pitter LK, 2016). Glucocorticoid is also well-known catabolic inducer as it frequently provokes steroid-induced diabetes mellitus (DM) as side effect.

Autophagy (literally meaning 'self-eating') is a vital catabolic mechanism where cells digest and recycle their own organelles for supplying energy and maintaining cellular homeostasis especially during starvation, growth factor deprivation, pathogen infections and endoplasmic reticulum stress (Moretti L, 2007, Gustafsson AB, 2008).

Thus, it is not surprising that dexamethasone affects autophagy in many cancer cell lines, which requires high energy consumption for rapid proliferation (Troncoso R, 2014)

Autophagy has been known to play both cytotoxic and cyto-protective roles in cancer development and progression via various signal pathways in determining the response of tumor cells to anticancer therapy (Hippert MM, 2006, Sharma K, 2014, and Tam S, 2017). Radiation-induced autophagy has been observed in almost every tumor types and attracted particular interest (Wilson EN, 2011, Classen F, 2016 and Wang L, 2018). However, whether radiation-induced autophagy triggers tumor cell survival or cell death remains unclear. As apoptosis was rarely observed in glioma cells after irradiation, the role of autophagy in programmed cell

death after irradiation has been suggested to be a responsible way to cell death (Gwak HS, 2015).

The exact mechanisms underlying glioma cell radio-resistance not well studied although many researchers believe to be associated with DNA repair defects due to a combination of mechanisms. These factors include gene mutations, epigenetic events, and alterations in signal pathways such as Epidermal growth factor receptor (EGFR) and phosphoinositide 3-kinase (PI3K)/ protein kinase B (PKB, also known as AKT) (Willers H, 2013). Akt promotes cell survival by inhibiting apoptosis through phosphorylation and inactivation of several targets.

Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN) suppresses the PI3K/Akt/mTOR pathway and is frequently mutated in glioblastoma and encodes a dual specificity phosphatase that impairs the PI3K/AKT/mechanistic target of rapamycin (mTOR) pathway, which is a key regulator of autophagy (Song M, 2012, Erraffiy R, 2013).

PI3K/Akt pathway plays an important role in cancer development and dysregulation of this pathway through loss of PTEN repression is a common occurrence in many aggressive cancers including malignant gliomas (Nowak D, 2016).

In this study, we try to evaluate how the catabolic effect of dexamethasone affects radiation-induced autophagy and related cell death in malignant glioma cells. In our study, we used two glioma cell lines of different PTEN functional status (U373 of PTEN-mt and LN229 of PTEN-wt) to explore how dexamethasone affected autophagy according to PTEN mutational status.

2. Materials and Methods

2.1 Cell culture and irradiation

Human glioblastoma cell lines, LN229 and U373 were obtained from American Type Culture Collection (ATCC). Cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% of penicillin/ streptomycin at 37°C, 5% CO2. Cells were treated with 10 μM dexamethasone sodium, and irradiated with a Gammacell 1000 Elite Cesium^{137} source (MDS Nordion, Ottawa, ON, Canada) at 24 hours after plating for a calculated dose by dose rate of 0.0416 Gy/second.

2.2 RNA interference

To suppress autophagosome formation, we knocked down autophagy related 5 (ATG5) protein. Knockdown of ATG5(Human) expression was

done using small interfering RNA (siRNA) and a negative control bought from Ambion (life technologies, USA) transfected into the glioma cells using lipofectamine 2000 reagent (Invitrogen, USA) in accordance with the manufacturer's instruction and 24 hours later cells were used in the experiment**.**

2.3 Cell survival assay

Two methods of cell viability were employed, cell-counting using automated cell counter after trypan blue staining and clonogenic assay. During survival assay, cells were plated on 60 mm culture plates (2×10^3 cells), after 24 hours 10 μM dexamethasone added, then 24 hours later subjected to different doses of radiation 0 Gy, 2 Gy, 5 Gy, 10 Gy, 15 Gy and 20 Gy. Cells were then incubated at 37°C with regular media changes for 8-10 days until control is 100% confluent. Cells were washed twice with DPBS, trypsinized with 1 ml trypsin-EDTA, stained with trypan blue and counted using automated cell counter. The percentage of viable cells calculated from the ratio of treated cells to normal control cells. Three (3) independent experiments were carried out and the mean determined.

For colony forming Assay, cells were plated at clonogenic density (1.0×10^3) in a 60 mm culture plate, and then treated with dexamethasone (10 μM) and 10 Gy radiation dose as per the experiment setup. Cells were

incubated for 8-10 days with regular media changes after treatment. Cells were fixed with 4% paraformaldehyde for 20 minutes, then stained with 0.4% crystal violet for 20 minutes, washed under running distilled water and allowed to dry for 2 hours. A cluster of 50 cells and more was scored as a colony.

2.4 Cell cycle analysis

Cells were plated on 60 mm plates (1×10^5 cells), and 24 hours after pretreatment with 10 μM dexamethasone and then subjected to 10 Gy radiation during the log growth phase. 72 hours after treatment, cells (including non-adherent cells) were collected, centrifuged twice at 1500 RPM for 5 minutes in PBS to remove trypsin then fixed slowly by adding drops of 70% cold ethanol and kept overnight at 4°C. Fixed cells washed twice with cold PBS at 2000 RPM for 10 minutes each. Fixed cells were then suspended in 500 μl of PI (50 μg/mL in phosphate buffered saline [PBS]) containing 100 μg/mL RNase A (CosmoGenetech, Seoul, Korea), then incubated in darkness at room temperature for 30 minutes. DNA content analyzed using a fluorescence-activated cell sorter (BD LSRFortessa, BD Biosciences) and the FACSDiva software (BD Biosciences).

2.5 Quantitative measurement of autophagy using acridine orange

For acidic vesicular organelle (AVO) staining, pre-treated cells as stated above were incubated at 37°C for 30 minutes in a pre-warmed culture media containing acridine orange (1 μg/mL). After trypsinization and washing twice in PBS, pelleted cells suspended in PBS and kept on ice, protected from light with aluminum foil cover and subjected to fluorescence-activated cell sorter (BD LSRfortessa, BD biosciences) and data analysis by BD FACSDiva software.

2.6 Autophagy measurement using GFP_LC3

Cells were transfected with a GFP_LC3 expression plasmid incorporated into the lentiviral vector using Lipofectamine 2000 reagent (Invitrogen Life Technologies). Selection with puromycin to establish stably transfected cells expressing GFP_LC3 fluorescence and was confirmed microscopically before experiment. Three days following pre-treatment with 10 μM dexamethasone and 10 Gy dose irradiation as described above, cells were observed under fluorescence microscope for the fluorescence of GFP_LC3 and LC3 punctuated spots cells were counted. The average percentage of autophagic cells was calculated from the ratio

of GFP_LC3 punctuated cells to normal cells only bearing GFP_LC3 fluorescence per high power field (x 200).

2.7 Western blot analysis

Cells were cultured on 60 mm plate (1×10^5 cells) treated and incubated for the required time as described above, washed and trypsinized, then centrifuged to form cell pellets. Cell pellets were lysed in a lysis buffer (RIPA with proteases and phosphatase inhibitor cocktail), for at least 30 minutes on ice, and then cleared by centrifugation for 20 minutes at 12,000 RPM/4°C. The concentration of protein was determined using bovine serum albumin (BSA) protein standard and measured with spectrophotometry at 562 wavelength. The protein was heated at 95°C for 5minutes and resolved by 12% and 15% polyacrylamide gel, electrophoresis using mini-PROTEAN and ran at 80V for first 30 minutes and later 125V for one (1) hour. The protein was transferred onto nitrocellulose membrane at 250A for 90 minutes at 4°C, blocked for 1hour with nonfat dry milk in TBST. The membrane incubated at 4°C overnight in different primary antibodies; anti-LC3, anti-AKT, pAKT, anti-ATG5 (Novus Biologicals, Littleton, CO), and anti–β-actin (Sigma-Aldrich). The membrane washed three (3) times in TBST for 10 minutes, and then incubated with horseradish peroxidase–conjugated secondary antibody for

1 hour at room temperature. The protein complex was detected using western blot detection Kit A and B mixed in the ratio of 1:1 and visualize using an enhanced chemo-luminescence, developed on an X-ray film.

2.8 Statistical analysis

Three independent experiments were carried out (triplicate) and results were expressed as the mean ±standard error of the mean. Statistical significance were calculated using a two-tailed unpaired Student's t test using GraphPad Prism (ver. 6.0, GraphPad software, La Jolla, CA). P-value less than 0.05 and was taken as statistically significant.

3. Results

3.1 Dexamethasone protects glioma cells from cell death after irradiation

We used clinically achievable dexamethasone dose of 10 μM that was determine from literatures (Mattern J et al, 2007) and pre-determined radiation dose from 2 Gy to 20 Gy was applied. Dexamethasone alone did not affect cell proliferation (Supplementary figure 1). The cell survival after irradiation was compared to evaluate the difference between with and without addition of dexamethasone (Figure 1 A and B). The addition of

dexamethasone increased the cell survival significantly in both U373 (p=0.02) and LN229 (p =0.03).

Based on these results, 5 Gy (for LN229) and 10 Gy (for U373) were used for further experiment to observe the cell death and autophagy as inhibitory dose 50 (IC_{50}).

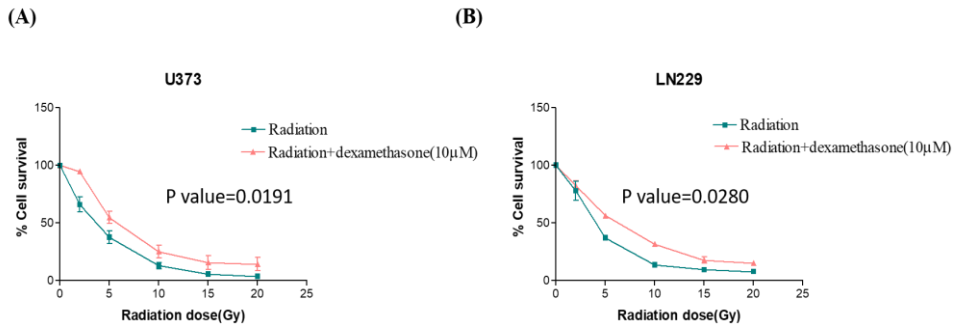


Figure 1: Dexamethasone increases survival in both U373 and LN229 glioma cell lines after irradiation. Malignant glioma cells were pre-treated with 10 μM of dexamethasone and different doses of irradiation. Both U373 (p=0.0191) and LN229 (p=0.0280), showed significant increase in survival. The red arrow showing the difference between the dexamethasone pre-treated and radiation only treated cells.

3.2 Cell cycle analysis using PI staining

To observe the effect of dexamethasone on cell cycle after irradiation, we prepared cells as per our experimental setup and performed flow cytometry analysis using PI staining, (Figure 2 A and B). The G2/M phase

accumulation was observed after 10 Gy irradiation and it was significantly increased with addition of dexamethasone in both U373 cell lines (38% vs. 45%) and LN229 (35% vs. 47%) (Figure 2. C, p=0.020 and 0.021, respectively). Meanwhile, subG1 accumulation after irradiation was significantly decreased compared to 10 Gy radiation only in both U373 and LN229 cells (p=0.019 and 0.03, respectively). These data together suggest that G2/M arrest and subG1 accumulation of cell population at lethal radiation dose is significantly interrupted by dexamethasone.

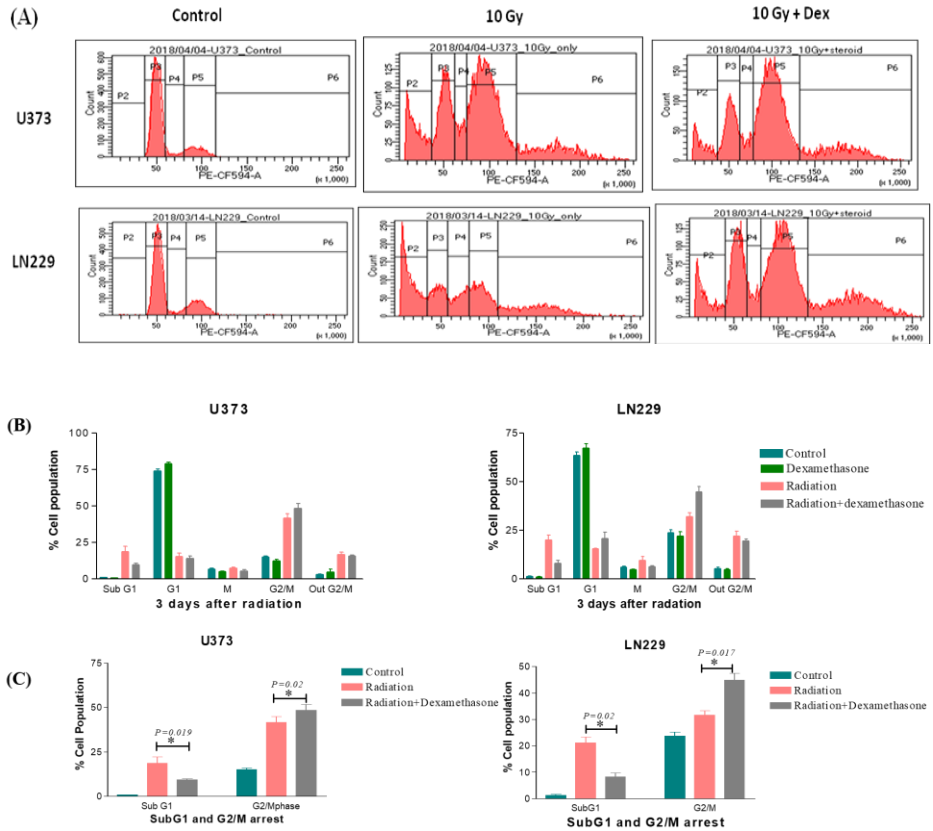


Figure 2: Dexamethasone increased G2/M arrest and decreased subG1 accumulation reducing apoptosis. (A) Cell cycle analysis of both U373 and LN229 after 3 days of incubation following treatment with both dexamethasone and irradiation. (B and C) Proportional analysis of cell cycle fraction revealed radiation-induced G2/M arrest and subG1 accumulation were interrupted significantly by the addition of dexamethasone in both U373 and LN229

3.3 Dexamethasone repressed autophagy in PTEN-mt U373 and increased autophagy in PTEN-wt LN229 cell lines

As autophagy was a main response to irradiation of MG cell lines (Gwak HS, 2015), we measured autophagy quantitatively to evaluate the influence of dexamethasone after irradiation. After acridine orange staining of acidic vascular organelles (AVO), we subjected cells to flow cytometry analysis to measure autophagy. In U373 cells, the AVO containing cells decrease significantly in dexamethasone treated cells compared to the radiation only treated cells (49% vs. 35%, p=0.01). Meanwhile, in LN229 cells, at 10 Gy AVO containing cells increases up to 40% compared to control (12%), but significantly increased after dexamethasone treatment compared to the radiation only treated cells (52%, p=0.01).

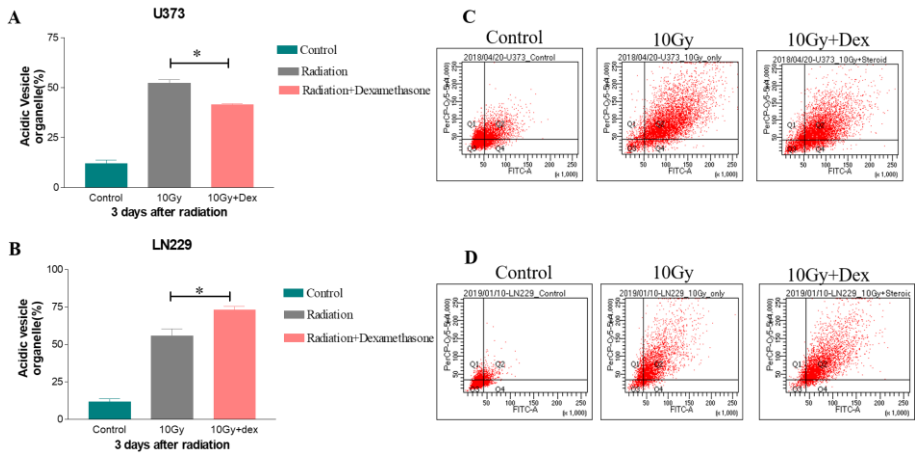


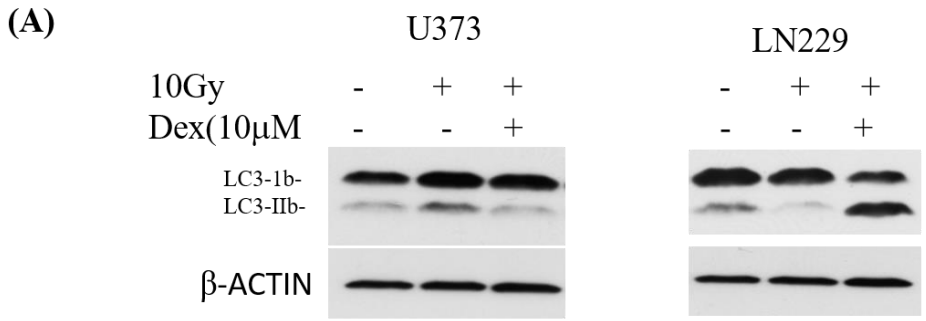
Figure 3: Measurement of autophagy using acridine orange staining and flow cytometry (A). Dexamethasone decreased autophagy in U373 (p=0.010) and (B). Increased autophagy in LN229 (p=0.010).

3.4 Dexamethasone interferes with autophagosome formation after irradiation.

Western blot evaluation of conversion of LC3-Ib to LC3-IIb conjugate is a standard marker/indicator of autophagy (Tanida I, 2008), In U373 cell lines; there was drastic reduction in LC3-IIb band on the western blot compared to the radiation only cells (Figure 4). Whereas, in LN229 there was significant increase of LC3-IIb in dexamethasone treated cells compared to the radiation only treated cells. These results are in accordance with quantitative measurement of AVO indicating autophagy.

These results were further confirmed using GFP_LC3 stably transfected U373 cells (Figure 4. B). The expression level of GFP_LC3 punctuated cells showed autophagy increased after irradiation, and after treatment with dexamethasone, there was significant reduction of the number of GFP_LC3 granules compared to the radiation only cells(Figure 4.C, p =0.016)

The data taken together suggest that dexamethasone interfere with autophagy after irradiation and the influence is different depending on the PTEN mutational status of the glioma cell



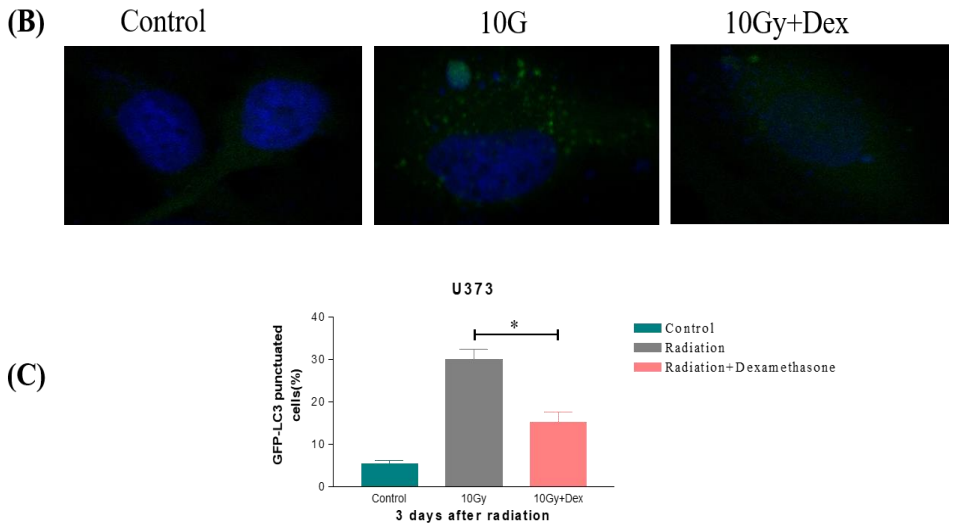


Figure 4: Illustration of dexamethasone effect on radiation induced autophagy in glioma cells. (A) Western blot analysis of LC3 shows an increase in LC3-IIb is indicative of autophagosome conjugation. (B). Fluorescence microscope pictures showing GFP_LC3 punctuated cells after treatment and incubated for the required time. (C). Graphs of U373 GFP_LC3 indicating reduced number of GFP_LC3 punctuated cells after treatment with dexamethasone (Average GFP-LC3 punctuated counted per field, 30 vs 15 respectively, P=0.0155). Three independent experiments were carried out and the mean taken as true value**.**

3.5 Dexamethasone-induced reduced cell death is reversed with phospho-Akt inhibitor in PTEN non-functional U373 after irradiation.

As PI3K/Akt, pathway activation is alleged cellular response to radiation-induced damage in glioma cells (Li HF, 2009), we adopted

phosphor-Akt inhibitor to evaluate if dexamethasone effect on radiation-induced cell death is related to interruption of Akt activation. We adopted colony forming assay to augment the partial effect of dexamethasone-induced decreased cell death after irradiation. The addition of phospho-akt inhibitor significantly reversed the dexamethasone-induced increased colony forming ability in U373, but provoked no significant change compared to dexamethasone-treated irradiated cells in LN229. Together these data demonstrate that dexamethasone protects glioma cells from apoptosis after irradiation and the effect is mediated via Akt activation in PTEN-mt glioma cells

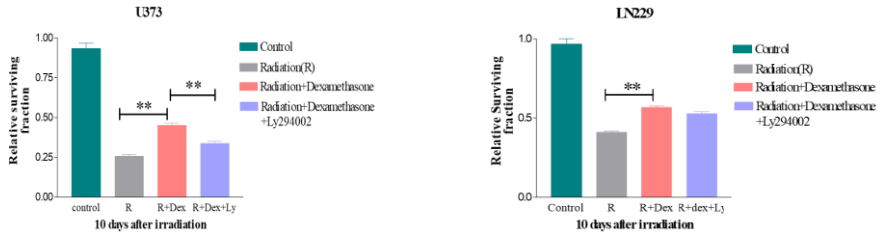


Figure 5: Colony forming assay after dexamethasone and radiation indicated reduced cell death. Combination of dexamethasone and Ly294002(Ly) inhibitor of Akt activation resulted into increased cell death in U373 (surviving fraction were 0.548 vs 0.336, p=0.0052) and no significant change (surviving fraction 0.562 vs 0.525, p>0.05) in LN229. 50 cells or more were counted as a colony. The experiment was in triplicate and the mean value calculated.

3.6 p-Akt inhibition interfering dexamethasone effect in U373 glioma cells after irradiation is mediated via autophagy formation

We tested if Akt inhibitor effect in U373 cells on dexamethasone-induced reduced cell death after irradiation is related to autophagy activity (Figure 6). Quantitative measurement of autophagy by AVO counting revealed significant decrease of autophagy activity by phospho-Akt inhibition in PTEN-mt U373 cells but no discernible change in PTEN-wt LN229 cells. This decreased autophagy by phosphor-Akt inhibitor in U373 cells appeared as a reverse of dexamethasone-induced reduced autophagy after irradiation. Together this data confirm that dexamethasone may be partly acting through PI3K/Akt/mTOR pathway of which Akt is the one negative regulator.

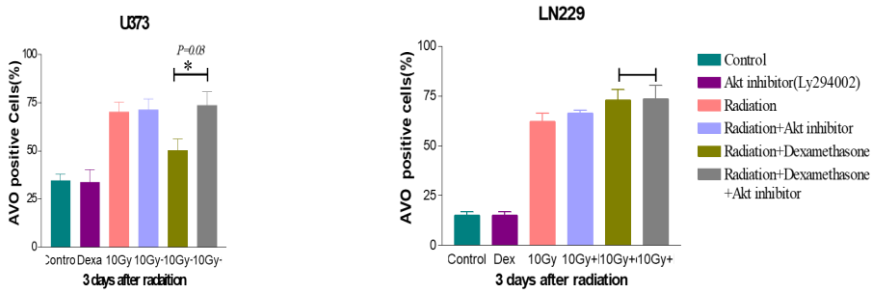


Figure 6: The effect of p-Akt inhibition on autophagy following dexamethasone interference. Cells were pre-treated with dexamethasone for 24 hours and then 5 μM of Ly294002(Ly) inhibitor added 30 minutes before irradiation. In U373,

there was significant increase (p=0.0377) in the AVO expression indicating increased autophagy. In LN229, there was no significant effect after blockage of p-AKT and hence no change in autophagy.

3.7 Atg5 inhibition decreased autophagy in both MG cell lines

To determine if the knockdown of autophagy would have an effect on dexamethasone treated cells, we knockdown autophagy with ATG5 siRNA. After confirming ATG5 knockdown by Western blot (supplementary figure 2), we measured autophagy activity by Western blot of LC3 protein.

As expected knock down of ATG5 significantly decreases LC3 II conjugation in both U373 and LN229 cells (Figure 7).

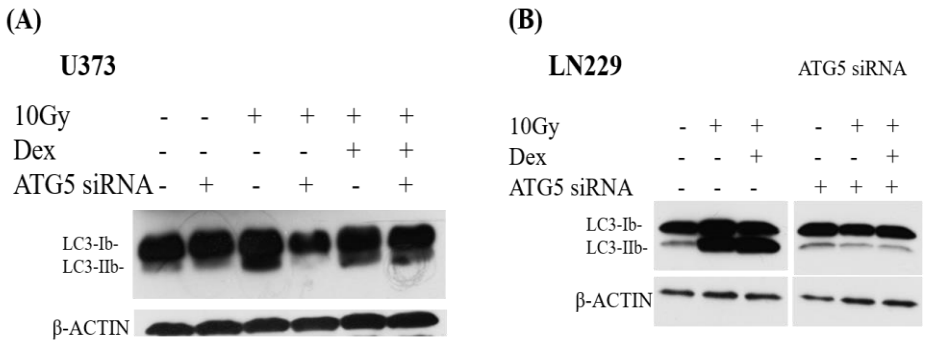


Figure 7: ATG5 knockdown decrease autophagy after irradiation in both U373 and LN229. (A) And (B) indicating the western blot result after knockdown of ATG5 on the ratio of LC3-IIb/LC3-Ib on both U373 and LN229. There was reduction in LC3-IIb migration from LC3-Ib.

3.8 Autophagy inhibition mitigate dexamethasone-induced reduced cell death after irradiation

To investigate whether inhibition of autophagy is related to cell death, we perform cell survival assay after transfecting cells with ATG5 small interfering RNA. The knockdown of Atg5 affects no discernible effect on clonogenic assay compared to control; it resulted in significant increased cell survival after irradiation in compared to radiation only in both cell lines (Figure 8). Addition of dexamethasone after autophagy inhibition also improves cell survival in the both cell lines.

Together these results indicated that inhibition of autophagy could as well increase cell survival and reduced cell death in our research.

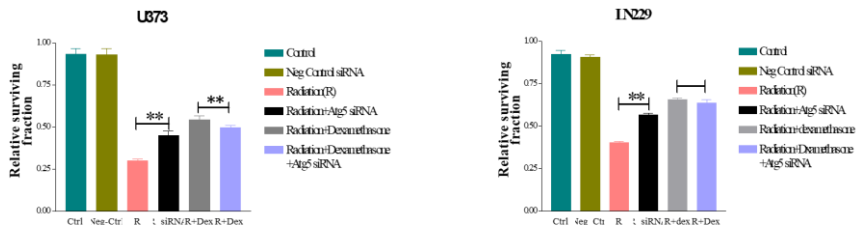


Figure 8. Increased cell survival after Autophagy inhibition-using Atg5 siRNA significantly decreased cell death of both U373 and LN229. In U373 after Atg5 knockdown, the surviving fraction of control compared to negative control (0.92 vs 0.90, p>0.05), radiation compared to radiation + Atg5 siRNA was significant (0.299 vs 0.45, p=0.0001) and radiation + dexamethasone compared to radiation + dexamethasone + Atg5 siRNA (0.543 vs 0.49, p=0.0012). In LN229 surviving

fractions were control compared to negative control (0.92 vs 0.90, p>0.05) radiation compared to radiation + Atg5 siRNA (0.402 vs 0.56, *p*=0.0045) and radiation + dexamethasone compared to radiation + dexamethasone + Atg5 siRNA (0.65 vs 0.63, *p*>0.05). Three independent replicate of data set used.

4. Discussions

In our study, we found out that dexamethasone increases cell viability against radiation effect in malignant glioma cells. It significantly reduced cell death, which was related to radio-resistance. Combination of selective phosphor-Akt inhibitor (Ly294002) and dexamethasone resulted to reverse of reduced cell death after radiation in PTEN-mt malignant glioma cells. Autophagy inhibition after knockdown of Atg5 in the glioma cells resulted into neutralization of reduced cell death following treatment with dexamethasone and irradiation.

4.1 Dexamethasone reduced cell death after irradiation of MG cells

Our study showed that dexamethasone use in radiation treatment for gliomas might interfere with cell death after irradiation. A study showed that glucocorticoid use during radiation therapy was controversial and may promote cell survival in glioblastoma (Pitter KL, 2016) as it protected solid tumors from cytotoxic effects of anticancer agents (Mattern J, 2007).

In another similar study, (Das A, 2004) suggested that dexamethasone protects tumor cells from apoptosis and promote resistance when used concurrently with radiotherapy or chemotherapy, which might result in an undesirable clinical outcome. In a clinical study (Shields LB, 2015) its administration during radiotherapy and temozolomide resulted into poor prognosis in newly diagnosed glioblastoma patients.

4.2. Possible mechanism of action of dexamethasone in protecting irradiated glioma cells

In our study, dexamethasone treatment resulted to reduced accumulation of subG1 phase, which is the late stage apoptosis and then increased G2/M arrest after irradiation. The G2/M checkpoint is an important cell cycle checkpoint that ensures cells do not initiate mitosis until damaged DNA or incompletely replicated DNA repair is sufficient.

Defective G2/M arrest could result into premature entry into mitosis before the DNA repair and hence damaged cells going into apoptosis. Increased accumulation of G2/M arrest in malignant glioma cells following dexamethasone and irradiation treatment we observed in our experiment could probably be due to delayed damage DNA repair mechanisms or processes hence decreased apoptosis and reduced cell death. Dexamethasone interferes with the cytotoxic and anti-proliferative

actions of most chemotherapeutic drugs (Mattern J, 2007), and has an antagonistic effect on temozolomide induced apoptosis in human glioblastoma U87MG cells (Das A, 2004).

Akt is known to promote DNA damage repair especially in irradiated cancer cells (Toulany M, 2012). PI3K/Akt/mTOR pathway negatively controlled autophagosome formation especially in PTEN non-functional cancer cells. Although we have observed the same increased G2/M arrest with the addition of dexamethasone into irradiated glioma cells, the autophagy response to radiation was different according to PTEN status, in LN229, PTEN-functional glioma cells, the increased G2/M arrest could induced prolonged stressful condition to cell's homeostasis than increased autophagy activity. However, in U373, PTEN-non-functional glioma cells, dysregulated PI3K/Akt/mTOR pathway might quench the stress from increased G2/M arrest into reduced autophagy as Akt phosphorylation is up-regulated in response to radiation damage.

4.3 Combination treatment of MG cells using dexamethasone and radiation affects autophagy according to PTEN functional status

Relevant clinical doses of radiation promote autophagy in tumor cells (Gewirtz AD, 2014), and there has been very strong evidence to

demonstrate that molecular responses to radiation induces autophagy in MG cells and is the way to cell death or apoptosis (Gwak HS, 2015, Yao CK, 2003). Our finding using the irradiation lethal dose of 10Gy confirmed the same hypothesis in MG cells. Some Studies have suggested that Autophagy may be a novel mechanism by which some cells can survive glucocorticoid exposure and provide a potential therapeutic target.

Dexamethasone induces autophagy in lymphocytes, although the mechanism was not fully elucidated (Molitoris JK, 2011) and activation of autophagy is secondary to dexamethasone induced mitochondrial fragmentation (Troncoso R, 2014). Meanwhile a controversial study suggested dexamethasone to inhibit level of autophagy in the injured nerve cells in a dose-dependent manner (Wang ZY, 2018).

We observed autophagy based on acridine orange staining, western blot and fluorescence GFP_LC3 after a combination treatment with dexamethasone and irradiation. LC3 is an important component of mammalian autophagosomes, and thus the GFP_LC3 fusion protein has been used as a reliable marker for autophagosome formation (Cao C, 2006). Measurement of autophagic activity by flow cytometry of acidic vesicle organelle (AVO), ratio of LC3 protein bands and observation of GFP_LC3. In U373 PTEN mutant, dexamethasone inhibits AVO formation after radiation and this was confirmed by decreased ratio of

LC3-IIb/LC3-Ib on western blot, and then reduced formation of GFP_LC3 punctuated cells observed under fluorescence microscope. However, in LN229 PTEN wild type cells, dexamethasone increases radio-resistance through increased autophagy. PTEN acts as a classical tumor suppressor, mainly involved in the homeostatic maintenance of the PI3K/AKT/mTOR cascade (Milella M, 2015) which is a key regulator of autophagy (Errafiy R, 2013). Akt is a known negative regulator of PI3K/mTOR pathway and inhibition or blockade can increase radio-sensitivity to autophagy. Dexamethasone dephosphorylated Akt (supplementary figure 3) and it is much documented to be an Akt deactivator.

However, inhibition of p-Akt using Ly294002 increases autophagy in PTEN-mt cells. Its inhibition caused a drastic increased in autophagy level measured by AVO formation. We hypothesize that dexamethasone could be acting through Glucocorticoid Receptor (GR) for the effect we observed. The effects of LY294002 on radiation examined in the PTEN mutant glioma cell line U251 MG and Low doses of p-Akt inhibitor sensitized U251 MG to clinically relevant doses of radiation (Nakamura JL, 2004).

In our study, although knockdown of autophagy related gene (ATG5) decreased autophagy after irradiated, there was no significant difference on level of autophagy after the addition of dexamethasone in both glioma

cells. Atg5 previously characterized as a protein specifically required for autophagy and it has a role in the formation of autophagosomes. However, an Atg5 fragment produced by calpain cleavage has pro-apoptotic properties (Codogno P, 2006).

Careful use of dexamethasone during radiotherapy could be important to minimize treatment failure in glioblastoma patients. Addition of Akt inhibitor could improve treatment in combination with dexamethasone to increase cancer cell death during radiotherapy especially in glioblastoma patients where PTEN is mutated.

5. Conclusion

Our findings demonstrated that combination treatment using dexamethasone and radiation of malignant glioma cells increased cell viability, which could be responsible for the radio-resistance normally observed during radiotherapy in MG patients. PTEN mutational status (PTEN-mt and PTEN-wt) of the cancer cells is responsible for the autophagy interference after dexamethasone and radiation. Autophagy inhibition after Atg5 knockdown reduce cell death. Dexamethasone interference of autophagy observed in malignant glioma cells during our experiment is partly mediated through PI3K/akt/mTOR pathway. Akt selective inhibitor like Ly294002 could be used to improve treatment

during radiotherapy in gliomas patients especially where PTEN is mutated.

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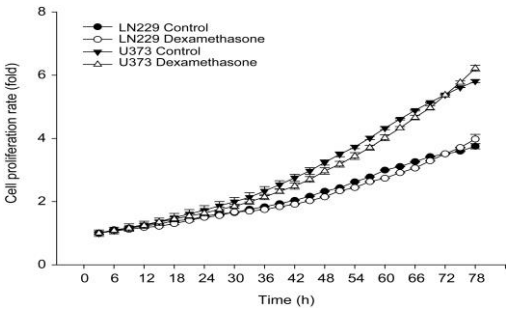
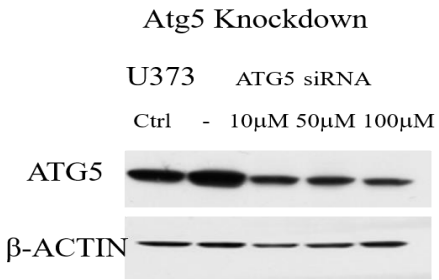
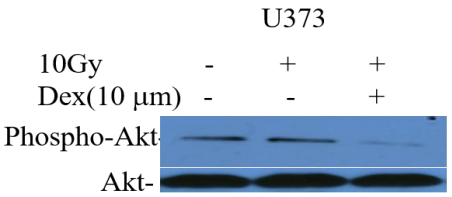
Supplementary Figures



Supplementary figure 1: Dexamethasone (10μM) had no significant effect on malignant glioma cells.



Supplementary figure 2: Atg5 knock down efficiency tested on U373 using different concentration of siRNA.



Supplementary figure 3: Dexamethasone dephosphorylated akt in PTEN_mt U373 glioma cell after radiation

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