Introduction

Glioma is one of the most dangerous and frequent malignant human brain tumors with poor prognosis. In fact, most patients die within approximately one year after diagnosis despite the most effective management, including adjuvant radiation therapy and adjuvant chemotherapy following initial surgery[1]. Radiation therapy, as a conventional treatment, is often given to patients after their initial surgery[2]. Although irradiation (IR) can prolong the overall survival of some patients, the resistance of glioma cells to radiation is a major therapeutic problem[3]. Therefore, improving the radiosensitivity of glioma cells is a promising approach for increasing the response to this treatment.

Accumulating evidence suggests that cathepsin L, which is a lysosomal cysteine protease[4, 5], may be involved in many key cellular functions that are linked to various illnesses such as neurodegenerative, metabolic, and infectious diseases, as well as cancer[6–12]. Cathepsin L is one of the most promising targets for anticancer therapy because it is overexpressed in a variety of malignant tumor cells[13]. For instance, cathepsin L knockout mice exhibit reduced tumor growth[8]. Navab et al suggested that cathepsin L inhibition reduces IGF-1 receptor responsiveness, thereby increasing apoptosis[14]. Another study revealed that cathepsin L inhibition greatly increases apoptosis via arsenite activity in U87 cells[15]. In cathepsin L antisense U87 cell clones, the apoptotic rate increases when induced by either intrinsic or extrinsic stimuli[16]. By contrast, other studies have also reported that increased cathepsin L activity is associated with cancer cell death[17, 18]. From these contradictory results, we considered that the effect of cathepsin L on tumors remains unclear. Because some in vitro experiments suggest that combining cathepsin L inhibition with regular chemotherapeutics is a promising therapeutic option for cancer therapy[19–21], we hypothesized that cathepsin L inhibition may also be a promising tool to improve radia-

Original Article

Cathepsin L suppression increases the radiosensitivity of human glioma U251 cells via G2/M cell cycle arrest and DNA damage

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Aim: Cathepsin L is a lysosomal cysteine protease that plays important roles in cancer tumorigenesis, proliferation and chemotherapy resistance. The aim of this study was to determine how cathepsin L regulated the radiosensitivity of human glioma cells in vitro.

Methods: Human glioma U251 cells (harboring the mutant type p53 gene) and U87 cells (harboring the wide type p53 gene) were irradiated with X-rays. The expression of cathepsin L was analyzed using Western blot and immunofluorescence assays. Cell survival and DNA damage were evaluated using clonogenic and comet assays, respectively. Flow cytometry was used to detect the cell cycle distribution. Apoptotic cells were observed using Hoechst 33258 staining and fluorescence microscopy.

Results: Irradiation significantly increased the cytoplasmic and nuclear levels of cathepsin L in U251 cells but not in U87 cells. Treatment with the specific cathepsin L inhibitor Z-FY-CHO (10 µmol/L) or transfection with cathepsin L shRNA significantly increased the radiosensitivity of U251 cells. Both suppression and knockdown of cathepsin L in U251 cells increased irradiation-induced DNA damage and G2/M phase cell cycle arrest. Both suppression and knockdown of cathepsin L in U251 cells also increased irradiation-induced apoptosis, as shown by the increased levels of Bax and decreased levels of Bcl-2.

Conclusion: Cathepsin L is involved in modulation of radiosensitivity in human glioma U251 cells (harboring the mutant type p53 gene) in vitro.

Keywords: cathepsin L; human glioma U251 cells; radiosensitivity; DNA damage; cell cycle arrest; apoptosis; irradiation

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tion therapy effectiveness. In this study, we investigated the effect of cathepsin L expression on functional status after IR in glioma cells. We also determined whether cathepsin L could regulate radioresistance in glioma cells. Our study revealed that cathepsin L inhibition could enhance the radiosensitivity of U251 cells. Therefore, cathepsin L may represent a novel therapeutic target for radiation therapy in a subset of glioma patients.

Materials and methods

Cell culture

Human glioma U251 cells and U87 cells (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) were maintained in Dulbecco’s modified Eagle’s media (DMEM)/F12 (Gibco Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco Life Technologies, Paisley, UK) and incubated at 37°C in the presence of 5% CO2.

Radiation treatment

The cells were irradiated with 6-MV X-rays from a Primus linear accelerator (Siemens, Malvern, PA, USA) at a dose rate of 198 cGy/min.

Reagents

A specific cathepsin L inhibitor, Z-FY-CHO, was purchased from Calbiochem (San Diego, CA, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, St Louis, MO, USA) to obtain a stock concentration of 20 mmol/L, which was aliquoted, stored at -80°C and then diluted to the desired final concentration in 20 mmol/L, which is 5% CO2.

Antibodies

The following antibodies were used in this study: cyclin B1 (1:200, Abcam, Cambridge, UK), Rad51 (1:1000, Abcam, Cambridge, UK), cathepsin L (1:1000, Abcam, MA, USA), γ-H2AX (1:500, Abcam, Cambridge, UK), cyclin A (1:750, Abcam, Cambridge, UK), Ku70 (1:200, Cell Signaling Technology, MA, USA), β-actin (1:1000, MultiSciences, Nanjing, China), Bcl-2 (1:500, Millipore, MA, USA), and Bax (1:500, Millipore, Billerica, MA, USA).

Construction of shRNA expression plasmids

Annealed sets of oligonucleotides encoding short hairpin transcripts that correspond to cathepsin L were ligated into a vector according to the manufacturer’s instructions (Ambion, Life Technologies, Austin, TX, USA) to generate the knockdown vector. The insert sequences used were as follows:

5'-GATCCAAAAAAGTATGACAACAGCCTCAAGTCTCTT-3';
5'-CACCGTATGACAACAGCCTCAAGTCTCTTCTTTTG-3' and
5'-GATCCAAAAAAGCGATGCACAACAGATTATACTCTC-3'.

A non-silencing RNA was used as the control treatment (5'-CACCGTATGACAACAGCCTCAAGTCTCTTCTTTTG-3').

Transfection and isolation of stable cell clones

To obtain stable clones, cells were transfected with control shRNA or cathepsin L shRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), with stably transfected cell clones designated U251-Con shRNA and U251-Cathepsin L shRNA, respectively. After the cells were transfected, they were allowed to recover for 48 h and then the growth medium was replaced with selection medium containing 300 μg/mL G418 (Roche, Indianapolis, IN, USA) for 2 weeks. After the cells were cultured under limiting dilution conditions with G418 selection, two clones from each transfection group were screened and used in this study.

Determination of cathepsin L mRNA levels by RT-PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. RNA was reverse-transcribed and amplified by PCR with the following primers:

cathepsin L upstream primer: 5'-AACACAGCTTCAACACG-3';
cathepsin L downstream primer: 5'-TTTGAAAGCCATTCACTACACGCTGC-3'.

The amplification products were analyzed by 1.0% agarose gel electrophoresis.

Clonogenic assays

The cells were seeded in six-well plates at a density of 3×10² cells per well. After the cells were incubated overnight, they were pretreated with Z-FY-CHO at 0, 1.25, 2.5, 5, and 10 μmol/L for 12 h and then irradiated with X-rays or left unirradiated. The colonies were grown for 2 weeks until colony formation was visible. Then, the plates were washed with phosphate-buffered saline (PBS), and the colonies were fixed in methanol for 15 min and stained with 0.5% crystal violet staining solution (Sigma Aldrich, St Louis, MO, USA). At least 3 independent experiments were performed, and Student’s t-test was performed on the surviving fraction (SF) values. The SF is calculated as follows: (mean colony count/inoculated cell count) x plating efficiency. The sensitization enhancement ratio (SER) is determined by calculating the ratio at the mean lethal dose. SER values greater than 1 indicate enhanced radiosensitivity.

Immunofluorescence analysis

After the cells had undergone IR, they were fixed in freshly prepared 4% paraformaldehyde solution for 10 min at 4°C and then permeabilized with 0.1% Triton X-100 for 10 min. Next, the cells were incubated in blocking buffer (1% bovine serum albumin, 0.1% Triton X-100) at 37°C for 30 min. Mouse anti-human cathepsin L (1:200, Abcam, MA, USA) was added, and the cells were incubated at 4°C overnight. Then, the cells were rinsed three times with PBS and incubated with Alexa Fluor 488-conjugated secondary antibody (1:200; Invitrogen, OR, USA) for 1 h at room temperature. The nuclei were counterstained with DAPI (0.5 ng/mL, KeyGen, Nanjing, China) for 15 min at room temperature. Coverslips were mounted
using a fluorescent mounting medium (Dako, CA, USA) and immediately observed using a confocal microscope to identify cathepsin L localization.

Western blot analysis
After the cells received the above-mentioned treatments, they were harvested and lysed with ice-cold lysis buffer. Fractionated nuclear and cytoplasmic extracts were harvested using an EpiQuik Nuclear Extraction Kit (Epigentek, NY, USA). Protein concentrations were determined using a bicinchoninic acid assay kit (Thermo, Rockford, USA). Equal amounts of protein were fractionated by 10% SDS-PAGE (Bio-Rad, CA, USA), and then the proteins were transferred to 0.45 µm nitrocellulose transfer membranes (Bio-Rad, CA, USA). The membranes were blocked in 5% BSA for 1 h and then incubated with primary antibodies at 4°C for 24 h. After the membranes were washed three times with TBST buffer (20 mmol/L Tris-buffered saline and 0.1% Tween 20), they were incubated with rabbit or mouse secondary antibodies for 1 h. Immunoblots were detected using an Odyssey Infrared Imaging System (LI-COR, NE, USA).

ELISAs
Human cathepsin L ELISA analysis was used to quantify secretory cathepsin L in the conditioned media of U251 cells and U87 cells according to the manufacturer’s instructions (R&D Systems, MN, USA). Cells were seeded in six-well plates at a density of 1×10⁶ cells per well. Cathepsin L was quantified in the conditioned medium 24 h after the cells were irradiated with X-rays or left unirradiated.

Alkaline comet assays
Cells were subjected to pretreatment (or untreated) with cathepsin L inhibitor Z-FY-CHO at 10 µmol/L for 12 h, treated with 8 Gy IR (or unirradiated) and then harvested at the indicated time for comet assays. U251 cells were isolated by trypsinization and centrifuged at 1200 rpm for 4 min. The cells were suspended in 130 µL of 0.8% low melting-point agarose/PBS (Sigma Aldrich, MO, USA) and then layered on a fully frosted slide. The slides were coated with 1% normal melting-point agarose/PBS and allowed to solidify. Then, the slides were treated with freshly prepared alkaline lysis buffer (4 mol/L NaCl, 500 mmol/L Na₂-EDTA, 500 mmol/L Tris-HCl, 1% sodium N-lauroyl sarcosinate, 1% Triton X-100 and 10% DMSO, pH 10) at 4°C for 1 h in the dark. After the slides were treated with freshly prepared electrophoresis buffer (1 mmol/L Na₂-EDTA and 300 mmol/L NaOH, pH 13) for 30 min to allow DNA unwinding, the samples were electrophoresed at 25 V and 300 mA at 4°C for 30 min, before subsequently being neutralized with 0.4 mol/L Tris buffer (pH 7.5) for 5 min and fixed in methanol for 10 min. Finally, the slides were stained with GelRed (Biotium) and observed using a fluorescence microscope. DNA damage in each cell was quantified as follows: the ratio of the maximum total length divided by the length of the comet tail.

Hoechst 33258 staining
The treated cells were analyzed using a Hoechst staining kit (Beyotime, Haimen, China). The staining was performed according to the manufacturer’s protocol. The cells were washed with PBS, treated with fixing solution for 10 min, and then stained with Hoechst 33258 fluorescent dye for 5 min at room temperature. Morphological nuclear changes were observed and captured using an inverted fluorescence microscope.

Flow cytometry analysis
The cell cycle phase distribution was determined using a Cell Apoptosis PI Detection Kit (KeyGen, Nanjing, China) and a FACScalibur flow cytometer (Epics XL, Beckman). The staining was performed according to the manufacturer’s protocol. Cells were harvested and fixed in 70% ethanol at -20°C overnight. Then, the samples were centrifuged, resuspended in 500 µL Buffer A with 250 µg/mL RNase A at 37°C for 30 min, and stained with 5 µL propidium iodide (PI) in the dark at room temperature for 30 min. The percentage of apoptotic cells was measured using an Annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis detection kit (KeyGen, Nanjing, China). Five thousand cells per sample were analyzed using a FACScalibur flow cytometer.

Statistical analysis
At least 3 independent experiments were performed. All quantitative data are presented as the means±SD and analyzed for statistical significance using independent samples t-test. The difference was considered statistically significant when P<0.05. All analyses were performed with GraphPad Prism 5.0.

Results
IR enhances cathepsin L expression in U251 glioma cells
To study the association between radiotherapy and cathepsin L expression, we measured the activity of this protease in human glioblastoma cell lines harboring the wild type (U87 cell line) and the mutant p53 gene (U251 cell line) after IR. Compared with U251 cells, a higher cathepsin L protein level was observed in U87 cells (Figure 1A). This result is consistent with the results of another group, who observed that the U87 cell line expressing wild type p53 exhibited significantly higher enzymatic activity and mRNA levels of cathepsin L compared with the mutant p53 glioblastoma cell line U373.[22] This group demonstrated that the p53 gene upregulates the cathepsin L gene transcription directly by binding to the cathepsin L gene promoter and indirectly by increasing the expression of another transcription factor, C/EBPα. Interestingly, we found that cathepsin L was present in the nuclei of certain U251 cells; after IR, cathepsin L expression increased in both nuclear and cytosolic fractions (Figure 1B). Moreover, the cathepsin L that accumulates in the nucleus of U251 cells is much more catalytically active,[23] suggesting that gliomas with mutant p53 expression have extremely different cathep-
sin L activity after IR. Mutant p53 genes may drive a mutator phenotype, resulting in radioresistance through differential p53 transactivation. We hypothesized that upregulated cathepsin L expression may be related to radioresistance in a mutant p53 glioblastoma cell line. Upon malignant transformation, cathepsin L often translocates to the cell surface and is secreted into the surrounding medium to modify the extracellular matrix. Therefore, we examined whether IR can influence cathepsin L secretion in glioma cells and thereby interfere with radioresistance. U251 and U87 cells were treated with IR, and the levels of cathepsin L secreted in the conditioned media were measured using an ELISA kit. The levels of secreted cathepsin L in the conditioned media of U251 cells treated with radiotherapy were higher compared to the levels in the untreated group. However, cathepsin L secretion in the conditioned medium of U87 cells treated with IR showed no significant correlation with the levels in the untreated group (Figure 1C). According to these results, we chose U251 cells for the following experiments.

U251 glioma cells are radiosensitized via cathepsin L suppression

As mentioned previously, cathepsin L expression is exclusively elevated in U251 cells and in the conditioned medium after IR, and cathepsin L expression levels may correlate with the degree of radioresistance. The crucial role of cathepsin L in the malignancy of brain tumors has led to the development of novel cathepsin L inhibition strategies. A specific cathepsin L inhibitor, Z-FY-CHO, was used to corroborate our findings. The effects of IR and Z-FY-CHO treatments on cell survival were determined by conducting clonogenic assays to identify the function of cathepsin L in cell viability. As shown in Figure 2A, the inhibition rate was lower than 10% when the cells were treated with Z-FY-CHO (2.5–10 μmol/L) alone, while the clonogenicity of U251 cells that were treated with both Z-FY-CHO and IR was significantly reduced. The analysis was performed by comparing the SF values for cells left untreated (SF=1), cells treated with the cathepsin L inhibitor Z-FY-CHO (SF=0.85), cells treated with radiation (SF=0.83) and cells treated with Z-FY-CHO+4 Gy IR (SF=0.22). The cells treated with both Z-FY-CHO and IR generated a reduced number of colonies compared with the Z-FY-CHO-treated cells (P<0.01). As shown in Figure 2A, our results indicated that the clonogenicity of U251 cells treated with IR and Z-FY-CHO was significantly reduced in an ionization dose-dependent manner compared with IR treatment alone (treatment with Z-FY-CHO+IR resulted in a SER of 1.31, P<0.01). To demonstrate the function of cathepsin L in U251 glioma cells after IR, endogenous cathepsin L was knocked down in cells using short hairpin RNA (shRNA) interference, and then RT-PCR and Western blot analyses were conducted to confirm that cathepsin L expression was reduced in the cathepsin L knockdown (cathepsin L shRNA) cells (Figure 2B). Next, the cells were exposed to increasing IR doses (0, 2, 4, 6, and 8 Gy), and a dose-dependent reduction in clonogenic survival was observed. The cathepsin L shRNA cells also exhibited higher radiosensitivity than the Con shRNA U251 cells (Figure 2A). The results were analyzed by comparing the SF values of cells left untreated (SF=1), cells treated with radiation (SF=0.140) and cells treated with cathepsin L shRNA+8 Gy IR (SF=0.018), and the difference in these values was statistically significant (P<0.01). The SER of cathepsin L shRNA U251 cells was 1.54 (Figure 2A). These results indicated that cathepsin L inhibition could sensitize U251 glioma cells to IR.

Cathepsin L inhibition prevents the repair of radiation-induced DNA damage

The repair of DNA damage, such as double-strand breaks (DSBs), is an important determinant of cellular radiosensitivity. We performed single-cell gel electrophoresis assays (alkaline comet assays) to study the DNA damage in U251 cells caused by cathepsin L inhibition. Figure 3 shows that a significantly faster DNA damage repair rate was observed in the Con shRNA cells compared with the Z-FY-CHO-pre-treated cells or post-IR cathepsin L shRNA-transfected cells. Over 90% of the repair was completed 30 h following IR in the Con shRNA cells, whereas the cathepsin L-suppressed cells remained unrepaired (65%). These data suggest that cathepsin L inhibition may affect the DNA repair level.

Phosphorylated H2AX (histone H2AX protein) is designated γ-H2AX; γ-H2AX, as an early response to DNA damage, is considered a marker of DSBs. Normally, the level of γ-H2AX is low in mammals, unless a DSB is introduced, eg, by ionizing radiation. As a key regulator of IR-induced nuclear foci formation, γ-H2AX functions as a barrier for the accumulation and retention of the central components of the signaling cascade initiated by DNA damage. Thus, high levels of γ-H2AX suppress DNA damage repair. Considering the important function of this protein in DNA repair, particularly after DNA damage is induced, γ-H2AX was quantified by Western blot. The level of γ-H2AX was increased in the IR alone group and particularly in the combined treatment group (Figure 4A and 4B) compared with the untreated group. Ku70, which is one of the key regulatory subunits of the DNA-dependent protein kinase, has an important function in the repair of DNA DSBs. Our results showed that the Ku70 protein level of the combined treatment group was significantly decreased compared with the IR alone group. These results suggest that the increased level of γ-H2AX and decreased level of Ku70 may be involved in the increased radiosensitivity induced by cathepsin L suppression.

Rad51, which is induced by ionizing radiation, assists in the repair of DNA DSBs and negatively correlates with tumor radiosensitivity. We analyzed Rad51 expression levels in U251 cells after different treatments were performed. The Rad51 protein level of cathepsin L-inhibited cells significantly decreased after IR treatment (Figure 4A) compared with the IR group. Based on the results of the SF value analysis and the DSB repair kinetics, cathepsin L inhibition clearly modulates radiosensitization.
Cathepsin L inhibition increases IR-induced G2/M phase arrest

Our previous study demonstrated that nuclear translocated cathepsin L can process the CDP/Cux transcription factor and revealed an important role for cathepsin L in controlling cell cycle progression[7]. We analyzed the cell cycle phase distribution by performing flow cytometry to assess whether cathepsin L inhibition-induced radiosensitivity in glioma cells is also associated with altered cell cycle distribution. Unlike the unirradiated controls, the cell percentages at G2/M phase were higher in the cathepsin L-suppressed U251 cells. Moreover, the G2/M cell cycle arrest in the cells treated with both cathepsin L inhibition and IR was more evident, particularly at the 48 h time point (Figure 5A), compared with that in the irradiated cells. These results may explain the cathepsin L suppression-induced radiosensitivity in glioma cells. However, the combined treatments for 24 and 72 h did not result in significant increases in the cell percentages at G2/M phase compared with IR alone (data not shown).

The results described above indicated that cathepsin L-inhibited, IR-treated U251 cells underwent cell cycle arrest. Therefore, we examined the expression of cell cycle-associated molecules. In particular, cyclins A and B1, which have a close

Figure 1. Changes in cellular cathepsin L following irradiation (IR). (A) Western blot analysis of cathepsin L protein levels in the nuclear fraction, cytosol fraction and total cell lysate at 48 h post-IR. (B) Cells were treated with 8 Gy IR and analyzed at 24 h post-IR by immunofluorescence using an anti-cathepsin L antibody. (C) The cathepsin L level in the conditioned medium at 24 h after irradiation was measured in triplicate, and the experiment was repeated at least twice to confirm the results. *P<0.05, **P<0.01 compared with the control group. The amount of secreted cathepsin L is presented in pg/mL. Con, control; IR, irradiation-treated samples.
with G2/M arrest, were selected for Western blot analysis. During G2/M phase transition, cyclin B1 binds to cell division cycle 2 (CDC2, also called cyclin-dependent kinase 1, CDK1) to form the mitosis-promoting factor that facilitates the transition from G2 to M phase of the cell cycle[30, 31]. Cyclin A downregulation by shRNA induces S and G2/M phase arrest with increased porcine circovirus type 2 (PCV2)[32]. Figure 5B shows that the protein levels of cyclins A and B1 increased after the cells were exposed to IR; however, the protein level of cyclin A was decreased compared with that in the IR-treated group when the cells were knocked down with cathepsin L or pretreated with Z-FY-CHO at 10 µmol/L for 12 h. In addition, cyclin B1 upregulation was observed after the cells were treated with Z-FY-CHO or transfected with cathepsin L shRNA after IR treatment. These data suggest that cathepsin L suppression induced cell cycle arrest at the G2/M phase in the irradiated U251 cells.

**The effect of cathepsin L inhibition on IR-induced apoptosis**

We assessed the ability of Z-FY-CHO+IR to induce apoptosis to study the mechanism by which cathepsin L inhibition affects the radiosensitivity of glioma cells. Figure 6A shows that U251 cells that were treated with IR underwent morphological changes, such as nuclear chromatin condensation, that are consistent with fragmented, apoptotic nuclei. Furthermore, the morphological changes in the Z-FY-CHO+IR-treated cells were more significant than those in the IR-treated cells (Figure 6A). Cotreatment with Z-FY-CHO and IR for 24 h did not result in significant morphological changes compared with the control group (data not shown).

Bax and Bcl-2, two of the most important proteins associated with apoptosis, have a ratio that is considered critical for cell survival or death[33–36]. To explore the mechanism by which cathepsin L inhibition promotes apoptosis, we examined the expression levels of Bcl-2 and Bax. Figure 6B shows that Bax was upregulated but Bcl-2 was downregulated when the cells were pretreated with Z-FY-CHO or transfected with the cathepsin L shRNA compared to the levels in the IR alone group.

To confirm the role of cathepsin L inhibition in apoptosis, the levels of apoptosis in U251 cells treated as described above were also measured by flow cytometry. We found that cathepsin L inhibition resulted in a higher percentage of early apoptotic cells at 72 h post-IR in U251 cells. However, an insignificant difference was observed between the Z-FY-CHO+IR treatment and IR alone treatment at the 48 h time
From these results, we conclude that the role of cathepsin L in apoptosis is uncertain. Furthermore, these data indicate that the enhanced radiosensitivity U251 cells upon cathepsin L inhibition likely only slightly correlated with its apoptotic response to radiation.

Discussion
In this study, we reported a previously unrecognized function of cathepsin L inhibition in enhancing radiosensitivity. We also described a possible mechanistic function by which cathepsin L inhibition contributes to the enhanced radiosensitivity of glioma cells. We discovered that radiation treatment increased cathepsin L expression and nuclear uptake in U251 cells but not U87 cells (Figure 1). In the last 20 years, many studies have reported that cathepsin L gene expression is regulated by transcription factors during malignant tumor progression. In 1997, Ishidoh K first indicated that early growth response (Egr) family proteins are involved in activating the cathepsin L gene in SR-3Y1-2 cells[37]. The Egr-1 gene is a member of the immediate early gene family, and its promoter can be rapidly induced by various stimuli such as irradiation[38]. Egr-1 gene transactivation contributes to mutant p53 gain of function[39]. The Egr-1 promoter induced the expression of downstream genes after irradiation. Srinaman V et al documented that three Sp1/Sp3 binding sites with one overlapping Egr-1 binding site in the promoter region are critical for cathepsin L transactivation[40]. Taken together, these findings suggest that Egr-1 plays an important role in mutant p53-regulated cathepsin L activation. Therefore, we hypothesized that wild type and mutant p53 differential regulation of Egr-1 transcription contributes to cathepsin L activation because an important difference of cathepsin L activity was found between the U251 and U87 cells. Based on these observations, further experimentation to determine the role of this active protease in tumor progression is needed.

Previously, the nuclear isoform of cathepsin L was shown to be able to regulate the proteolytic processing of CDP/Cux, while two short CDP/Cux isoforms, p75 and p110, were found to be responsible for malignancies in several organs.
and cell types[24]. Additionally, cathepsin L was observed in the nucleus after G1 phase of the cell cycle. All these results indicate that increased cathepsin L expression in the nucleus may also be involved in its regulation of cancer progression, including radiosensitivity. Considering that increased cathepsin L expression and activity in the nucleus have important functions in cell proliferation and that cathepsin L inhibition or downregulation can lead to cell death induced by anticancer agents[13], we determined whether increased cathepsin L expression is involved in cell viability following IR. A previous study revealed that cathepsin L silencing in p53 wild type glioma cells results in a significant increase in the proportion of apoptotic cells, whereas cathepsin L silencing in p53-mutant U251 cells slightly increases the proportion of apoptotic cells[41]. However, our clonogenic assay results revealed that cathepsin L inhibition was able to sensitize p53-mutant U251 cells in response to IR (Figure 2), indicating that cathepsin L could be critical in mediating U251 glioma cell radiosensitivity.

Further experiments are needed to elucidate the mechanism by which cathepsin L inhibition enhances radiosensitivity. Radiation interferes with cell division by causing DNA damage to induce relevant toxic lesions[42]. Cells have evolved complex and well-designed mechanisms to detect DNA damage and subsequently initiate DNA repair. If the repair fails, then apoptosis pathways are triggered to eliminate the damaged cells. These processes ensure the genomic integrity and stability of cells[43]. In our study, alkaline gel electrophoresis comet assays revealed that the combined cathepsin L suppression and IR treatment in U251 cells resulted in distinct DNA comet tails and that this DNA damage was maintained, whereas the IR-induced DNA damage was eventually repaired (Figure 3A). Thus, the slower repair kinetics due to cathepsin L inhibition may be a major factor that contributes to radiosensitivity enhancement. In addition, γ-H2AX, which promotes the enrichment of DNA repair proteins at damaged DNA sites, is positively associated with radiosensitivity[44, 45]. γ-H2AX levels increased more significantly after the combined treatment at 6 h post-IR compared with the IR treatment (Figure 3B). Because high levels of γ-H2AX suppress DNA damage repair, the combined treatment group indicated a poor ability to repair DNA damage. This indication was consistent with...
Figure 5. Effect of cathepsin L inhibition on irradiation (IR)-induced G$_2$/M phase arrest. (A) Cell cycle phase distribution of cells that were pretreated with Z-FY-CHO or transfected with Con shRNA or cathepsin L shRNA. Then, the cells were treated with 8 Gy IR or unirradiated. (B) The cells were pretreated with Z-FY-CHO at 10 µmol/L (or untreated) for 12 h or transfected with cathepsin L shRNA or Con shRNA. The cells were analyzed at 48 h post-IR by Western blot to determine cyclin B1 and A protein levels. At least 3 independent experiments were performed. Mean±SD. n=3. $^b$P<0.05, $^c$P<0.01 compared with the control group. $^e$P<0.05, $^f$P<0.01 compared with the IR group.
with the results in Figure 3A that >90% of DSBs were repaired 30 h after irradiation, while only 35% of the DSB repair was completed in the combined treatment group within the same period. By contrast, Rad51 and Ku70, which have important functions in double-strand DNA repair, decreased after the combined treatment was administered compared with those in the IR treatment (Figure 3B). However, the combined treatment for 1 h post-IR exhibited a similar but less significant result (data not shown). Thus, these results indicated that cathepsin L inhibition was able to decrease cell viability and increase the sensitivity of U251 cells to IR. This finding likely elucidated the further mechanism of action in U251 cells.

Cell cycle checkpoints are another defense mechanism to protect cells from DNA damage. Many anticancer agents and radiotherapy treatments regulate cancer cell growth via cell cycle arrest at G0/G1, S, or G2/M phases, and then induce apoptotic cell death [46–49]. Previous studies have indicated that various anticancer drugs and radiotherapy treatments induce cell cycle arrest at the G2/M phase [50, 51]. Cyclins A and B1 are known to regulate G2/M cell cycle progression [52]. High levels of cyclin A usually correspond to a high proliferation rate, whereas a decrease in cyclin A induces G2/M phase...

Figure 6. Effect of cathepsin L inhibition on irradiation (IR)-induced expression of Bax and Bcl-2 proteins. (A) Hoechst staining of the cells that were pretreated with Z-FY-CHO at 10 µmol/L (or untreated) for 12 h. The resulting cells were treated with 8 Gy IR and analyzed at 48 and 72 h post-IR. (B) The cells were pretreated with Z-FY-CHO at 10 µmol/L (or untreated) for 12 h or transfected with cathepsin L shRNA (or Con shRNA). The cells were treated with IR and analyzed at 72 h post-IR by Western blot analysis of Bax and Bcl-2 protein levels. Mean±SD. n=3. *P<0.05, **P<0.01 compared with the control group. †P<0.05, ‡P<0.01 compared with the IR group.
arrest\textsuperscript{[32, 33]}. Cyclin B1 shuttles between the cytoplasm and nucleus until the activation of cyclin B1/CDC2 complexes, which are required for G\textsubscript{2}/M phase arrest of the cell cycle\textsuperscript{[54]}. In our study, more evident cell cycle arrest in the G\textsubscript{2}/M phase was observed in the combined treatment group at 48 h post-IR than in the IR treatment group (Figure 4A). Moreover, the radiation treatment likely induced cyclin B1 expression, which was higher in cells treated with Z-FY-CHO+IR or transfected with cathepsin L shRNA (Figure 4B). Although cyclin B1 is required for mitosis, cyclin B1 degradation is required for cells to leave mitosis\textsuperscript{[50]}. In our study, cathepsin L inhibition and radiation likely induced irreversible G\textsubscript{2}/M arrest.

Cathepsin L inhibition is also able to delay G\textsubscript{1} phase progression and S phase entry by downregulating the genes that are expressed and upregulated during S phase, such as the cyclin A gene\textsuperscript{[55]}. A high cyclin A level was observed in the irradiated cells, whereas the cyclin A level decreased in the cells pretreated with Z-FY-CHO or transfected with cathepsin L shRNA (Figure 4B). These data indicated that decreased cyclin A levels also have an important function in the enhanced G\textsubscript{2}/M phase arrest and delayed G\textsubscript{1}/S transition.

In addition, apoptosis is genetically regulated programmed cell death that has two major pathways: the extrinsic pathway and the intrinsic pathway\textsuperscript{[56, 57]}. The Bcl-2 family of proteins regulates the mitochondria-apoptosome-mediated apoptotic intrinsic pathway. Furthermore, the balance between the expression levels of Bax and Bcl-2 is considered critical in determining cell survival or death\textsuperscript{[59]}. Faulty apoptosis is also a commonly known mechanism that leads to radiation therapy resistance\textsuperscript{[59]}. In our study, Hoechst 33258 staining and Bax and Bcl-2 expression levels showed that radiation sensitization in the combined treatment group was due to apoptosis induced by cathepsin L inhibition. Somewhat unexpectedly, Annexin V-FITC/PI staining revealed that the higher rates
of apoptosis in the combined treatment were not significant, particularly at 48 h post-IR. The role of cathepsin L in apoptosis is seemingly contradictory to the results of other studies. No definitive conclusions can be drawn because of the lack of other studies using human cell lines\(^{[13]}\). Therefore, we infer that the effect of cathepsin L inhibition on radiosensitivity is only slightly related to its apoptotic response to radiation.

In summary, our study demonstrated that cathepsin L is involved in radiosensitivity modulation. In particular, we demonstrated that cathepsin L inhibition caused by exogenously added inhibitors or by shRNA-mediated silencing resulted in enhanced radiosensitivity of the p53-mutant glioma cell line U251. We also revealed that this mechanism may be associated with enhanced G\(_2\)/M phase cell cycle arrest and reduced DNA damage repair activities. These findings may expand the current knowledge regarding cathepsin L and radiosensitivity regulation in glioma cells. In general, our results suggest that cathepsin L suppression can be potentially applied to future radiotherapy.

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**Author contribution**

Qing-qing ZHANG and Wen-juan WANG conducted the studies, analyzed the data and prepared the manuscript; Jun LI and Neng YANG participated in the experiment operation; Gang CHEN and Zhong WANG participated in the statistical analyses; Zhong-qin LIANG designed study and critically revised the manuscript.

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