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Original Articles

Knockdown of Cathepsin L promotes radiosensitivity of glioma stem cells both in vivo and in vitro

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ABSTRACT

The presence of glioma stem cells (GSCs) in tumor is relevant for glioma treatment resistance. This study assessed whether knockdown of Cathepsin L can influence GSC growth, tumor radiosensitivity, and clinical outcome. Protein levels of Cathepsin L and stem cell markers (CD133 and Nestin) were analyzed in samples from 90 gliomas of different WHO grades and 6 normal brain tissues by immunohistochemistry. Two glioma stem cell lines with overexpressed Cathepsin L were stably transfected with Cathepsin L short hairpin RNA expression vectors. The effects of Cathepsin L inhibition on radiosensitivity, self-renewal, stemness, DNA damage, and apoptosis were evaluated. In addition, an intracranial animal model and subcutaneous tumor xenografts in nude mice were used to assess tumor response to Cathepsin L inhibition in vivo. Our results proved that expressions of Cathepsin L and CD133, but not of Nestin, correlated with malignant grades of glioma tissues. GSCs with high Cathepsin L and CD133 co-expression were extraordinarily radioresistant. Cathepsin L inhibition with radiotherapy significantly reduced GSC growth, promoted apoptosis, and improved radiosensitivity. Knockdown of Cathepsin L resulted in a dramatic reduction of CD133 expression, as well as the decreased phosphorylation of DNA repair checkpoint proteins (ATM and DNA-PKcs). Furthermore, combination of Cathepsin L inhibition and radiotherapy potently blocked tumor growth and decreased blood vessel formation in vivo. Taken together, these findings suggest Cathepsin L as a promising therapeutic target for clinical therapy in GBM patients.

Introduction

Glioblastoma (GBM) is the most aggressive and lethal primary brain tumor in adults; this disease recurs in a large number of patients despite the standard of care treatment. Nevertheless, radiotherapy remains the major form of therapy administered post-surgery for GBM [1,2]. In recent years, many studies have demonstrated that radioresistance in GBM is attributable to a small population of glioma cells called glioma stem cells (GSCs), which exhibit self-renewal and multipotent differentiation, and are suggested to be the cause of GBM recurrence [3]. Despite controversial results, CD133 is the most accredited stem cell marker that is enriched after radiotherapy [4,5].

Most of the recent studies being conducted focus on targeting these radioresistant GSCs. One proposed attractive approach to directly target GSCs is by inducing differentiation, which makes the cells more amenable to radiotherapy [6,7]. Our prior observations have confirmed that induction of autophagy promotes differentiation of GSCs and their resultant radiosensitivity through cotreatment with rapamycin and ionizing radiation (IR) both in vivo and in vitro [8]. Another potential strategy would be to exploit molecular biological techniques that inactivate the DNA damage checkpoint response and decrease the DNA repair capacity of GSCs [9,10]. Similarly, we have previously shown that downregulation of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) radiosensitizes GSCs by inducing autophagy [11]. Furthermore, we used a dual PI3K/mTOR inhibitor, NVP-BEZ235, which increases the radiosensitivity of GSCs in vitro by activating autophagy associated with synergistic increase of apoptosis and decrease of DNA repair capacity [12]. In particular, our recent work has shown that inhibition of Cathepsin L, a lysosomal cysteine proteinase, can radiosensitize U251 cells by regulating NF-κB signaling [13,14]. This finding may offer a selective therapeutic window to overcome the radioresistance of GBM.

Cathepsin L has been proven to be dramatically upregulated in malignant gliomas and correlates with the malignant progression

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of human GBM [15,16]. This cysteine proteinase is translated as preprocathepsin L, transported via the Golgi apparatus as procathepsin L, stored as mature Cathepsin L in lysosomes, and proteolytically active when present in the acidic lysosomal compartment [17]. Various processes are involved in activating Cathepsin L, including cellular transformation [18], differentiation [19], motility [20], apoptosis [21], and angiogenesis [22]. In the past two decades, research has focused on Cathepsin L and its role in human malignant gliomas. Previous evidence has shown that downregulation of Cathepsin L by antisense oligonucleotides significantly impaired glioma cell invasion in vitro and markedly induced glioma cell apoptosis [23,24]. Inhibition of Cathepsin L may lower the apoptotic threshold of GBM cells by upregulation of p53 and transcription of caspases 3 and 7 [25]. Moreover, Cathepsin L knockout mice exhibited decreased cell proliferation and tumor growth [26]. Overall, targeting the elevated levels of Cathepsin L in human brain cancer by administering Cathepsin L inhibitors or by genetically manipulating its expression would render glioma cells more vulnerable to radiotherapy and may provide a potential therapeutic target for GBM treatment [27]. We have previously confirmed the radiosensitive effect of Cathepsin L inhibition in glioma cells in vitro [13,14], and we have continued our work both in vivo and in vitro to target the relatively more radioresistant GSCs.

Materials and methods

Patients and tumor specimens

The tissue microarray used in the study was provided by Professor Huang Qiang of the Department of Neurosurgery in the Second Affiliated Hospital of Soochow University, Suzhou, China [28]. This study was carried out in accordance with the principles of the Declaration of Helsinki and approved by the Ethics Committee of Soochow University Medical School. Signed informed consent was obtained from the patients prior to sample acquisition. Each tissue microarray slide contained 90 glioma samples with different grades, including tissues from 12 pilocytic astrocytoma (grade I), 32 diffuse astrocytoma (grade II), 23 anaplastic astrocytoma (grade III), and 23 glioblastoma multiforme (grade IV). The samples were classified according to the 2007 World Health Organization (WHO) Classification of Tumors of the Central Nervous System. Meanwhile, 6 normal brain tissue samples were obtained from cerebral decompression surgery. All of the 96 tissue specimens were obtained from the Second Affiliated Hospital of Soochow University.

Immunohistochemistry

The paraffin embedded tissue microarray and xenografted tumors were sectioned at 4 μm for identification of indicated proteins using a microtome (Leica). Immunostaining was conducted using Vectastain ABC kit (Vector) following the manufacturer's instructions. Briefly, slides were deparaffinized, rehydrated, and treated with citric acid solution to prepare for immunohistochemical studies. After blocking endogenous peroxidase activity with preincubation in 3% hydrogen peroxide solution, the slides were incubated in blocking solution (PBS, 3% bovine serum albumin) and sequentially incubated with primary antibodies, including anti-Cathepsin L (Abcam, ab6314), anti-CD133 (Millipore, MAB4399), anti-Nestin (Santa Cruz Biotechnology, sc-23927), and anti-CD31 (Abcam, ab76533). The sections were counterstained with hematoxylin (Sigma) for nuclear staining. Negative control slides without the primary antibodies did not exhibit nonspecific staining. In this study, these slides were independently evaluated by two investigators who were blinded to clinical information. To evaluate the expression levels of the protein, the intensity of staining was scored according to a semiquantitative 4-grade scale as follows: 0, none (no positive tumor cells); 1, weak (<33% positive tumor cells); 2, moderate (33–67% positive tumor cells); 3, strong (>67% positive tumor cells). An intensity score of ≥ or = 2 was considered as high expression, whereas <2 was regarded as low expression.

Cell line and culture conditions

SU2 and SU3 human glioma stem cell lines were obtained from Professor Huang Qiang of the Department of Neurosurgery, at the Second Affiliated Hospital of Soochow University, Suzhou, China [8,11,29–31]. These two cell lines were grown at 37 °C in a humidified incubator with 5% CO_2 in serum-free Dulbecco's modified Eagle's media (DMEM)/F12 (Gibco Life Technologies) supplemented with recombinant human fibroblast growth factor (bFGF, 20 ng/mL; Invitrogen), recombinant human epidermal growth factor (EGF, 20 ng/mL; Invitrogen), and 1% N_2 supplement (Gibco Life Technologies).

Construction of shRNA expression plasmids

A pGPU6/Neo plasmid (Ambion) was used in the construction of the shRNA expressing vector according to the manufacturer's instructions. The annealed oligonucleotide fragments encoding short hairpin transcripts corresponding to Cathepsin L were designed as follows: (5′-CACCGCGATGCACAACAGATTATACTTCAAGAGATATAATCTGTTGTGCATCGCTTTTTTG-3′; 5′-GATCCAAAAAAGCGATGCACAACAGATTATACTCTCTTGAAGTATAATCTGTTGTGCATCGC-3′).

The non-targeting empty plasmid was used as the control shRNA plasmid.

Transfection and isolation of stable cell clones

The human glioma stem cell lines SU2 and SU3 were transfected separately with control shRNA plasmid or the Cathepsin L shRNA plasmid using Lipofectamine 2000 reagent (Invitrogen). The entire process was conducted according to the manufacturer's guidelines. After 2 days of transfection, the medium was replaced with DMEM/F12 cells containing growth factors and 600 μg/mL G418 (Roche) for 2 weeks. After the dilution culture was limited under the pressure of G418, several clones of each transfection group were selected and screened for low Cathepsin L expression by Western blot and RT-PCR. Four stable transfected cell clones were selected for further experiments and each designated as SU2-Con shRNA, SU2-Cathepsin L shRNA, SU3-Con shRNA and SU3-Cathepsin L shRNA.

Reverse transcription-PCR

Cathepsin L mRNA levels were quantified using reverse transcription-PCR (RT-PCR). The total RNA of the cells was isolated with TRIzol reagent (Invitrogen) and 0.5 μg of total RNA was used to synthesize cDNA using an RT-PCR kit (Takara). For Cathepsin L mRNA, the primer pair, 5′-AAACACAGCTTCACAATGGCC-3′ and 5′-TTTGAAAGCCATTCATCACCTG-3′, was used. For amplification of GAPDH mRNA, the primer pair, 5′-TACTAGCGGTTTTACGGGCG-3′ and 5′-TCGAACAGGAGGAGCAGAGAGCGA-3′, was used. The amplification products were electrophoresed in 1.0% agarose gel. Cathepsin L levels were normalized to GAPDH levels within the same sample.

Western blot

Western blot was conducted as previously described [12]. The primary antibodies used in this study are as follows: anti-Cathepsin L (Abcam, ab6314), anti-DNA-PKcs (Abcam, ab32566), anti-pDNA-PKcs (phospho S2056, Abcam, ab124918), anti-ATM (Abcam, ab32420), anti-pATM (Ser1981, Millipore, 05-740), and loading control anti-GAPDH (Abcam, ab181602). Finally, the primary antibodies were probed with rabbit or mouse secondary antibodies labeled with DyLight 800 (KPL) and scanned with the Odyssey Infrared Imaging System (LI-COR).

Irradiation condition

The cells and mice were irradiated using a 6-MV X-ray linear accelerator (model: PRIMUS, D. E., Siemens, Malvern, PA, USA) at a dose rate of 198 cGy/min. After irradiation, cells were placed in the incubator and samples were collected at the indicated time points (0, 1, 12, 24 h).

Clonogenic survival assay

Cells were seeded in triplicate in 6-well plates at a density of 5×10^2 cells per well. The next day, the plates were exposed to 0 (sham), 2, 4, 6, or 8 Gy of IR. After incubation for 2 weeks, colonies were washed twice with PBS, fixed with methanol, and stained with 0.5% crystal violet (Sigma Aldrich). The colonies containing more than 50 cells were counted as surviving clones. Surviving fraction (SF) was determined by normalizing the average plating efficiency for each dose to the plating efficiency at 0 Gy. The sensitization enhancement ratio (SER) was calculated by taking the ratio at the mean lethal dose (GSCs-Con shRNA radiation dose divided by the GSCs-Cathepsin L shRNA radiation dose). SER values > 1 indicate enhancement of radiosensitivity.

Neurosphere formation assay

The cells were plated at a density of 1×10^3 cells per well in 12-well plates and irradiated for 8 Gy. 7 days later, plates were examined for neurosphere formation using an inverted microscope. Neurospheres with diameter > 100 μm were counted.

Immunofluorescence staining

Cells were plated on glass coverslips (Fisher Scientific). After treatment with 8 Gy of irradiation, cells were fixed with 4% paraformaldehyde. Following the PBS wash, cells were permeabilized using 0.2% Triton X-100, incubated in a blocking solution (PBS, 3% bovine serum albumin), and further incubated overnight at 4 °C with primary antibodies, including anti-Cathepsin L (Santa Cruz Biotechnology, sc-6500), anti-CD133 (Millipore, MAB4399), and anti-γ-H2AX (phospho S139, Abcam, ab18311). Thereafter, biotinylated horse anti-goat IgG antibodies (Vector Labs) were added and