RESEARCH ARTICLE

β-elemene Induces Caspase-dependent Apoptosis in Human Glioma Cells *in vitro* through the Upregulation of Bax and Fas/FasL and Downregulation of Bcl-2

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Abstract

Background: β-elemene, extracted from herb medicine *Curcuma wenyujin* has potent anti-tumor effects in various cancer cell lines. However, the activity of β-elemene against glioma cells remains unclear. In the present study, we assessed effects of β-elemene on human glioma cells and explored the underlying mechanism. Materials and Methods: Human glioma U87 cells were used. Cell proliferation was determined with MTT assay and colony formation assay to detect the effect of β-elemene at different doses and times. Fluorescence microscopy was used to observe cell apoptosis with Hoechst 33258 staining and change of glioma apoptosis and cell cycling were analyzed by flow cytometry. Real-time quantitative PCR and Western-blotting assay were performed to investigated the influence of β-elemene on expression levels of Fas/FasL, caspase-3, Bcl-2 and Bax. The experiment was divided into two groups: the blank control group and β-elemne treatment group. Results: With increase in the concentration of β-elemene, cytotoxic effects were enhanced in the glioma cell line and the concentration of inhibited cell viability (IC_{50}) was 48.5 μg/mL for 24h. β-elemene could induce cell cycle arrest in the G0/G1 phase. With Hoechst 33258 staining, apoptotic nuclear morphological changes were observed. Activation of caspase-3,-8 and -9 was increased and the pro-apoptotic factors Fas/FasL and Bax were upregulated, while the anti-apoptotic Bcl-2 was downregulated after treatment with β-elemene at both mRNA and protein levels. Furthermore, proliferation and colony formation by U87 cells were inhibited by β-elemene in a time and does-dependent manner. Conclusions: Our results indicate that β-elemene inhibits growth and induces apoptosis of human glioma cells *in vitro*. The induction of apoptosis appears to be related with the upregulation of Fas/FasL and Bax, activation of caspase-3,-8 and -9 and downregulation of Bcl-2, which then trigger major apoptotic cascades.

Keywords: β-elemene - glioma - Fas/FasL - proliferation - apoptosis

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Introduction

Gliomas are the most common and malignant type of primary brain tumor in adult central nervous system, representing over 50% of all primary intracranial tumors. Despite recent advances in the development of surgery and adjuvant therapy, it remains difficult to completely remove gliomas by conventional approaches. And the prognosis for patients with glioma is only 10-14 months on average (Louis et al., 2007; Buonerba et al., 2011). Elemene, extracted from a traditional Chinese herb medicine *Curcuma wenyujin*, is a mixture of β-, γ-, and δ-elemene; the chemical structure of β-elemene was showed in Figure 1A. β-elemene is the active component (molecular formula C15H24, molecular weight 204.34), which has strong anti-tumor effects *in vitro* and *in vivo* (Wang et al., 2005; Yu et al., 2011; Li et al., 2011). The antitumor capability of β-elemene has been shown to display various types of cancers, such as glioma, breast carcinoma, liver cancer, leukemia, laryngeal cancer and ovarian cancer (Li et al., 2010; 2013; Zhao et al., 2012). However, the mechanism of β-elemene induces apoptosis of gliomas remains unclear.

Apoptosis is a physiological process which possessed

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biochemical and morphologic characteristics such as development, homeostasis of tissues and elimination of malignant cells (Thompson et al., 1995). Two main apoptotic pathways have been mentioned as follows: the extrinsic death receptor pathway and the intrinsic mitochondrion pathway (Zhang et al., 2010). Both pathways involve mitochondria and Bcl-2 family protein. The activation of Caspase is regulated by various cellular factors, including pro-apoptotic Bcl-2 family members, and/or the Fas/Fas ligand (FasL) system. The Fas and its Fas ligand (FasL) system play a key role in apoptotic signaling in many cell lines and stimulation of Fas leads to initiating the extrinsic pathway of apoptosis (Wang et al., 2012). Fas is a member of the TNF family which is expressed in a variety of tissues. FasL is a type II transmembrane protein which binds to its surface receptor Fas to induce apoptotic cell death (Zhang et al., 2010). FADD and caspase-8 form death-inducting signal complex which mediates Fas-induced cell death and then activated caspase-3,-6 and -7, leading to the hydrolysis of cytosolic and substrates (Kupcinskas et al., 2011).

However, the roles of β-elemene in human glioma remain largely undefined. The aim of present study was designed to investigate the antitumor effect of β-elemene on human glioma U87 cells and the molecular mechanism involved. Our result showed that the effect of anti-proliferation and induction of apoptosis were triggered by the induction of Fas/FasL and the pro-apoptotic molecules Bax and downregulation of Bcl-2.

Materials and Methods

Cell cultures and treatments

Human glioma cell line U87 was purchased from the Chinese Academy of Sciences Cell Bank. All cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) under the conditions of 37°C, 5% CO_2. Cells were routinely passaged 2-3days at 80% confluence. β-Elemene (98% purity) was purchased from Dalian Holley Kingkong Pharmaceutical Co, Ltd (Dalian, China).

Cytotoxicity assay

Cells were seeded in 96-well plates at 5000-8000 cells/well and cultured overnight. Various concentrations of β-elemene (Dalian Jingang Pharmaceutical Co. China) were used for 20h.Then 5 mg/ml MTT (Sigma-Aldrich, USA) was added to each well for another 4h as reported previously (Du WZ et al., 2013). The amount of formazan was assessed by measuring the absorbance using TECAN microplate reader at 490nm. All measurements were performed three times.

Cell cycle and apoptosis assay

U87 cells were cultured on a 6-well plate (5×10^5 cells/well) for 24h and then β-elemene was added to the medium at various concentrations. Cells were resuspended in 50mg/mL PI (Sigma-Aldrich, USA) and 10mg/mL RNase A (Sigma-Aldrich) for 30 min in the dark. The samples used for apoptosis analysis were adjusted to a concentration of 1×10^6 cells/mL with 4°C PBS 400ul binding buffer was added to each tube. Then 5ul of Annexin V-FITC (BD Pharmingen, San Jose, CA, USA) and 10uL PI (BD Pharmingen) were added and incubated for 20mins (Bai et al., 2014). The flow cytometry (BD Biosciences Clontech, USA) was used to analyze the samples.

Quantitative real-time PCR

U87 cells were cultured in 6cm culture capsules and treated with various concentration of β-elemene for 48h.The ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) was used for real-time reverse transcription polymerase chain reaction (RT-PCR) assays. Total RNA was extracted from U87 cells using TRizol reagent (Invitrogen, USA) (Liu et al., 2013). Fas/FasL, caspase-3, Bcl-2 and Bax mRNA levels were using SYBR@ primescript RT-PCR Kit (TaKara, Japan).

Western blot analysis

U87 cells were treated with various concentration of β-elemene (40 μg/mL and 60 μg/mL) for 48h. Samples were scraped in Thermo scientific RIPA buffer (Pierce, USA) with protease inhibitors. Protein concentration was determined using the PIERCE BCA protein assay kit. The membranes were blocked with 5% skimmed milk at room temperature for 1h and incubated separately with mouse antihuman Fas (1:1000,Cell Signaling Technology, USA), mouse antihuman FasL (1:1000, Cell Signaling Technology, USA), mouse antihuman Bcl-2 (1:500, Santa Cruz USA) Rabbit antihuman Bax (1:500, Santa Cruz), Rabbit antihuman Caspase-3 (1:1000, Cell Signaling Technology, USA) and mouse antihuman β-actin (1:500, Santa Cruz, USA), and followed by incubation in HRP-labeled secondary antibody for 1h (Invitrogen) (Zhao et al., 2012). Immunoreactive proteins were scanned with the ECL system and quantified by Gel Doc 2000 (Bio-Rad).

Detection of caspases-3,-8 and -9 activity

The activity of caspase-3,-8 and -9 were measured using the caspase activity kit (Beyotime, China) according to the manufacture's recommendation. Cells (1×10^6 cells/well) were treated with β-elemene at the concentration of 0, 40 μg/mL and 60 μg/mL for 24h. The standard curve was determined by detecting the absorbance of samples at 490nm (Zhu et al., 2013). The U87 cells were collected and lysed in caspase assay buffer and supernatant was collected. The activity of caspase-3,-8 and -9 were read as optical density at 405nm with microplate reader.

Detection of morphological apoptosis with hoechst 33258 staining

U87 cells were grown on glass coverslips overnight (1×10^5 per well). Then they were treated with β-elemene (40 μg/mL and 60 μg/mL) for 24h. The cells were fixed in 4% paraformaldehyde for 20min at room temperature. After washed with PBS, samples were stained with 200μM Hoechst 33258 for 10min, and the changes in the nuclei of cells were scanned using a fluorescence microscope (Olympus, BX-60, Japan) (Liang et al., 2012).

Cell proliferation assay and colony formation assay

The methyl thiazolyl tetrazolium (MTT) assay was

used to detected cell viability. After cell were seeded in to 96-well plate at a concentration of 5×10^3 cells/hole and treated with various concentrations of β-elemene to the need of the experiment. Absorbance of formazan was quantified using TECAN microplate reader at 490nm.

Cells were cultured on a 6 cm dish (0.5×10^3cells per well) and grown overnight. Then, the cells were treated with various concentration β-elemene for 24h. The medium containing β-elemene was then removed, and the samples were washed in PBS. Cells were cultured for 12 days with complete medium. Then the colonies obtained were washed with PBS and fix in 10% formalin for 10min at room temperature (Du et al., 2013). Samples were stained with Giemsa stain and the number of colonies with more than 50 cells was counted.

Statistical analysis

Experiments were performed at least 3 times for data analysis. Data with mean±SEM were performed with GraphPad Prism software (version 5.0). Different treatments were made using paired t-test. *P* values<0.05 were considered statistically significant.

Results

β-elemene had cytotoxic effects on U87 glioma cells

We investigated the cytotoxic effect of β-elemene (Figure.1B) on U87 cells using the MTT assay. The study showed dose-dependent effect of β-elemene on glioma cell line (U87). The concentration of inhibited cell viability (IC_{50}) was 48.52 μg/mL. The result showed that β-elemene was cytotoxic against U87 glioma cell lines. Then we chose two doses of β-elemene for U87 cell line (40 μg/mL and 60 μg/mL).

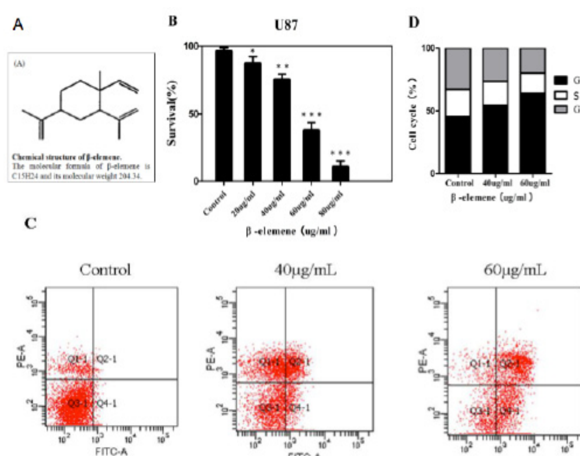

Figure 1. β-elemene Induces Apoptosis Of Human Glioma Cells and G0 /G1 Arrest. (A) Chemical structure and molecular weight of β-elemene; **(B)** Cytotoxicity effect of β-elemene on human glioma cells. Cell death was measured by using MTT assay. The error bars represent standard error ($p<0.05$); **(C)** U87 cell apoptosis was examined by flow cytometry using Annexin V-FITC/PI staining; **(D)** Cell cycle was measured by flow cytometry following propidium iodide staining for DNA content. Histograms shown are representative of three independent experiments. Data are shown as mean±SEM for the three replicates. Statistical significance levels are indicated as:*$p<0.05$; **$p<0.01$ and ***$p<0.001$

β-elemene induced glioma cell apoptosis and G0/G1 arrest

In this experiment, we performed the Annexin V/PI staining to confirm that β-elemene induced apoptosis of glioma cells. Our data showed that the production of apoptotic cells was increased to 22.15±3.1% and 50.69±5.71% after treatment with 40 μg/mL and 60 μg/mLof β-elemene for 24h compared with control cells 2.08±0.42%. Flow cytometry analysis showed that β-elemene induced the apoptosis of glioma cells in a dose-dependent manner (Figure 1C). To further understand the mechanism that β-elemene inhibited the growth of human glioma cells, the effect of β-elemene on cell cycle were measured using FCM. The data revealed that different dose of β-elemene induced a significant G0/G1 phrase arrest in U87 cells, simultaneous decreased at the G2/M (Figure. 1D). These data suggested that β-elemene could induce cell cycle arrest at the G0/G1 phase in glioma cells.

β-elemene promoted Fas/FasL, caspase-3 and bax expression and suppressed Bcl-2 expression in glioma cells

Fas (CD95/APO-1) was a cell surface receptor protein which induced apoptosis on binding FasL (Lee et al., 2012). RT-PCR was used to identify the expression of Fas and FasL genes. The results showed that a increase in both Fas and FasL mRNA expression with different concentrations of β-elemene ($p<0.05$) (Figure 2C). After treatment with various concentration of β-elemene, the expression level of Fas and FasL and Bax were significantly increased but the expression of Bcl-2 was decreased compared with the control group ($p<0.05$) (Figure 2A). We also found a correlation between the levels of caspase-3 and Fas proteins Figure 2B. Moreover, Fas and FasL expression gradually increased as the concentration of β-elemene increased ($p<0.05$) .The data indicated that β-elemene

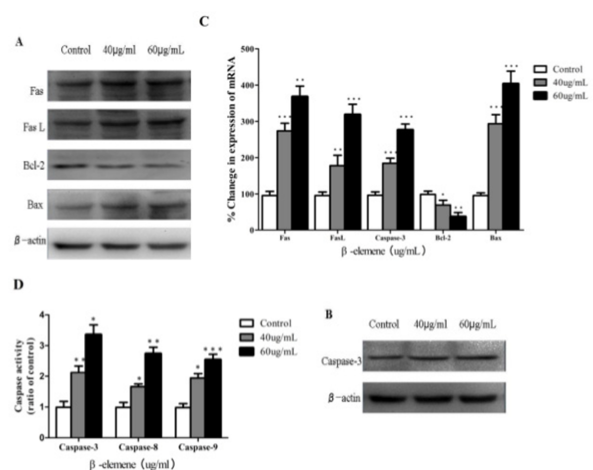

Figure 2. β-elemene Induces the Activation of Caspase-3, -8, and -9, Upregulates Fas, FasL, Caspase-3 and Bax, and Downregulates Bcl-2 of Human U87 Glioma Cells in a Dose-dependent Manner. (A-B) Western blotting analysis of Fas, FasL, caspase-3, Bcl-2 and Bax in U87 cells treated with 40 μg/mL and 60 μg/mL of β-elemene for 48h; **(C)** Real-time RT–PCR analysis for quantitative evaluation of the mRNA expression of target genes; **(D)** Caspase-3,-8 and -9 were measured by colorimetric assay as described. The data are mean±SD of three samples. *$p<0.05$; **$p<0.01$ and ***$p<0.001$.

induced a dose-dependent manner and triggers apoptosis *in vitro* by decreasing the rate of Bcl-2/Bax.

β-elemene increased activity of caspase enzyme

When U87 cells were treated with different concentration of β-elemene for 24h, the caspase-3,-8 and -9 enzyme activity was significantly enhanced compared to control ($p<0.01$). There was a positive correlation between caspase-3,-8 and -9 enzyme activity and apoptosis of U87 cells (Figure 2D). The result indicated that the activitation of caspase-dependent apoptosis signaling pathways might be involved in β-elemene-induced apoptosis in human glioma cells.

Measurement of Apoptosis of U87 Cells by Hoechst33258 Staining

After treatment with different doses of β-elemene for 24h, U87 cells were stained with Hoechst 33258. Then cells were observed under a fluorescence microscope (Olympus, Fluoview 400, Japan). Hoechst 33258 staining exhibited morphological changes of cell apoptosis, such as condensation of chromatin and nuclear fragmentation. Apoptotic bodies began to appear in the β-elemene treated groups, while few in the control group (Figure 3). The percentage of apoptotic cells increased at higher β-elemene concentration in U87 cells. These result indicated that β-elemene induced a dose-dependent manner.

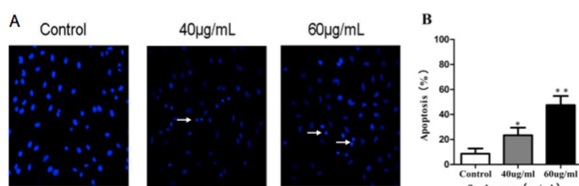

Figure 3. Cell Apoptosis Observed by Hoechst 33258 Staining Using a Fluorescence Microscope (200×). After cells were treated with indicated concentration of β-elemene for 24h, Hoechst 33258 staining was used to observe the apoptotic cells under a fluorescence microscope. The data are mean ±SD of three independent experiments. *$p<0.05$; **$p<0.01$ and ***$p<0.001$. The arrow shows apoptotic cells

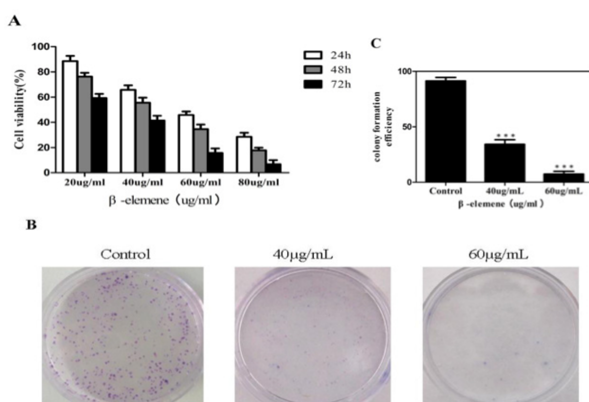

Figure 4. β-elemene Suppressed Proliferation of U87 Cells. (A) Cell viability was measured using the MTT assay. The result was expressed as the percentage of cell viability rate compared with the control. The data are mean±SD of three independent experiments; **(B)** β-elemene inhibited colony formation. Human glioma cells were planted with indicated concentration of β-elemene for 24h and allowed to grow into colonies for 12 days. Treatment with β-elemene inhibits colony formation. Data are mean±SEM for the three replicates. *$p<0.05$; **$p<0.01$ and ***$p<0.001$

β-elemene inhibits proliferation in U87 glioma cells

MTT assay was used to evaluate the anti-proliferative effect of β-elemene on U87 cells. The effect of β-elemene with different concentration on cell growth after 72h was shown in Figure.4A. MTT assay confirmed that the inhibitory effect of β-elemene is does and time dependent. Similarly, colony formation was suppressed by β-elemene treatment in U87 glioma cells (Figure 4B). And the inhibition of formation capacity was significantly at a high concentration of β-elemene Figure 4C, suggesting that the effect of anti-proliferation was irreversible.

Discussion

Malignant glioma is the most common and most deadly form of intracranial tumor, having an extremely high recurrence. In present, approaches for treatment of malignant glioma have a rapid development. But there are still a series of difficulties to be further addressed, and the median survival time of the patients is merely about 14 months (Tabatabai et al., 2012). Therefore, chemotherapy of glioma becomes a promising way of treatment.

Curcuma aromatica Salisb is a type of traditional Chinese medicine plant and the essential oils of this plant are widely used in the treatment of cancer (Lu et al., 2012). β-elemene is a sesquiterpene extracted from the soil and is a non-toxic anti-tumor drug where the production is independent research and development in our country. β-elemene can get through the blood-brain barrier and used in the treatment of brain tumor. And the anticancer effect is usually mediated by the inhibition of proliferation, cell cycle arrest and the induction of apoptosis. The anti-tumor effect of β-elemene has been shown in some types of tumor, but the mechanism remains unclear. It has been reported that beta-elemene arrests chemoresistant ovarian carcinoma at the G2-M phase and inhibits the proliferation of cells (Li et al., 2005). Other reported that β-elemene could induce apoptosis in prostate cancer cells and lung tumor cells (Li et al., 2009; 2010). β-elemene also inhibits the proliferation of HEp-2 laryngeal cancer cells and induces apoptosis *in vitro* and *in vivo*. Researches have found that β-elemene showed strong antitumor activity in glioblastoma cell lines from the human and rat (Zhao et al., 2012). β-elemene induced apoptosis in human renal-cell carcinoma cells by regulating MAPK/ERK and PI3K/Akt/mTOR signaling pathways (Zhan et al., 2012). The result indicated a protective autophagy induced by β-elemene, and inhibition of autophagy might be an effective approach to enhance the anti-tumor effect of β-elemene.

Apoptosis is a kind of biological character of programmed cell death. Anti-tumor medicine could induce the apoptosis of tumor cells through cell cycle arrest and oxidative stress. There are two important apoptotic pathways: the extrinsic (receptor-midiated pathway) and the intrinsic (mitochondrial pathway).β-elemene could induce the regression of human hepatoma HepG2 cells by

inducing apoptosis via Fas ligand interaction (Xu et al., 2005). Accumulating evidence showed that upregulation of Fas expression in glioma tissues may have evidence with the apoptotic activity of glioma cells.

In the present study, we demonstrate that β-elemene increase the expression of Fas and FasL in U87 cells and increased the activation of the extrinsic pathway. It has been demonstrated that FasL expression involved in oral tumorigenesis and act as a marker for early neoplasia (Fang et al., 2013). This may be an effective way in choosing patients for early interventional therapies, include chemoprevention and/or nutritional therapies. Death receptors, such as Fas, Fas ligand, and TNF receptors is triggered by cellular stress or death signals and leads to the recruitment o Fas-associated death domain (FADD) which then induce the activation of Caspase-8 and -10 (Zhang et al., 2010). Caspase-3 acts as a key executor in apoptosis and plays important role in programmed cell death. This leads to the activation of Bax, which then binds to Bak to induce the the release of cytochrome C. The release of cytochrome C is related to the cleavage of caspase-9. Interaction between Caspase-9 and caspase-3,-6 and -7 activates the latter capases in the cells (Thorburn, 2004). Finally the activated caspase-3 causes DNA breakage and induces cell apoptosis. In this study, β-elemene increased caspase-3,-8 and-9 activation through the induction of Bax and Fas expression and up-regulated caspase-3 and confirmed that caspase-dependent pathway is related to β-elemene-induce apoptosis. Furthermore, treatment with β-elemene is attributed to enhanced active caspase-8 signaling and levels of Fas or caspase-3. These finding suggested that β-elemene -induced apoptosis might activate Fas-mediated caspase-dependent pathway in U87 cells.

The intrinsic mitochondrion pathway is associated with the Bcl-2 family. Bcl-2 could further cell survival by limiting the pro-apoptotic effects of Bax and suppress the release of cytochrome C from mitochondria (Ackler et al., 2010) and the Bcl-2/Bax ratio could induce the apoptosis of cells. It has been indicated that the Bcl-2/Bax ratio may be an important way compared with either promoter alone in determining apoptosis (Simin et al., 2014). Our results exhibit that β-elemene may inhibit the proliferation of U87 cells *in vitro* by suppressing Bcl-2 and inducing Bax. These findings suggest that β-elemene induces apoptosis in human U87 glioma cells by activating both death receptor and mitochondrial apoptotic pathways.

In conclusion, the present study demonstrates that β-elemene suppressed the proliferation of U87 glioma cells *in vitro*. It reveals that β-elemene induces apoptosis by increasing the expression of Fas/FasL and caspase-3 and activating caspase-3,-8 and -9 signaling and suppressing the Bcl-2/Bax ratio. These results indicate that β-elemene maybe used as a promising chemotherapeutic therapeutic agent in treatment of patients with glioma.

Acknowledgements

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