Suppression of autophagy augments the radiosensitizing effects of STAT3 inhibition on human glioma cells

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Article Chronology:
Received 16 June 2014
Received in revised form 29 August 2014
Accepted 3 September 2014
Available online 16 September 2014

Keywords:
Autophagy
Glioma cell
Radiosensitivity
Signal transducer and activator of transcription 3
WP1066

Abstract
Radiotherapy is an essential component of the standard therapy for newly diagnosed glioblastoma. To increase the radiosensitivity of glioma cells is a feasible solution to improve the therapeutic effects. It has been suggested that inhibition of signal transducer and activator of transcription 3 (STAT3) can radiosensitize glioma cells, probably via the activation of mitochondrial apoptotic pathway. In this study, human malignant glioma cells, U251 and A172, were treated with an STAT3 inhibitor, WP1066, or a short hairpin RNA plasmid targeting STAT3 to suppress the activation of STAT3 signaling. The radiosensitizing effects of STAT3 inhibition were confirmed in glioma cells. Intriguingly, combination of ionizing radiation exposure and STAT3 inhibition triggered a pronounced increase of autophagy flux. To explore the role of autophagy, glioma cells were treated with 3-methyladenine or siRNA for autophagy-related gene 5, and it was demonstrated that inhibition of autophagy further strengthened the radiosensitizing effects of STAT3 inhibition. Accordingly, more apoptotic cells were induced by the dual inhibition of autophagy and STAT3 signaling. In conclusion, our data revealed a protective role of autophagy in the radiosensitizing effects of STAT3 inhibition, and inhibition of both autophagy and STAT3 might be a potential therapeutic strategy to increase the radiosensitivity of glioma cells.

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Introduction
Signal transducer and activator of transcription 3 (STAT3), a multifunctional intracellular signaling pathway, regulates cell proliferation, survival, differentiation, angiogenesis, immune function and malignant transformation [1]. In response to cytokines and growth factors, STAT3 is activated by phosphorylating the tyrosine residue in the transactivation domain, after which it dimerizes and translocates into the nucleus, then collaborates with other transcriptional factors to mediate the expressions of a variety
of genes. As a convergence point of many oncogenic signaling pathways, constitutive activation of STAT3 has been reported in 50–90% of human cancers [2]. In glioblastoma, STAT3 is also engaged in tumorigenesis and may be a potential candidate as a prognostic parameter [3]. Therefore, STAT3 represents an attractive therapeutic target for malignant brain tumors. An increasing number of studies have reported a collection of small molecule inhibitors of STAT3 for the treatment of glioblastoma [4]. Currently, the treatment of glioblastoma is based on radiotherapy combined with temozolomide [5]. In spite of the improvement in therapeutic approaches, the overall prognosis of glioblastoma patients remains poor, partially due to the radioresistance of glioma cells. It has been shown that STAT3 inhibition is capable to enhance the radiosensitivity of glioma cells both in vitro and in vivo [6].

The radiosensitizing effects of STAT3 inhibition are related to the activation of the mitochondrial apoptotic pathway.

The importance of radiation-induced apoptosis in radiotherapy has been recognized for decades, and better outcome for glioblastoma patients may be achieved through manipulating of apoptotic cascades [7]. Nevertheless, autophagy has recently emerged as an essential mechanism in human glioma cells to orchestrate cellular response to ionizing radiation (IR) [8]. Autophagy refers to a catabolic mechanism able to promote lysosomal degradation of cytoplasmic components and organelles [8].

Autophagy is an evolutionarily conserved process characterized by a prominent formation of double-membrane autophagic vacuoles that progressively engulf cytoplasmic components and deliver to the lysosomes for degradation. Recently, it has been pointed out that autophagy may be a key mediator of tumor initiation and progression [9]. The role of autophagy in cancer cells might be protective or deleterious, which depends on the cellular context and external microenvironment. The baseline autophagy contributes to the maintenance of cellular homeostasis, and autophagy flux can be upregulated in response to IR or cytotoxic agents. Unsurprisingly, autophagy also plays an essential part in glioblastoma formation and determining the response of glioma cells to chemotherapy and radiotherapy [10].

In this regard, modulation of autophagy could be an adjuvant modality to improve radiotherapy effects.

In this study, we focused on the radiosensitizing effects of STAT3 inhibition on human glioma cells. Treatment of STAT3 inhibitor or shRNA for STAT3 dramatically upregulated IR-induced autophagy. Suppression of autophagy by pharmacological or genetic approach further strengthened the radiosensitizing effects, accompanied by reduced clonogenic ability and increased level of cell apoptosis. Our data revealed a protective role of autophagy in the radiosensitizing effects of STAT3 inhibition in glioma cells.

**Materials and methods**

**Chemicals**

Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone (Logan, UT). An inhibitor of STAT3 WP1066 was purchased from Selleck Chemicals (Houston, TX) and dissolved in dimethyl sulfoxide to obtain a stock concentration of 40 mg/ml, stored at –20 °C and diluted to the desired concentration in fresh medium immediately before use.

Autophagy inhibitor 3-methyladenine (3-MA) was purchased from Sigma Aldrich (St Louis, MO) and was used at a final concentration of 1 mM.

**Cell culture and irradiation administration**

Human malignant glioma cells U251 were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2 humidified atmosphere. Cells were irradiated with 160 kV X-ray with a dose rate of 1.15 Gy/min by a biological research irradiator (Rad Source Technologies, Suwanee, GA).

**Plasmids, siRNA and transfection**

A plasmid bearing a short hairpin RNA (shRNA) designed against STAT3 (shSTAT3) and the corresponding control vector were purchased from Genechem (Shanghai, China). siRNA for ATG5 (sense 5’-GACGUGGUAACUGACAAATT-3’) and scramble siRNA were also obtained from Genechem. For each well of 6-well plates to be transfected, 2 μg plasmid and/or 50 pmol siRNA duplexes were transfected cells using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. Two days after transfection, whole cell lysates were extracted and analyzed by western blotting to examine the knockdown efficiency.

**Clonogenic survival assay**

Cells were plated in triplicate into six-well plate and subjected to various doses of X-ray irradiation. Cells were further cultured for 14 days, and then fixed with methanol and stained with 1% crystal violet. Colonies consisting of more than 50 cells were counted as a single colony. The plating efficiency and the radiation-surviving fraction were measured. The combined effects of STAT3 inhibition with autophagy inhibition were evaluated by median-effect plot analyses, which is a well-established methodology described by Chou and Talalay [11]. The combination index (CI) values were obtained from the data using a CalcuSyn 2.0 software (Biosoft, Cambridge, UK). The CI values were interpreted as follows: CI < 0.8 indicates synergism; CI 0.8–1.2 indicates additive; and CI > 1.2 indicates antagonism.

**Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining**

U251 cells were irradiated with 8 Gy X-ray. After 24 h, U251 cells were fixed with 4% paraformaldehyde for 40 min, and permeabilized using 0.2% Triton X-100. Endogenous peroxidase was inactivated by incubating with 0.2% H2O2 in methanol for 20 min. Then cells were incubated with terminal deoxynucleotidyl transferase end-labeling cocktail for 60 min. After washing, cells were incubated with streptavidin-HRP solution for 30 min.

**Analysis of apoptosis by flow cytometry**

U251 cells were washed with ice-cold phosphate-buffered saline and further stained with Annexin V-phycoerythrin. The percentage of apoptotic cells was monitored by a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA).
Western blotting

Cells were lysed by RIPA lysis buffer on ice for 15 min and centrifuged at 13,200 rpm for 10 min at 4°C. Cell lysate containing equal amount of protein were subjected to 10% SDS-PAGE, and electrically transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA). Nonspecific binding was blocked with Tris-buffered saline containing 5% (w/v) skim milk for 2 h at room temperature. The membranes were then incubated with primary antibody for p-STAT3 (Y705), STAT3 (Epitomics, Burlingame, CA), caspase-3, poly (ADP-ribose) polymerase (PARP1), Beclin 1, microtubule-associated protein light chain 3 (LC3), autophagy-related gene 5 (ATG5) (Proteintech, Chicago, IL) and β-actin (Santa Cruz Biochemicals, Santa Cruz, CA) and then incubated with horse-radish peroxidase (HRP)-conjugated second antibody. β-actin was used as a loading control. Immunoblotting signals were detected using an enhanced chemiluminescence method.

Fluorescent LC3 plasmid transfection

U251 cells were transfected with a pEX-GFP-LC3 expressing plasmid (GenePharma, Shanghai, China) by Lipofectamine 2000 Reagent. The formation of green fluorescence protein (GFP)-LC3 punctate structures was examined using a Leica DM IL LED microscope (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

Data were expressed as mean ± standard deviation. Analysis was performed by one-way ANOVA following multiple comparisons using SPSS 18.0 software (SPSS Inc., Chicago, IL). Results with a p Value less than 0.05 were considered statistically significant.

Results

STAT3 inhibitor WP1066 radiosensitizes U251 glioma cells

Downregulation of STAT3 by RNA interference was shown to enhance radiosensitivity of glioma cells [6], but inhibition of STAT3 by a chemical inhibitor, cucurbitacin, or a neutralizing gp130 antibody failed to increase radiosensitivity of glioma cells [12]. In the present study, we used WP1066, an inhibitor of STAT3 which has the capacity to penetrate an intact blood–brain barrier.
U251 cells were irradiated with different doses of X-ray and incubated with or without 1 μM WP1066 for 1 day. As shown in Fig. 1A, clonogenic survival assay shows that the surviving fractions of WP1066-treated group are significantly lower compared to those of the control group after 6 or 8 Gy irradiation, suggesting WP1066 can exert radiosensitizing effects on U251 cells. The expression level of p-STAT3 was upregulated by IR administration, whereas WP1066 decreased p-STAT3 levels in a dose-dependent manner (Fig. 1D). WP1066 treatment also promoted IR-induced apoptosis of U251 cells, confirmed by TUNEL staining and Annexin V staining (Fig. 1B and C). Accordingly, the apoptosis markers, cleaved caspase-3 and cleaved PARP1, were markedly induced (Fig. 1D). As a previous report [6], treatment of 1 μM WP1066 for 1 day alone could also induce a slight but significant increase of apoptosis cells, indicating the anti-apoptotic function of STAT3 pathway in glioma cells. These data clearly demonstrated radiosensitizing effects of WP1066 on U251 cells, which might be owing, at least in part, to a greater amount of cell apoptosis.

**Knockdown of STAT3 radiosensitizes glioma cells**

Next, an shRNA plasmid targeting STAT3 was transfected to U251 cells. Two days after transfection, the expression of STAT3 was remarkably decreased as shown in Fig. 2A, upper panel. The activation of STAT3 signaling in response to IR exposure was also...
repressed by shSTAT3 transfection (Fig. 2D). Similar to the data demonstrated by pharmacological inhibitor of STAT3, diminishing of STAT3 expression by shSTAT3 reduced the surviving fraction of U251 cells after 6 or 8 Gy irradiation (Fig. 2A, lower panel). In another human glioma cell line A172, shSTAT3 transfection also significantly increased radiosensitivity (Fig. S1D). In addition, shSTAT3-transfected group exhibited the maximum percentage of apoptosis cells, as well as the highest expression levels of apoptosis-related proteins (Fig. 2B–D). Taken together, down-regulation of STAT3 expression could restrain the phosphorylation of STAT3 after irradiation, and increase the radiosensitivity of glioma cells.

**Inhibition of STAT3 in glioma cells promotes autophagy induced by IR**

Autophagy is considered to play an important part in the response of radiation therapy [8]. And one of the mechanisms by which STAT3 inhibition exert antineoplastic function is through induction of autophagy [15,16]. To examine whether autophagy was induced by IR treatment and/or STAT3 inhibition in U251 cells, western blot analysis was performed to show the conversion of LC3-I (18 kDa), the cytoplasmic form, to LC3-II (16 kDa), the autophagosome form, and the expression of Beclin 1, an activated autophagy-related gene. As shown in Fig. 3A and B, Beclin 1 and LC3-II expression were upregulated by IR exposure, as well as WP1066 or shSTAT3 treatment. When combined IR administration and STAT3 inhibition, these autophagic markers were more markedly increased. Similarly, the autophagy-upregulating effects of STAT3 inhibition were also observed in irradiated A172 cells (Fig. S1A and B). Next, transient transfection of GFP-LC3 was used to show the aggregation of LC3 in U251 cells. The control cells displayed a diffuse cytoplasmic pattern of GFP-LC3 (Fig. 3C). WP1066 or IR-treated cells display a punctuate expression of autophagosome. A more punctuate fluorescence was observed in the U251 cells treated with WP1066 and IR together. These data confirmed the induction of autophagy in glioma cells by IR or STAT3 inhibition, and IR-induced autophagy flux could be greatly triggered by STAT3 inhibition.

In addition, it has been reported that WP1066 could suppress the activation of extracellular signal-regulated kinase (ERK1/2) in erythroid cells [17]. In U251 cells, IR-induced ERK1/2 phosphorylation could also be suppressed by treatment of 1 μM WP1066 (Fig. S2A), though the inhibitory effectiveness of WP1066 on ERK signaling was slighter than that on STAT3 signaling. To examine whether ERK signaling participated in the regulation of autophagy in glioma cells, PD98059, an ERK1/2 inhibitor, was used to treat U251 cells together with IR administration. The dose-dependent suppressive effects of PD98059 on ERK1/2 phosphorylation were demonstrated in Fig. S2B. However, treatment of 5 μM PD98059 did not induce the conversion of LC3-I to LC3-II in U251 cells (Fig. S2C), indicating that upregulation of autophagy by WP1066 might be unrelated to ERK signaling.

**Inhibition of autophagy by 3-MA promotes the radiosensitizing effects of STAT3 inhibition**

To determine the function of autophagy in the radiosensitizing effects of STAT3 inhibition, 1 mM 3-MA, an autophagy inhibitor, was used to treat U251 cells together with WP1066. The expressions of autophagy-related proteins were substantially reduced by 3-MA treatment (Fig. 4A). Clonogenic survival assay revealed a further radiosensitizing effects on U251 cells treated with 3-MA and WP1066 (Fig. 4B). 3-MA treatment also suppressed
shSTAT3-induced autophagy (Fig. 4C), and enhanced the radiosensitivity of U251 cells treated with shSTAT3 (Fig. 4D). The CI values were 0.332 and 0.275 for the combined treatment of WP1066 and 3-MA, shSTAT3 and 3-MA, respectively, suggesting 3-MA treatment and STAT3 inhibition had synergistic radiosensitizing effects. These data meant that autophagy might serve a protective role in the response to IR exposure and STAT3 inhibition.

siATG5 transfection promotes the radiosensitizing effects of STAT3 inhibition

3-MA blocks autophagosome formation via the inhibition of phosphatidylinositol 3-kinases (PI3K). Since a functional link between PI3K and STAT3 pathway in human cancer cells has been described [18], 3-MA treatment might interfere with the STAT3 signaling in U251 cells. Concerning the probable unspecific effects of 3-MA, we used RNA interference to diminish the expression of ATG5, a key mediator for autophagosome elongation [19]. The knockdown efficiency of ATG5 and suppression of autophagy in glioma cells by siATG5 transfection were confirmed by western blot analysis (Figs. 5A, C and S1C). In accordance with the results of 3-MA, siATG5-transfected glioma cells showed the minimum surviving fraction among different groups (Figs. 5B, D and S1D). In U251 cells, the CI values were 0.256 and 0.113 for the combined treatment of WP1066 and siATG5, shSTAT3 and siATG5, respectively.

Inhibition of autophagy enhances apoptosis induced by IR and STAT3 inhibition

To further explore the protective role of autophagy, apoptotic cells were quantified using Annexin V staining. As shown in Fig. 6, pharmacological or genetic blockage of autophagy, in combination with WP1066 or shSTAT3 treatment, induced more apoptotic cells after IR administration compared to other groups. In agreement with the percentage of apoptosis, the IR-induced expressions of cleaved caspase-3 and cleaved PARP1 were greatly upregulated by dual inhibition of STAT3 signaling and autophagy. These data proved that suppression of autophagy further radiosensitized STAT3-inhibited U251 cells via shifting from autophagy to apoptosis.
Discussion

Aberrant activation of STAT3 signaling in glioblastoma has drawn increasing attention to the therapeutic potential of STAT3 inhibitors [3,4,20]. In the present study, it was demonstrated that STAT3 inhibitor WP1066, as well as downregulation of STAT3 by RNA interference, had radiosensitizing effects on glioma cells. In fact, treatment of WP1066 alone could effectively induce apoptosis in U87-MG and U373-MG glioma cells by activating proapoptotic proteins and downregulating antiapoptotic proteins [21]. It is noteworthy that the phosphatase and tensin homolog (PTEN)-mutant U251 cells and PTEN-null A172 cells were used in current study, and STAT3 could behave as a tumor suppressor in PTEN-deficient glioma cells by preventing tumor growth and invasion [22]. However, our data suggested that STAT3 activation following irradiation conferred radioresistance in these PTEN-deficient glioma cells. Inhibition of STAT3 signaling was also shown to radiosensitize many other kinds of cancers, including squamous cell carcinoma [23], laryngeal squamous carcinoma [24,25], head and neck carcinoma [26], non-small cell lung cancer [27,28], gastric cancer [29], nasopharyngeal carcinoma [30], hepatocellular carcinoma [31,32], suggesting a universal radioresistance-promoting function of STAT3 in cancer cells. Most of these studies came to the conclusion that the radiosensitizing effects of STAT3 inhibition might be a consequence of the induction of apoptosis pathway. Consistently, upon IR exposure, STAT3 signaling was activated in U251 cells, and STAT3-inhibited U251 cells exhibited elevated levels of IR-induced apoptosis, which might contribute to the increased radiosensitivity.

Interestingly, when glioma cells exposed to IR and STAT3 inhibition, a pronounced increase of autophagy flux was observed, evidenced by conversion of LC3-I to LC3-II, increased expression of Beclin 1 and accumulation of GFP-LC3 punctuates. Autophagy is originally recognized a highly conserved cellular homeostatic mechanism from yeast to mammals [33]. Recently, autophagy has been found to involve in numerous biological
aspects of tumor development and progression [9]. And both radiotherapy and chemotherapy could trigger autophagy in malignant glioma cells [34,35]. A genome-wide siRNA screen identified STAT3 pathway as a negative regulator of autophagy via inhibiting type III PI3K [36]. An analysis of immunohistochemical data from surgical glioma samples showed that the phosphorylation of STAT3 was inversely correlated with Beclin 1 expression [37]. Intriguingly, the autophagy-modulated function of STAT3 seems to be independent on its transcriptional activity [38]. A natural selective inhibitor of STAT3, cucurbitacin I was reported to induce autophagy and apoptosis simultaneously in glioma cells [39]. Here, WP1066 and shSTAT3 treatment remarkably upregulated autophagy in IR-treated glioma cells, suggesting that activation of STAT3 pathway prevented IR-induced autophagy.

There are dual functions of autophagy in the response of cancer cells to anticancer treatment [8]. On one hand, autophagy may function as a cytoprotective mechanism, probably through preserving nutrient and energy homeostasis, and removing aggregated proteins and defective organelles. Once the protective autophagy is inhibited, cancer cells would be susceptible to cytotoxic agent and IR. For example, inhibition of IR-induced autophagy by 3-MA or bafilomycin A1 radiosensitized glioma U373-MG cells [34]. Researches concerning the antitumor mechanism of resveratrol [40], metformin [15] and ZD6474 (an inhibitor of tyrosine kinase) [41] demonstrated a prosurvival role of autophagy. On the other hand, prolonged excessive autophagy could be a cell death pathway, which is termed as programmed cell death type II. So induction of autophagy might account for the cytotoxic effects of radiation. Administration of berberine increased radiosensitivity both in vitro and in vivo models of lung cancer, and only a small fraction of apoptotic cells was detected [42]. Further investigation showed that the radiosensitizing effects of berberine were due to the autophagy-related cell death, which could be blocked by 3-MA and Beclin 1 siRNA.

Regardless of the paradoxical roles of autophagy, it is generally accepted that autophagy plays an important role in the response of cancer cell to radiation. Here, to clarify the role of IR-autophagy augmented by STAT3 inhibition, 3-MA or siRNA targeting ATG5 was used to treat glioma cells together with WP1066 or shSTAT3. It was shown that STAT3 inhibition induced a protective autophagy, since autophagy inhibition further attenuated the clonogenic ability of glioma cells. Another STAT3 inhibitor, cucurbitacin I, could also induce protective autophagy in glioma cells [39]. More apoptotic cells caused by inhibition of autophagy and STAT3 were inspected in IR-treated U251 cells, suggesting that autophagy exerted its protective effects through inactivation of apoptosis. Induction of autophagy seems to be a compensatory mechanism to antagonize the disadvantageous circumstances, probably via removal of damaged mitochondria and reducing the abundance of proapoptotic proteins [43], therefore allowing the restoration of cellular damage.

Collectively, STAT3 suppression could radiosensitize glioma cells and induce significant upregulation of autophagy. Inhibition of autophagy further promotes radiosensitizing effects of STAT3 suppression and induces more cells to undergo apoptosis, indicating a cytoprotective role of autophagy in glioma cells. Our results
suggest a possible combination use of inhibition of STAT3 and autophagy, which are capable to act synergistically to improve the radiosensitivity of glioma cells.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (No. 31270897, 81202149, and 81271682), Graduate Education Innovation Project of Jiangsu Province and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2014.09.006.

REFERENCES


