Epigallocatechin-3-gallate induces apoptosis and proliferation inhibition of glioma cell through suppressing JAK2/STAT3 signaling pathway

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Abstract: Excess proliferation and apoptosis inhibition are the major pathologic feature of glioma. Epigallocatechin-3-gallate (EGCG), a major polyphenol in green tea, has been considered a potential therapeutic and chemo-preventive agent for cancer. However, its effect and mechanism on glioma remain to be elucidated. In this study, the effect of Epigallocatechin-3-gallate (EGCG) on gliomas and its mechanism were investigated in cultured U251 cells by the methylthiazoletetrazolium (MTT), flow cytometry and western blotting. The results showed that treatment with EGCG can significantly suppress the U251 cell proliferation and induce the apoptosis of U251 cells. The mechanism of EGCG inducing apoptosis and proliferation inhibition of U251 cell were associated with suppressing JAK2/STAT3 signaling activation. Taken together, EGCG contribute to the favorable effects of treatment in U251 cell by suppressing JAK2/STAT3 signaling pathways activation.

Keywords: Glioma, epigallocatechin-3-gallate, proliferation, apoptosis, JAK2/STAT3

Introduction

Gliomas are one of the most widespread malignant cancers rooted in astroglial or astrocytes cells and can damage the central nervous system [1]. Conventional treatment incorporates the surgical resection, radiation, chemotherapy, and biological treatment. However, conventional therapies for Gliomas are ineffective or insensitive; Therefore, the survival rate is quite low, less than 1 year after standard treatment [2, 3]. Therefore, it is an urgent need to develop more therapy strategies or seek for safety and effective agents for Gliomas.

Epigallocatechin-3-gallate (EGCG) is the major component of polyphenols in green tea and is widely investigated due to its ability to anti-inflammatory, anti-proliferative, and anti-carcinogenic activity [4-6]; however, its molecular mechanisms of its action in Gliomas is still unknown.

Janus-activated kinase-2 (JAK2) Signal transducer and activator of transcription-3 (STAT3) signaling have been demonstrated that play an important role in proliferation, apoptosis of gliomas [7, 8] and EGCG have anti-tumor activation in skin tumors by suppressing JAK2/STAT3 signaling [9].

In this study, we investigated the anti-tumor effects of the EGCG on gliomas and the involvement of JAK2/STAT3 signaling in this process. We demonstrate that inhibition of JAK2/STAT3 signaling by EGCG may contribute to its anti-tumor action in gliomas.

Materials and methods

Reagents

EGCG was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The annexin V-FITC apoptosis detection kit was from Beckman Coulter (Fullerton, CA). Primary antibodies to survivin, Bcl-2, Bax, p-caspase-3, and p-PARP-poly (ADP-ribosyl) polymerase (PARP), b-actin and secondary antibodies were purchased from Santa-Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to JAK2, p-JAK2, STAT3, and p-STAT3 were purchased from Cell Signaling Technology (Beverly, MA). Human p-STAT3 small interfering RNA (siRNA) and control siRNA were all purchased from Santa Cruz Biotechnology.
Cells
Human gliomas cell lines U251 was from ATCC and was grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). To obtain the STAT3C-expressing cells, U251 cells were transiently transfected with plasmids containing pRC/CMV-vector and pRC/CMV-STAT3C-Flag using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). The murine cell line Renca was also obtained from ATCC and was grown in RPMI 1640 supplemented with 10% FBS.

MTT assay of cell proliferation
Cells, 3×10⁵ per well, were seeded in 96-well culture plates the day before EGCG treatment. After the treatment, 20 μl of MTT reagent, 5 mg/ml, was added to each well and incubated for 4 h at 37°C. At the end of incubation, the media were carefully removed by aspiration. One hundred microlitre of DMSO was then added to each well. The plate was gently vortexed for 30 min at room temperature. The absorbance of each well was measured at 490 nm. All experiments were repeated at least three times.

Apoptosis assay
U251 cell were seeded in 60-mm culture dishes in RPMI-1640 with 1% FBS. The following day, cells were treated with indicated concentrations of EGCG for 24-hours. After treatment, floating and attached cells were collected and stained with PI and Annexin V-FITC Apoptosis Detection kit (BD Biosciences) in FACS Wash Buffer (HBSS2/2 containing 2% FBS) according to the manufacturer’s instruction. Viable and apoptotic cells were analyzed by flow cytometry (Accuri C6). Data was analyzed using FlowJo software (Treestar).

Western blot
Total protein (20 mg) was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Membranes were blocked for 1 hour at ambient room temperature (ART) in 10% non-fat dry milk in TBST (16TBS with 0.1% Tween 20) followed by an overnight incubation at 4uC with primary antibodies in TBST with 5% BSA. Horseradish peroxidase-labeled antimouse or anti-rabbit secondary antibodies were added for 1 hour at ART and detected with Super Signal West Pico substrate (Pierce). Bands were measured as optical density using ImageJ software. The optical density of each band was normalized by b-actin optical density.

Plasmid transfection
U251 cells were transiently transfected with human STAT3 siRNA and control siRNA using LipofectamineTM 2000 (Invitrogen). After 24 hours transfection, cells were treated with EGCG or DMSO control for 24 hours and cell viability was measured siRNA and control siRNA using LipofectamineTM 2000 (Invitrogen).
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**Statistical analysis**

All experiments were performed in triplicates. The results are expressed as mean ± SD. For statistical analysis, Student’s T-tests were performed using SPSS software. Statistical significance was accepted at the level of $P < 0.05$.

**Results**

**EGCG inhibits the proliferation of U251 cells**

To determine whether EGCG has direct anti-tumor effects in gliomas cells, the U251 cells was treated with different concentrations of EGCG by MTT assay. As shown in Figure 1A, EGCG showed significant inhibition of cell proliferation in a concentration-dependent manner and proliferation inhibition is over 40% at 10 µg/ml (Figure 1A). Western blotting was also performed to determine the downstream factors mediating the effects of EGCG on U251 cells. The results showed that EGCG treatment of U251 can reduce the expression of key pro-proliferative proteins, survivin.

**EGCG induces apoptosis in U251 cells**

We next investigated whether EGCG induced the apoptosis of U251 cells. After treatment with EGCG for 72 hours, 50% U251 tumor cells were Annexin-V positive as defined by flow cytometry (Figure 2A). To further confirm the activation of EGCG in inducing apoptosis of U251 cells, we detected the expression levels of Bax, Bcl-2, activated caspase-3 and PARP cleavage. EGCG can significantly increase the expression of Bax, cleaved-caspase-3 and cleaved PARP, along with decreasing the expression of Bcl-2 in a concentration-dependent manner (Figure 2A). Collectively, these data indicated that EGCG has potent anti-proliferation and pro-apoptotic effects on human U251 cells.

**EGCG inhibits JAK2/STAT3 signaling in U251 cells**

To explore the underlying mechanisms of EGCG anti-tumor effect on U251 cells, we selected the JAK2/STAT3 signalling. STAT3 is constitutively activated in diverse cancers, including U251, we assessed whether EGCG inducing the apoptosis of U251 was associated with STAT3 inhibition. Theses results showed that EGCG had no effects on total STAT3 protein levels in U251.
Epigallocatechin-3-gallate on glioma cells

In this study, we evaluated the therapeutic potential of EGCG against gliomas and its potential mechanism of action. In line with previous study that EGCG has the anti-tumor action in the gliomas by suppressing the proliferation and inducing apoptosis of U251 cell [11, 12]. It is well known that activated STAT3 promotes tumorigenesis by inducing apoptosis and inhibiting proliferation [13, 14]. In various cancer types, including leukemias and solid cancers of the colon, prostate and pancreas, aberrant activation of STAT3 crucially contributes to cancer progression [15]. STAT3 is constitutively activated in human gliomas and is an independent prognostic indicator which indicated STAT3 represents a promising therapeutic target for the treatment of gliomas [16-18]. Several study showed several small molecule inhibitors of STAT3 could decrease the proliferation and induce apoptosis in tumor cell. But whether this inhibitor required STAT3 for its anti-tumor effects was not directly assessed. EGCG, a novel a major polyphenol in green tea, has been recently reported with anticancer effects, inhibiting growth of breast cancer, skin cancer and chronic myeloid leukemia cells [13-15]. Our results demonstrate that EGCG could suppress STAT3 activation, in part through inactivation of upstream JAK2 in U251 cell lines. JAK2 phosphorylate leads to the recruitment and activation of the STAT3, which then leads to STAT3-mediated transcriptional regulation. In our

EGCG-inducing apoptosis is regulated by STAT3 signaling in U251 cells

To further investigate whether STAT3 activity directly influences the biological effects of EGCG in U251 cells, an expression vector encoding a constitutively-active STAT3 mutant, STAT3C [10] or an empty control vector (vector) were transfected into U251 cells. Transfected cells were confirmed by Western blot analysis (Figure 4A Left). Expression of constitutively-active STAT3 in U251 cell promoted resistance to the anti-proliferative and pro-apoptotic effects of EGCG (Figure 4A-C Right). Our initial results (Figure 1B) showed that EGCG treatment inhibited several STAT3-regulated proteins important for tumor cell survival and proliferation. In agreement with this finding, siRNA-mediated knockdown of STAT3 in U251 cells significantly reduced the expression of known STAT3 downstream genes, survive (Figure 4B). We further demonstrated that siRNA-mediated knockdown of STAT3 sensitized U251 cells to the anti-proliferative and inducing-apoptosis effects of EGCG (Figure 4D-F).

**Discussion**

In this study, we evaluated the therapeutic potential of EGCG against gliomas and its potential mechanism of action. In line with previous study that EGCG has the anti-tumor action in the gliomas by suppressing the proliferation and inducing apoptosis of U251 cell [11, 12]. It is well known that activated STAT3 promotes tumorigenesis by inducing apoptosis and inhibiting proliferation [13, 14]. In various cancer types, including leukemias and solid cancers of the colon, prostate and pancreas, aberrant activation of STAT3 crucially contributes to cancer progression [15]. STAT3 is constitutively activated in human gliomas and is an independent prognostic indicator which indicated STAT3 represents a promising therapeutic target for the treatment of gliomas [16-18]. Several study showed several small molecule inhibitors of STAT3 could decrease the proliferation and induce apoptosis in tumor cell. But whether this inhibitor required STAT3 for its anti-tumor effects was not directly assessed. EGCG, a novel a major polyphenol in green tea, has been recently reported with anticancer effects, inhibiting growth of breast cancer, skin cancer and chronic myeloid leukemia cells [13-15]. Our results demonstrate that EGCG could suppress STAT3 activation, in part through inactivation of upstream JAK2 in U251 cell lines. JAK2 phosphorylate leads to the recruitment and activation of the STAT3, which then leads to STAT3-mediated transcriptional regulation. In our
Epigallocatechin-3-gallate on glioma cell

In the study, we found that the U251 cell is constitutive activation of STAT3 and EGCG treatment dramatically inhibits STAT3 activity, associated with upstream JAK2 inhibition. We further demonstrated that the anti-proliferative and pro-apoptotic effect of EGCG in U251 cells was mediated, in part, by inhibition of STAT3 activation. Activated STAT3 has been shown to protect tumor cells from apoptosis by inducing proliferation/survival genes and blunting pro-apoptotic genes [19].

Conclusions

In summary, we demonstrated convincing evidence that EGCG had the effects of inducing...
apoptosis and inhibiting proliferation in U251 cells in vitro through inhibiting JAK2/STAT3 signaling activation. All together, our data supports that EGCG could be a good alternative therapy for treatment of gliomas in the clinical practice.

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Disclosure of conflict of interest

None.

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Epigallocatechin-3-gallate on glioma cell
