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·Original Article·

Epigallocatechin-3-gallate induces apoptosis, inhibits proliferation and decreases invasion of glioma cell

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ABSTRACT

Epigallocatechin-3-gallate (EGCG), a major polyphenol in green tea, has been considered a potential therapeutic and chemopreventive agent for cancer. Glioma is a malignant tumor with high mortality but effective therapy has not yet been developed. In this study, we found that EGCG induced apoptosis in U251 glioma cells *via* the laminin receptor (molecular weight 67kDa) in a time- and dose-dependent manner, decreased their invasiveness and inhibited their proliferation. The mitogen-activated protein kinase pathway was shown to be involved in glioma cell apoptosis and proliferation. Furthermore, the mRNA levels of matrix metalloproteinase (MMP)-2 and MMP-9 were reduced after EGCG treatment. These results suggest that EGCG has important therapeutic effects with low toxicity and side-effects, and could be used in cancer chemoprevention.

Keywords: glioma; epigallocatechin-3-gallate; proliferation; apoptosis; invasion

INTRODUCTION

Gliomas are one of the most prevalent malignant cancers derived from astrocytes or astroglial cells and can fatally damage the central nervous system[1]. Glioblastoma is grade IV, the most aggressive growth stage, based on histological characteristics. Glioblastoma was classified as a potent infiltrative tumor by the World Health Organization (WHO) in 2007. Anaplastic astrocytoma, a WHO grade III astrocytoma, has a relatively lower invasion rate while diffused and pilocytic astrocytomas are grades II and I respectively, with low invasion rates[2, 3]. However, standard therapies such as surgical resection, chemotherapy, and radiation are ineffective in treating high-grade gliomas. Furthermore, the survival rate is very low, less than 1 year after conventional treatment[4].

Green tea is one of the most popular beverages worldwide and is known for its anti-inflammatory, anti-mutagenic, anti-proliferative, and anti-carcinogenic properties[5-7]. The most abundant components extracted from green tea is epigallocatechin-3-gallate (EGCG). In contrast to other components, EGCG is a remarkably bioactive compound exhibiting anti-cancer activity. EGCG has been considered a strong chemotherapeutic anti-cancer drug; however, its molecular mechanisms of action remain unknown.

The laminin receptor with a molecular weight of 67 kDa (67LR) was simultaneously purified in three different laboratories in 1983[8-10]. It is frequently overexpressed in tumor cells including gliomas[11, 12]. Studies have also confirmed that tumor invasion and poor prognosis are associated with 67LR overexpression[13-15]. However, 67LR is also an effective receptor for EGCG, through which

EGCG shows strong anti-tumor activity in lung cancer cell lines[16].

It has been reported that matrix metalloproteinases (MMPs) are associated with tumor cell invasion and extracellular matrix degradation, as well as being involved in the proteolytic activation of factors that affect various tumor types[11, 12]. Moreover, MMPs are overexpressed and excessively activated in almost all types of human cancers compared with normal tissues and indicate a poor prognosis[17-19].

Studies have shown that apoptosis is induced and cell proliferation is considerably suppressed in the U251 cell line after EGCG treatment[20]. EGCG also inhibits tumor cell growth *via* the mitogen-activated protein kinase (MAPK) pathway[21]. This was also demonstrated in our study. Extracellular signal-regulated kinases (ERKs), c-Jun NH_2-terminal kinase (JNK), and P38 are likely involved in EGCG-induced apoptosis and EGCG-inhibited proliferation. In this study we investigated the effect of EGCG on proliferation, apoptosis, and invasion of glioma cells, and the possible mechnisms.

MATERIALS AND METHODS

Reagents and Cell Lines

EGCG was from Sigma Chemical Co. (St. Louis, MO). EGCG was dissolved with Dulbecco's modified Eagle's medium (DMEM; Sigma) at different concentrations (0, 25, 50, 75, 100, 125 and 150 μg/mL) before use. The treatment durations of EGCG on U251 cells were 48 and 72 h. The antibodies anti-ERK, anti-phospho-ERK, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-JNK, and anti-phospho-JNK were from Cell Signaling Technology (Bevery, MA). The human malignant glioma cell line U251 was from Shanghai Institutes for Biological Sciences (China). Cells were cultured in DMEM containing 10% fetal bovine serum (USA) at 37°C with 5% CO_2. Proliferation was assessed using the cell counting kit-8 (CCK-8; Dojindo, Japan). A Matrigel invasion chamber was used for cell invasion assays (Becton Dickinson Labware, Bedford). The Annexin V-FITC apoptosis detection kit was from Bipec Biopharma Corp, Lexington, USA.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from U251 cells and then reverse-transcribed to synthesize cDNA using standard procedures. The primers were as follows: MMP-2 5′-GTGCTGAAGGACACACTAAAGAAGA-3′ and 5′-TTGCCATCCTTCTCAAAGTTGTAGG-3′; MMP-9 5′-CGCAGACATCGTCATCCAGT-3′ and 5′-GGATTGGCCTTGGAAGATGA-3′; β-actin 5′-CCAAGGCCAACCGCGAGAAGATGAC-3′ and 5′-AGGGTACATGGTGGTGCCGCCAGAC-3′. β-Actin served as an internal control. The PCR products were electrophoresed on 1% agarose gel, treated with ethidium bromide, and analyzed.

Constructs and Transfection

shRNA-67LR (Allele Biotechnology, San Diego, CA) was transfected to knockdown the expression of 67LR in U251 cells. Random siRNA plasmid, shRNA-67LR-SV (Allele Biotechnology) was used as negative control.

U251 cells were seeded in six-well plates at 1×10^6/well and allowed to attach for 24 h before transfection. Then plasmids were transfected by Transfection Reagent (Promega, Madison, WI) according to the protocol. After incubation at 37ºC for 48 h, cells were collected and down-regulation of 67LR was testified by Western blot analysis.

Western Blot for ERK, p38 MAPK, JNK, and Their Phosphorylated Forms

Total proteins were extracted from U251 cells, resolved in 10% SDS-PAGE and transferred to PVDF membrane. The membrane was incubated at 4ºC overnight with non-fat milk, followed by successive incubations with primary and secondary antibodies. The proteins were visualized with an enhanced chemiluminescenece detection system.

Proliferation Assays *in vitro*

CCK-8 was used to assess cell proliferation at an absorbance of 450 nm. The cells were seeded in 96-well plates at 1×10^4 cells per well. In each well, the total volume of cell culture medium was 0.2 mL. The cells were treated with different concentrations of EGCG for 48 or 72 h.

Apoptosis Assays *in vitro*

To count the number of apoptotic cells, 4×10^5 cells were collected and centrifuged. Annexin V-FITC was then added and the resulting culture was incubated at room temperature for 5 min in the dark. The apoptotic cells were

quantified by green fluorescence in the plasma membrane under a fluorescence microscopy (Leica, DM2500, Germany) in five random areas.

Invasion Assay *in vitro*

The Matrigel invasion chambers contained a polyethylene glycol terephthalate (PET) membrane with a pore size of 8 μm and a thin layer of Matrigel basement membrane matrix. In our experiment, 5×10^4 cells were seeded in the upper chamber without fetal bovine serum. Complete medium was placed in the lower chamber. At 24 h or longer, U251 cells with invasiveness are able to detach themselves and invade through the Matrigel. The cells on the lower surface of the membrane of chamber are stained with crystal violet hydrate solution. Light and electron microscopy (Leica, DM2500, Germany) were performed to assess the number of cells that invaded the PET membrane at random five areas of each chamber.

Statistical Analysis

Data are presented as mean ± SEM and were analyzed with one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

EGCG Induces Apoptosis of U251 Cells

Previous studies showed that EGCG induces apoptosis in carcinoma cells without affecting normal cells[22]. Annexin V assays were performed to investigate the apoptosis after U251 cells were treated with EGCG. The apoptotic rate of EGCG-treated U251 cells was higher than that of untreated cells in a dose-dependent manner (Fig. 1A and 1B).

Previous research demonstrated that reduction of cancer cell migration occurs through the downregulation of 67LR in human glioma cells *in vitro*[15]. 67LR has also been identified as an EGCG receptor that regulates an anti-cancer action in human lung tumor[16]. To verify whether or not EGCG induces glioma cell apoptosis *via* 67LR, the cells were transfected with shRNA-67LR or shRNA-67LR-SV (negative control) before treatment with EGCG for 48 h. The apoptotic rate of cells transfected with shRNA-67LR was lower than that of shRNA-67LR-SV or without transfection (Fig. 1C). These findings indicated that EGCG induces glioma cell apoptosis *via* 67LR.

EGCG Inhibits the Proliferation of U251 Cells

Previous research showed that EGCG suppresses the proliferation of human ovarian cancer cells *in vitro*[23]. Here, we found that EGCG (25, 50, 75, and 100 μg/mL) significantly suppressed the proliferation of glioma cells. After 72 h of EGCG treatment (100 μg/mL), the maximum suppression of proliferation rate was reached, ~71.52% compared with the 0 μg/mL group. EGCG also decreased cell survival in a dose-dependent manner (Fig. 2A and B).

EGCG Affects the Phosphorylation of ERK, JNK, and P38 in U251 Cells

The MAPK signaling pathway is involved in several physiological processes, including cell proliferation, differentiation, and death. To elucidate the molecular mechanism of EGCG-induced apoptosis and inhibition of proliferation of U251 cells, the levels of phosphorylated P38, ERK1/2, and JNK were evaluated by Western blot. The results showed that the phosphorylation of MAPKs changed irregularly after treatment with EGCG for 48 h (Fig. 3). We found that the activation of P38 and JNK signaling after 72 h EGCG treatment was dose-dependent. The phosphorylation level of ERK1/2 was similarly upregulated at 25 μg/mL EGCG. In contrast, 50, 75, and 100 μg/mL EGCG decreased the level of ERK1/2. No changes were found in total MAPK protein levels.

U251 Cell Invasion Decreases after EGCG Treatment

It has been shown that EGCG can reduce the invasion of human breast cancer cells *in vitro*[24]. Here, we found that 25 and 50 μg/mL EGCG also decreased the number of cells that invaded the lower membrane compared with untreated U251 cells (Fig. 4B and C). These results indicated that the invasiveness of U251 cells was weakened after EGCG treatment.

EGCG Inhibits MMP-2 and MMP-9 Expression

We further used RT-PCR to examine the expression of the regulatory genes MMP-2 and MMP-9 that are involved in cell invasion. Their mRNA expression levels were significantly suppressed by EGCG (Fig. 5). Therefore, EGCG regulated MMP-2 and MMP-9 expression in the U251 glioma cell line.

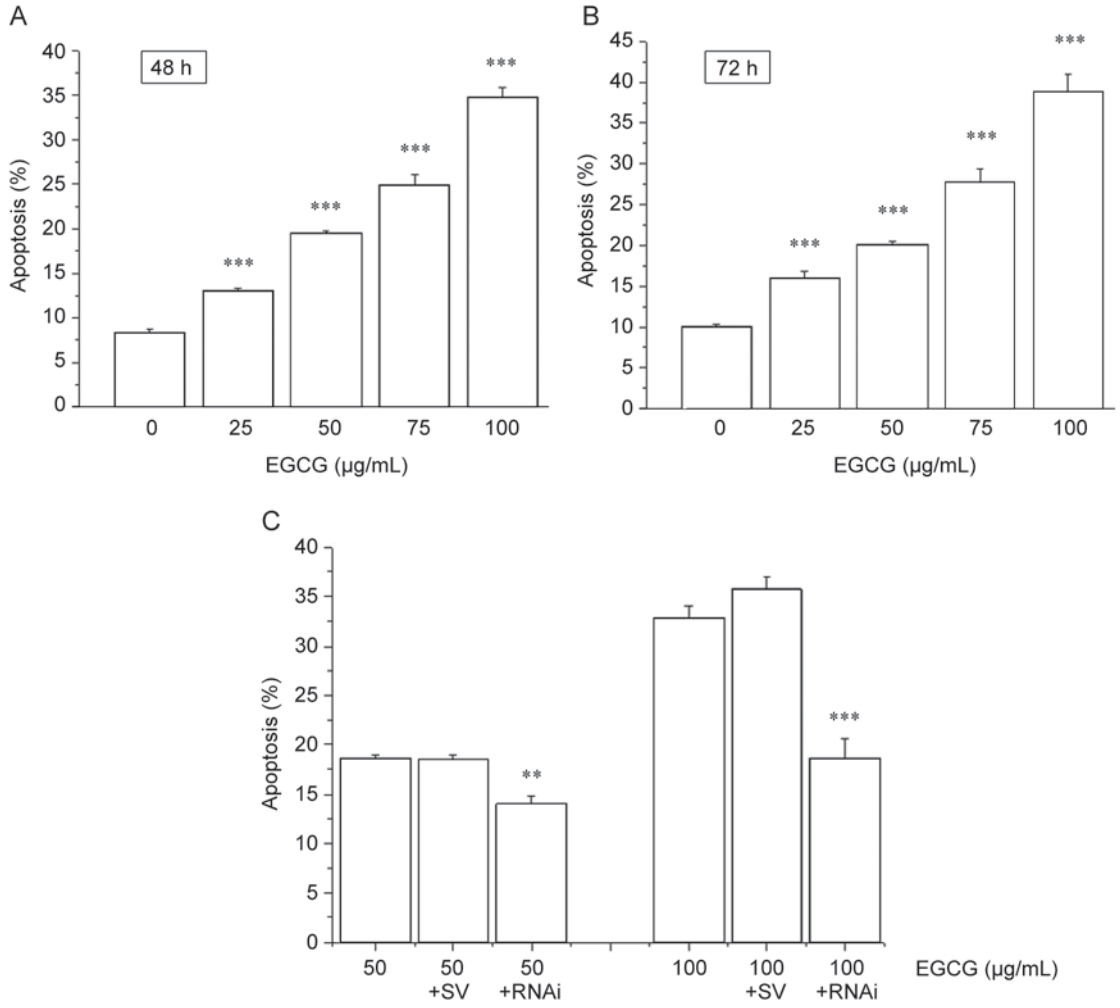

Fig. 1. EGCG induces apoptosis of U251 cells. U251 cells were treated with EGCG for 48 h (A) and 72 h (B). C: EGCG induced apoptosis through the 67-kDa laminin receptor (P* <0.05, ***P* <0.01 ****P* <0.001). Cells were transfected with shRNA-67LR or shRNA-67LR-SV for 48 h, followed by 24-h incubation with 50 or 100 μg/mL EGCG. Data represent three individual experiments.**

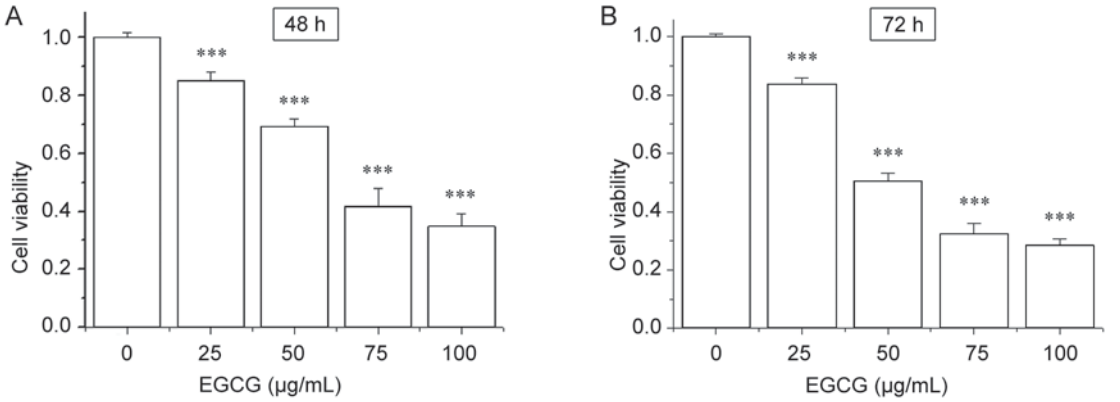

Fig. 2. Effect of EGCG on proliferation. EGCG treatment for 48 h (A) and 72 h (B) inhibited the proliferation of U251 cells in a dose-dependent manner. ****P* <0.001. Data are from three individual experiments.

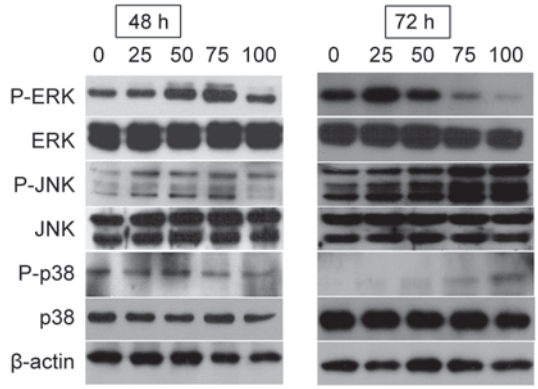

Fig. 3. Effects of EGCG (25, 50, 75 and 100 μg/mL) treatment for 48 h and 72 h on the phosphorylation of ERK, JNK, and p38, detected by Western blot.

DISCUSSION

Although it is one of the most prevalent brain tumors in the central nervous system, glioma remains poorly treated by chemotherapy and traditional therapies[25]. In this study, we investigated the effect of EGCG on malignant U251 glioma cells and found that it induced apoptosis, inhibited cell proliferation, and reduced the invasion of these aggressive cells. Furthermore, the apoptotic traits induced by EGCG occurred *via* 67LR, a target of EGCG. However, further studies are needed to determine whether or not 67LR mediates other beneficial effects of EGCG.

We investigated the MAPK signaling pathway and found that the phosphorylation of the ERK1/2, JNK, and

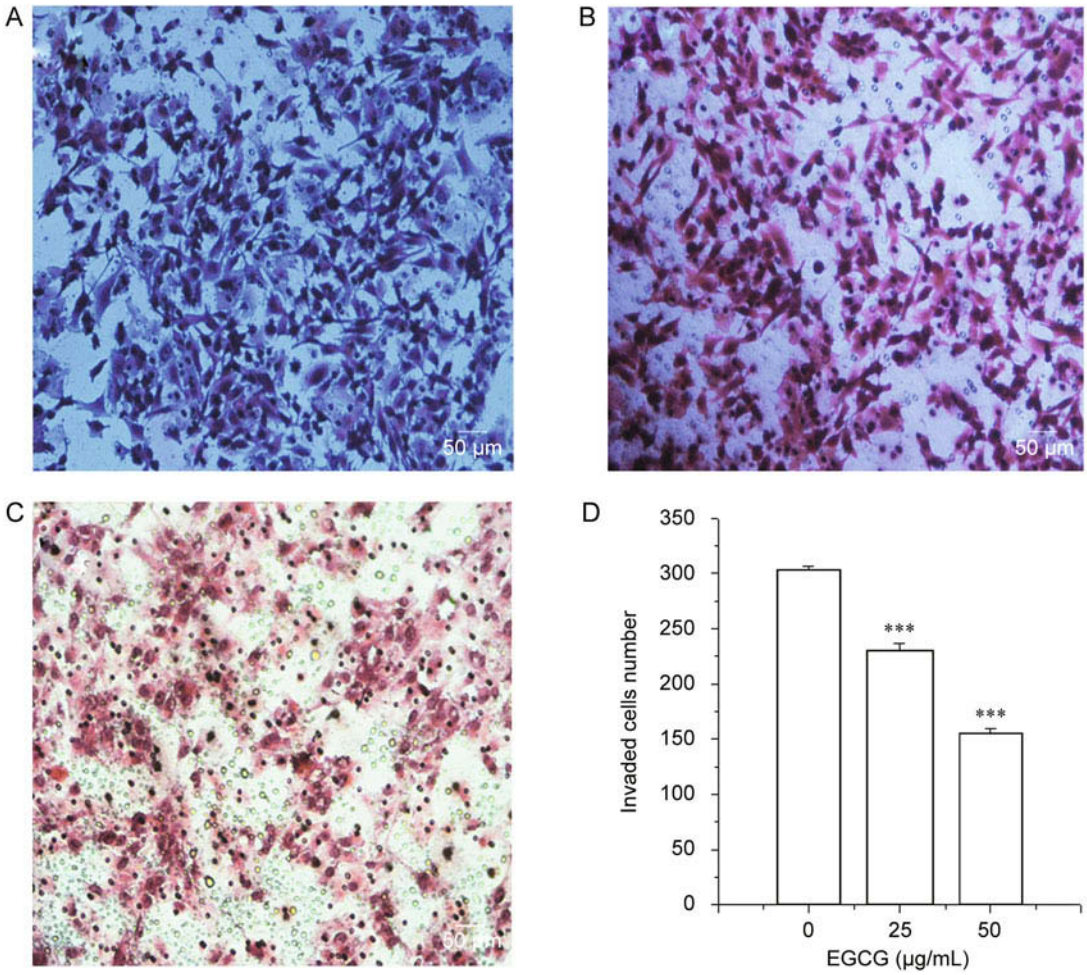

Fig. 4. Effects of EGCG on invasion of U251 glioma cells. A: U251 cells without EGCG treatment. B and C: U251 cells treated with 25 μg/mL (B) and 50 μg/mL EGCG (C). D: Quantification of invading cells (*P <0.001). Scale bars, 50 μm.**

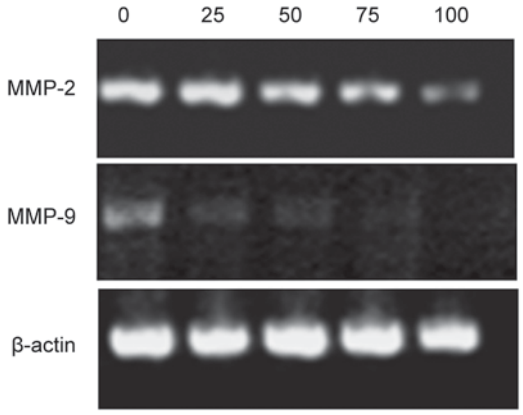

Fig. 5. EGCG at different concentrations (0, 25, 50, 75, 100 μg/mL) inhibited MMP-2 and MMP-9 mRNA expression, as detected by RT-PCR.

p38 proteins changed after 48 h of EGCG treatment. In particular, treatment for 72 h increased *P*38 and JNK phosphorylation dose-dependently. The same abnormal protein expression in the MAPK pathway has also been reported in lung adenocarcinoma SPC-A-1 cells[26]. Therefore, the EGCG-induced apoptosis and inhibition of proliferation may be associated with the MAPK signaling cascade in human glioma cells.

To investigate the mechanism by which EGCG reduced cancer cell invasion, we explored MMP-9 and MMP-2 expression after EGCG treatment. An apparent association between tumor aggression and enhanced MMP-9 and MMP-2 levels has been reported in various experimental and clinical studies[27-29]. For instance, MMP-2 expression is inhibited by Δ^9-tetrahydrocannabinol in glioma cells, resulting in a significant decrease in invasiveness[30]. In human breast tumor cells, EGCG effectively decreases MMP-9 expression[24]. Here, out data also suggested that MMP-2 and MMP-9 are involved in the EGCG-induced decrease in invasion of human glioma cells.

EGCG is a major polyphenol component of green tea that has gained increasing attention because of its cancer therapeutic and chemopreventive properties. The present study adds to the current knowledge of EGCG, providing evidence that it has anti-tumor activity in human glioma cells and the mechanism was explored. Further studies should be conducted *in vivo* to test these findings *in vitro*. Besides, other chemotherapeutic effects of EGCG against glioma should also be investigated to provide additional information on molecular therapeutic methods in cancer treatment.

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