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Original Article

Dying glioma cells establish a proangiogenic microenvironment through a caspase 3 dependent mechanism

Xiao Feng [a, 1], Yang Yu [a, 1], Sijia He [a], Jin Cheng [a], Yanping Gong [a], Zhengxiang Zhang [a], Xuguang Yang [a], Bing Xu [a], Xinjian Liu [a], Chuan-Yuan Li [b, ***], Ling Tian [c, **], Qian Huang [a, *]

[a] The Comprehensive Cancer Center and Shanghai Key Laboratory for Pancreatic Diseases, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 201620, China
[b] The Department of Dermatology, Duke University Medical Center, Durham, NC 27710, USA
[c] Institute of Translational Medicine and Shanghai Key Laboratory for Pancreatic Diseases, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 201620, China

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ABSTRACT

Vascular recovery or re-angiogenesis after radiotherapy plays a significant role in tumor recurrence, whereas molecular mechanisms of this process remain elusive. In this work, we found that dying glioma cells promoted post-irradiation angiogenesis through a caspase 3 dependent mechanism. Evidence *in vitro* and *in vivo* indicated that caspase 3 inhibition undermined proangiogenic effects of dying glioma cells. Proteolytic inactivation of caspase 3 in glioma cells reduced tumorigenicity. Importantly, we identified that NF-κB/COX-2/PGE_2 axis acted as downstream signaling of caspase 3, mediating proangiogenic response after irradiation. Additionally, VEGF-A, regulated by caspase 3 possibly through phosphorylated eIF4E, was recognized as another downstream factor participating in the proangiogenic response. In conclusion, these data demonstrated that caspase 3 in dying glioma cells supported the proangiogenic response after irradiation by governing NF-κB/COX-2/PGE_2 axis and p-eIF4E/VEGF-A signaling. While inducing caspase 3 activation has been a generally-adopted notion in cancer therapeutics, our study counterintuitively illustrated that caspase 3 activation in dying glioma cells unfavorably supported post-irradiation angiogenesis. This double-edged role of caspase 3 suggested that taming caspase 3 from the opposite side, not always activating it, may provide novel therapeutic strategies due to restricted post-irradiation angiogenesis.

Introduction

Gliomas, accounting for almost 80% of primary malignant brain tumors [1], are a devastating disease with uncontrollable proliferation and invasion, damage to surrounding brain tissue and profound neurological dysfunction [2,3]. In spite of endeavors to exploit new therapeutic strategies for gliomas [4–7], gliomas cunningly develop different compensatory mechanisms [8–11], leading to therapy resistance and unfavorable prognosis. For example, astrocytomas, including glioblastomas (GBMs), can establish microtube-dependent cell interconnection and form functional multicellular network, which protect tumor cells from radiotherapy-induced cell death and develop radioresistance [12].

Though radioresistance widely exists in gliomas, radiotherapy has long been the primary therapeutic modality for unresectable gliomas and also acts as the standard adjuvant approach in glioma treatment [13]. Nonetheless, in almost all patients with malignant gliomas, recurrence following initial treatment, including radiotherapy, inevitably occurs and represents grim outcomes [14]. Glioma growth and progression is heavily reliable on angiogenesis [15]. Thus, it is conceivable that vascular recovery or re-angiogenesis plays a crucial role in glioma recurrence following radiotherapy. For instance, evidence from both mice [16] and human specimens [17] suggested that the angiogenic pattern of

Abbreviations: COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2; eIF4E, eukaryotic initiation factor 4E; VEGF-A, vascular endothelial growth factor-A; GBM, glioblastoma.

* Corresponding author. Fax: +86 21 63240825.
** Corresponding author. Fax: +86 21 63240825.
*** Corresponding author. Fax: +86 21 63240825.
E-mail addresses: chuan.li@duke.edu (C.-Y. Li), tl09168@hotmail.com (L. Tian), qhuang@sjtu.edu.cn (Q. Huang).

[1] These authors contributed equally to this work.

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CXCL12-CXCR4 pathway may be responsible for GBM recurrence after radiotherapy.

Therefore, unveiling the proangiogenic mechanisms of glioma after radiotherapy is of great importance, because it would aid us in developing more useful strategies to reduce glioma recurrence following radiotherapy. One group reported that ionizing irradiation-induced MMP-9 upregulation promoted medulloblastoma angiogenesis by enhancing syndecan-1 shedding [18]. Another study demonstrated that depletion of DNA-dependent protein kinase catalytic subunit in GBM cells partly diminished irradiation induced-angiogenesis, with decreased VEGF secretion [19].

While these studies discovered important mechanisms underlying post-irradiation angiogenesis in gliomas, we still hope to identify the initial proangiogenic factor buried in the irradiated glioma microenvironment. Because ionizing irradiation induces a vast amount of glioma cell death, we hypothesize that these dying glioma cells may act as supporters, inflicting strong proangiogenic impacts on surrounding microenvironment.

Caspase 3 has been well established to function as executioner during cell apoptosis. However, accumulating interesting studies have identified growth-promoting roles of caspase 3 under various circumstances, such as fibrosis [20], wound healing and tissue regeneration [21], tumor repopulation [22], osteoclastogenesis [23] and oncogenic transformation [24]. Here, we therefore investigated whether caspase 3 in dying glioma cell mediated the proangiogenic effects following irradiation. We hope this caspase 3-mediated proangiogenic pathway could provide new therapeutic strategies to reduce glioma recurrence after radiotherapy.

Materials and methods

Cell culture and irradiation

U-87 MG (U87), human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HMEC-1) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, MA, USA) with supplementation of 10% fetal bovine serum (FBS) (Gibco, life technologies, Auckland, NZ). X-ray irradiation for cells was performed with an ONCOR linear accelerator (Siemens, Amberg, Germany), whose dose rate is 3.6 Gy/min.

Gene transduction

The pLEX lentiviral vector system (Open Biosystem, Huntsville, AL, USA) was used to transduce exogenous genes into target cells. The firefly luciferase (Fluc) and green fluorescent protein (GFP) fusion gene [22] and dominant-negative caspase 3 [22,24] with a key cysteine mutation in the catalytic domain of caspase 3 (C163A) were provided by Prof. Chuan-Yuan Li. Lentiviral vectors were packaged into live, replication-deficient lentivirus in 293T cells following the manufacturer's instructions. HUVEC-Fluc, HMEC-1-Fluc and U87 CASP3DN were constructed through lentivirus infection and subsequent puromycin selection at 3 μg/ml.

Measurement of endothelial cell proliferation with bioluminescence imaging

We used bioluminescence imaging to measure endothelial cell proliferation, because luciferase activity of Fluc-labeled endothelial cells was validated to be tightly correlated cell numbers. HUVEC-Fluc or HMEC-1-Fluc (100 cells) were seeded onto (1.5×10^4) of differentially irradiated U87 cells within 24 h after irradiation in 24-well plates. For transwell assay, HUVEC-Fluc were seeded onto hanging cell culture inserts of 0.4 μm pore size (PIHT12R48; Millipore, MA, USA). During the coculture period (7–10 days), culture medium was replaced by fresh 2% FBS DMEM every 2 days. Finally, to measure luciferase activity of HUVEC-Fluc and HMEC-1-Fluc, D-Luciferin potassium (bc219; Synchem UG & Co. KG, Felsberg/Altenburg, Germany) diluted in PBS (0.15 mg/ml) was added into each well before bioluminescence imaging.

Machines for bioluminescence imaging used in this study were NC100 instrument (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany), SPECTRAL Ami X (Spectral Instruments Imaging, Tucson, AZ, USA) and IVIS Lumina Series III (PerkinElmer, USA).

Conditioned medium preparation

Equal numbers of tumor cells were seeded into cell culture dishes overnight. Culture medium was replaced by low serum medium (2% FBS) before irradiation. 48 h after irradiation, culture medium was collected, centrifuged at 3000 rpm for 10 min, filtered with 0.22 μm filter unit and stored at −80 °C until use.

HUVEC migration assay

HUVEC migration was conducted with hanging cell culture inserts of 8 μm pore size (PIEP12R48; Millipore) for 24-well plates. Briefly, 600 μl conditioned medium was added into the lower chamber of every well and 200 μl serum free DMEM containing HUVECs (3×10^4) added on the top of inserts. 16–18 h later, HUVECs staying in the inserts were gently removed with cotton swabs. Migratory HUVECs were fixed with 4% paraformaldehyde and stained with crystal violet. Number of migratory HUVECs was measured by counting cells from five random fields under microscope.

Flow cytometric analysis

Cell apoptosis was detected by Accuri C6 Flow cytometer (BD Biosciences, CA, USA) with FITC Annexin V apoptosis detection kit (556547; BD Pharmingen™, San Diego, CA, USA). Procedures were conducted in accordance with the technical data sheet from the kit.

Confocal microscopy for phosphorylated histone H2A.X and cleaved caspase 3

Confocal microscopy was mainly performed as described [20]. Primary antibodies used here were phosphorylated histone H2A.X and cleaved caspase 3 (#9718; #9664; Cell Signaling Technology, MA, USA).

Western blot

Western blot analysis was performed as previously described [25]. Primary antibodies were against HA-tag, β-actin, COX-2, phospho-p65, caspase 3, cleaved caspase 3 and phospho-eIF4E (#3724; #4967; #12282; #3033; #9662; #9664; #9741; Cell Signaling Technology).

Matrigel plug assay

To perform matrigel plug assay, we used female nude mice at 4–6 weeks old. For each mice, 500 μl matrigel mixed with 2×10^6 either U87 or U87 CASP3DN cells treated with 10 Gy irradiation was subcutaneously implanted into either flank of three mice, respectively. 12 days after implantation, three mice were sacrificed and six plugs (three plugs containing irradiated U87 cells and the other three containing irradiated U87 CASP3DN cells) were collected. Skin vasculature formation adjacent to plugs was photographed (the number of independent plug-adjoining vasculature is three because there are three plug replicates) and then length of vessels and diameter of major vessels were evaluated with the software ImageJ.

Immunohistochemistry analysis

Immunohistochemistry analysis was conducted as previously described [26]. Primary antibody used here was against CD34 (#3569; Cell Signaling Technology) and VWF (sc-14014; Santa Cruz Biotechnology). The number of analyzed plug sections for immunohistochemistry analysis is five. The area of vessels was quantified with the software ImageJ.

Tumor xenograft assay

Female nude mice at 4–6 weeks old were utilized for tumor xenograft experiment. 100 μl PBS containing 2.5×10^6 U87 or U87 CASP3DN was subcutaneously injected into either hind leg, respectively. Every week tumor size was measured and tumor volume (V) was calculated according to the formula: $V = 0.5 \times \text{length} \times \text{width}^2$.

In silico analysis of correlation between CASP3 and angiogenesis markers

The 169 RNA-seq data from 161 GBM cases were acquired from TCGA (The Cancer Genome Atlas, http://cancergenome.nih.gov/). The original count data of RNA-seq were normalized with edgeR package [27] in R.

We also downloaded the microarray data from 220 glioma patients from CGGA (the Chinese Glioma Genome Atlas, http://www.cgga.org.cn/index.php?m=Page&a=index&id=42).

Enzyme-linked immunosorbent assay (ELISA)

To measure the PGE_2 concentration of cell culture supernatants, we used Prostaglandin E_2 Express ELISA Kit (500141; Cayman Chemical, MI, USA). To detect the VEGF-A concentration of supernatants, we used Human VEGF Valukine ELISA Kit (VAL106; R&D Systems, MN, USA). Procedures were carried out according to the guidebooks in the kits.

Other drugs

NS-398 and Z-DEVD-FMK were bought from Cayman Chemical and Ki8751 was from Selleckchem (TX, USA).