β-elemene induces glioma cell apoptosis by downregulating survivin and its interaction with hepatitis B X-interacting protein

HANG ZHANG[1], FENG XU[3], TIAN XIE[2], HONGCHUAN JIN[4] and LIYUN SHI[1]

[1]Department of Basic Medical Science, [2]Research Center for Biomedicine and Health, Hangzhou Normal University; [3]Department of Respiratory Medicine, Second Affiliated Hospital, [4]Laboratory of Cancer Epigenetics, Key Laboratory of Biotherapy in Zhejiang Province, Biomedical Research Center, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, P.R. China

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Abstract. β-elemene, extracted from the ginger plant, possesses antitumor activity against a broad range of cancers clinically. However, the mechanism underlying β-elemene-induced cytotoxicity remains incompletely understood. Here, we show that β-elemene promoted apoptotic cell death in human glioma cells, downregulated survivin gene expression, and induced caspase-9, -3 and -7 activities. Induction of apoptosis was associated with inhibition of survivin gene expression, and restoration of survivin levels remarkably attenuated β-elemene-induced glioma cell death. Moreover, we found that the interaction between surviving and HBXIP, a critical regulator of caspase-9 activity, was impaired by β-elemene treatment. The results, therefore, reveal a caspase-mediated apoptotic pathway induced by β-elemene in human glioma cells, which is associated with downregulation of survivin itself and the interaction between survivin and HBXP.

Introduction

Malignant glioma is one of the most common brain tumors, and its incidence rate tends to be increased worldwide, especially in large cities (1). The overall survival rates of malignant glioma remains very poor. Fewer than 50% of glioma patients estimated to survive for longer than 5 years after diagnosis (2). Although ionizing radiation and chemotherapy constitute the major treatment for malignant gliomas (3,4), it is critical to identify potential drugs for the disease.

β-elemene is one of the active components extracted from the traditional Chinese medicinal herb *Rhizoma zedoariae* (5). Due to its effectiveness in retarding growth in various tumor cells both *in vitro* and *in vivo*, β-elemene has been utilized in treating certain types of tumors, including lung cancer, colorectal cancer and glioblastoma (5,6). Previous studies showed that β-elemene inhibited cell proliferation by inducing cell apoptosis as well as arresting the cell cycle (7,8), and Bcl-2, Bcl-X(L) and XIAP was involved in this process (3,9). However, the mechanisms by which β-elemene induces cell apoptosis are not completely understood, and further studies need to be done to uncover the molecular mechanisms underlying its antitumor activity.

Survivin (*BIRC5*), a protein of 16.5 kDa, constitutes the smallest member of the inhibitor of apoptosis (*IAP*) gene family (10). Highly expressed during embryonic and fetal development, survivin however is almost not or only barely detectable in most adult tissues (11,12). Conversely, survivin was found to be expressed in abundance in various cancer tissues and cancer cell lines. In human gliomas, high levels of survivin expression revealed to be correlated with a poor prognosis (13,14), indicating its beneficial effects for glioma cells survival and/or growth. Mechanically, survivin is able to inhibit the second mitochondria-derived apoptosis-inducing factor Smac/DIABLO (15) and other apoptosis-inducing factors (16). Also there are data supporting that survivin plays a unique role in regulating mitotic events and TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis (17-20). Survivin contains a single baculoviral IAP repeat domain, the structure supposed to be able to block caspase-3 and-9 activities. However, this molecule lacks the RING domain, which can act as a ubiquitin ligase to facilitate the proteasomal degradation of caspases and retard the apoptotic process. Probably to complement to this, a novel complex formed between survivin and hepatitis B X-interacting protein (HBXIP) was identified, which was shown to be essential in regulating caspase-9-mediated apoptotic signaling (21,22). It suggested that survivin-HBXIP complexes, but neither survivin nor HBXIP individually, are able to bind to pro-caspase-9, blocking the recruitment of caspase-9 to Apaf1,

Correspondence to: Dr Liyun Shi, Department of Basic Medical Science, Hangzhou Normal University, no. 16 Xuelin St., Hangzhou Xiasha Higher Educational Zone, Hangzhou, Zhejiang 310036, P.R. China
E-mail: shi_liyun@msn.com

Abbreviations: β-ELE, β-elemene; HBXIP, hepatitis B X-interacting protein; IAP, inhibitor of apoptosis protein; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; FITC, fluoresceine isothiocyanate; TUNEL, terminal deoxynucleotidyltransferase-mediated deoxy-UTP-fluorescein nick end-labeling

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and subsequently suppressing the apoptosis initiated via the mitochondria/cytochrome c pathway (13,23).

In this study, we report that β-elemene induced apoptosis in human glioma cancer cells. Induction of the apoptosis was associated with inhibition of survivin gene expression, and overexpression of survivin gene reduced β-elemene-induced apoptosis. Furthermore, β-elemene inhibited the direct interaction between survivin and HBXIP, which enhanced the activation of caspase-9 and promoted glioma apoptosis.

Materials and methods

Chemicals, reagents and antibodies. Q-VD-OPH and Q-LEHD-OPH were from MP Biomedicals (Aurora, OH, USA), Ac-DEVD-CHO was purchased from Promega Co. β-elemene was purchased from Zhejiang Institute for Food and Drug Control (Hangzhou, China). Anti-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-capase-3, anti-cleaved caspase-3, anti-caspase-7, anti-cleaved caspase-7, anti-caspase-9, anti-cleaved caspase-9, anti-PARP, anti-cleaved PARP, and anti-survivin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. U251 and A172 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in recommended growth media. U251 cells were transfected with pMEG06-3HA-survivin (U251-survivin) or pMEG06-3HA vector as control (U251-HA). Stable cell lines were established by G418 selection (0.6 mg/ml, Sigma-Aldrich).

Plasmid construction. pMEG06-3HA-survivin plasmid, which contains the ORF of survivin gene, a 3HA-Tag and the N-terminal, was purchased from FuGENE Gene Co. Total cDNA was generated by reverse-transcription from U251 mRNA using RT-kits (Takara), and the ORF of HBXIP was amplified by PCR, the specific primers for PCR were as follows: 5'-GAC GAATTCATGGAGGCGACCTTGGAGCA-3' (forward) and 5'-GATCTCGAGTCAAGAGGCCATTTTGTGCA-3' (reverse) (23). The resultant cDNA fragments were ligated into pCMV-Tag2B vector, which contains a FLAG-Tag and the N-terminal, for mammalian cell expression.

Analysis of apoptosis by annexin V staining. To determine apoptosis, U251 and A172 cells were stained with annexin V and propidium iodide (PI) using a Vybrant Apoptosis Assay kit (Invitrogen, Carlsbad, CA). In brief, cells were collected by trypsinization and washed with ice-cold phosphate-buffered saline (PBS). Then cells were resuspended in 500 μl binding buffer, and incubated with 5 μl of annexin V-FITC and 10 μl of the 100 μg/ml PI working solution at room temperature for 5 min in the dark. Stained cells were analyzed for apoptosis by flow cytometry. FITC was detected at 518 nm and PI was detected at 620 nm.

Terminal deoxynucleotidyltransferase-mediated deoxy-UTP-fluorescein nick end-labeling (TUNEL) assay. For TUNEL assay, a DNA Fragmentation Detection kit, Fluorescent-TdT Enzyme (Cabiochem, San Diego, CA), was used to detect DNA fragmentation. In brief, cells were treated with β-elemene, harvested, and resuspended in 80% ethanol at 4˚C overnight. The next day cells were washed with Tris-buffered saline (TBS) and incubated with 20 μg/ml proteinase K for 5 min at room temperature. Cells were then washed three times with PBS, incubated with 100 μl TdT equilibration buffer at room temperature for 30 min, and incubated in the dark with 60 μl of TdT-labeling reaction mixture at 37˚C for 60 min. Finally, pellet cells were washed three times with PBS and analyzed by a flow cytometer. The emission wavelength of fluorescein is 517 nm.

Isolation of RNA and RT-PCR analysis. Total RNA was extracted using TRIzol (Invitrogen), and after DNase I treatment, cDNAs were synthesized by using the cDNA reverse-transcription kit (Takara). Survivin and GAPDH mRNA was detected by RT-PCR, the specific primers for RT-PCR were as follows: survivin: 5'-ACCAGG TGAGAAGTGAGGGA-3' (forward) and 5'-AACAGTAGAGGAGCCAGGGA-3' (reverse) (24); GAPDH: 5'-CATGG GTTCAACATGCCAAGTGGT-3' (forward) and 5'-TCCACGGCAGCATTAATCACAGGA-3' (reverse).

Immunoprecipitation and western blot analysis. For immunoprecipitation, after transfection, cells were lysed in 1 ml of 1% Nonidet P-40, 25 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, pH 8.0, a 1:50 dilution of a protease inhibitor mixture (Sigma) for 30 min on ice. Cell lysates were centrifuged at 14,000 g for 5 min to pellet cell debris and incubated with primary antibodies overnight at 4˚C with rotation followed by the addition of protein A-agarose (60 μl of 50% slurry) for 1 h at 4˚C with rotation to capture antibody-antigen complex. The antibody-antigen complex was washed, and samples were mixed with SDS-PAGE sample buffer, boiled for 5 min, electrophoresed on an SDS-polyacrylamide gel, and electroblotted onto a nitrocellulose membrane (Millipore, Bedford, MA). For western blot detection, proteins were resolved on SDS-PAGE, transferred to a nitrocellulose membrane. The membrane was blocked in PBS containing 5% skimmed milk powder and 0.02% Tween-20, and then probed with specific antibodies at 4˚C overnight. After washing, the membrane was incubated with a 1:20,000 dilution of peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG in PBS containing 5% skimmed milk powder. The blot was then developed using the ECL detection kit (Amersham Biosciences) to produce a chemiluminescence signal, which was captured on X-ray film.

Results

β-elemene induced glioma cell apoptosis. In this experiment, we detected β-elemene-induced apoptosis by annexin V staining and TUNEL assay in human glioma cells. Human glioma U251 and A172 cells were treated with β-elemene at concentrations of 0, 100, 200 and 300 μg/ml for 24 h. Flow cytometry analysis showed a significant increase in the apoptotic population of the cells treated with β-elemene for 24 h when compared with that of the cells without treatment. The most striking increase in the percentage of apoptotic cells occurred at the concentration of 300 μg/ml (Fig. 1A). TUNEL assay further confirmed that apoptosis was induced by β-elemene (Fig. 1B).

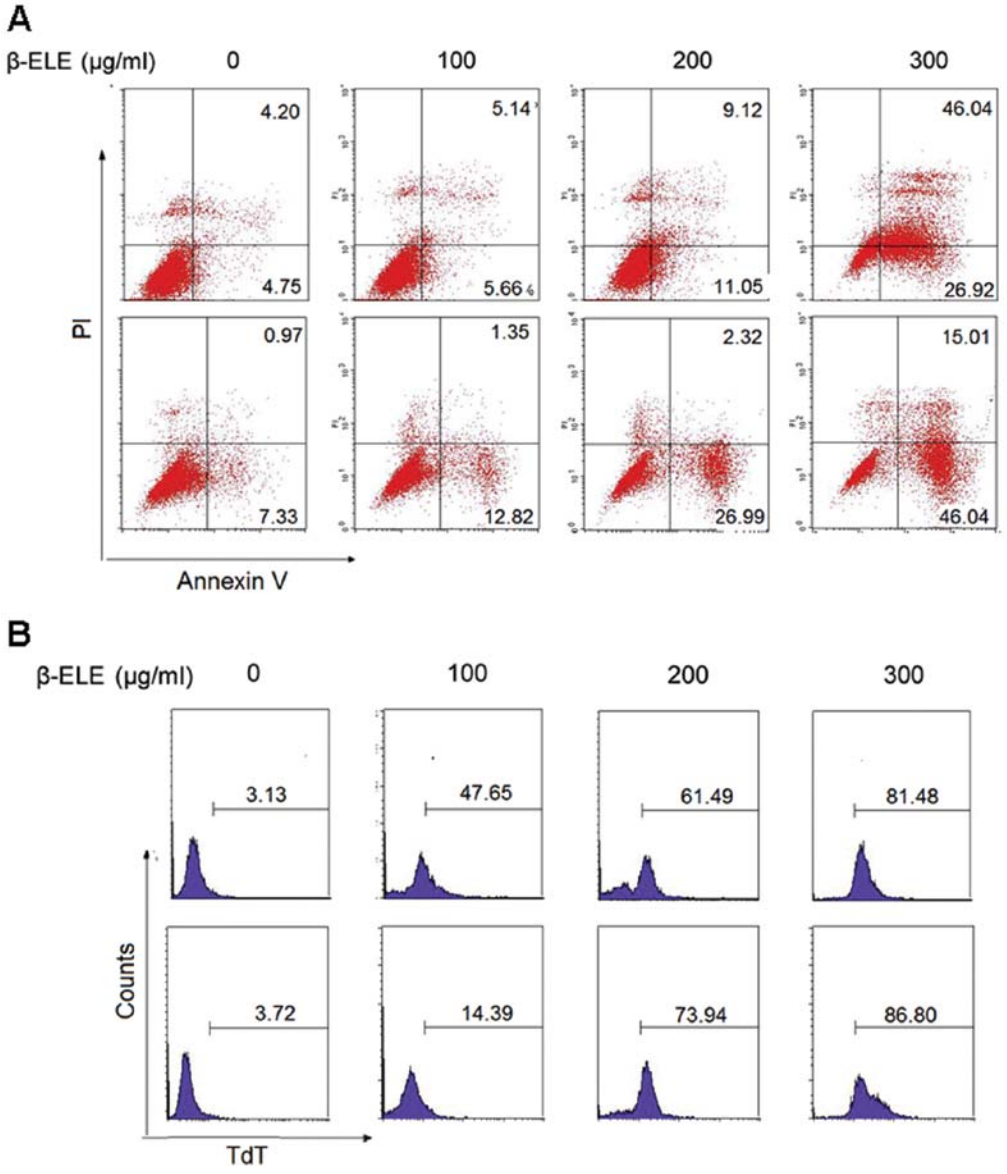

Figure 1. Induction of apoptosis of glioma cells by β-elemene. U251 (upper) and A172 (lower) cells were treated with 0, 100, 200 and 300 μg/ml of β-elemene for 24 h. Apopttic cells were stained with annexin V/PI (A) or determined with a TUNEL assay (B) and analyzed by flow cytometry. The data are representative of at least three independent experiments.

Next we tested the activities of caspase-9, -3 and -7 in glioma cells when stimulated with β-elemene. As shown in Fig. 2A and B, β-elemene clearly induced the cleavage of pro-caspase-9, -3, -7 to their active forms in human U251 and A172 glioma cells. Moreover, poly(ADP-ribose) polymerase (PARP), a specific substrate of caspase-9, -3 and -7, was also cleaved into its active form in a dose-dependent manner, indicating that caspase-9, -3, -7-mediated pathways was involved in β-elemene-induced glioma apoptosis. To further prove that, we examined the effect of caspase inhibitors on the β-elemene-induced apoptosis in U251 cells. The results showed that both of pan-caspase inhibitors, Q-VD-OPH and Q-LEHD-OPH, markedly inhibited β-elemene-induced apoptosis. Treatment with a specific caspase-3 inhibitor Ac-DEVD-CHO provided the protection for U251 cells against apoptotic death (Fig. 2C). These results suggested that β-elemene induced caspase-dependent apoptosis of glioma cells.

β-elemene inhibits the expression of survivin gene in glioma cells. As anti-apoptotic protein survivin is expressed abundantly in human gliomas and related with poor prognosis, we investigated whether proapoptotic effect of β-elemene can be mediated by regulation of survivin levels. We firstly determined the expression of survivin in glioma cells with or without β-elemene treatment. The results indicated that β-elemene induced a dose- and time-dependent reduction of survivin expression either in mRNA (Fig. 3A and C) or protein levels (Fig. 3B and D), suggesting a potential role for survivin in β-elemene-induced glioma cell death.

β-elemene-induced glioma cell apoptosis is blocked by restoring survivin expression. Next, we examined whether β-elemene-induced downregulation of survivin was associated with apoptosis in glioma cells. We established stable U251 cell lines with forced expression of HA-tagged survivin and

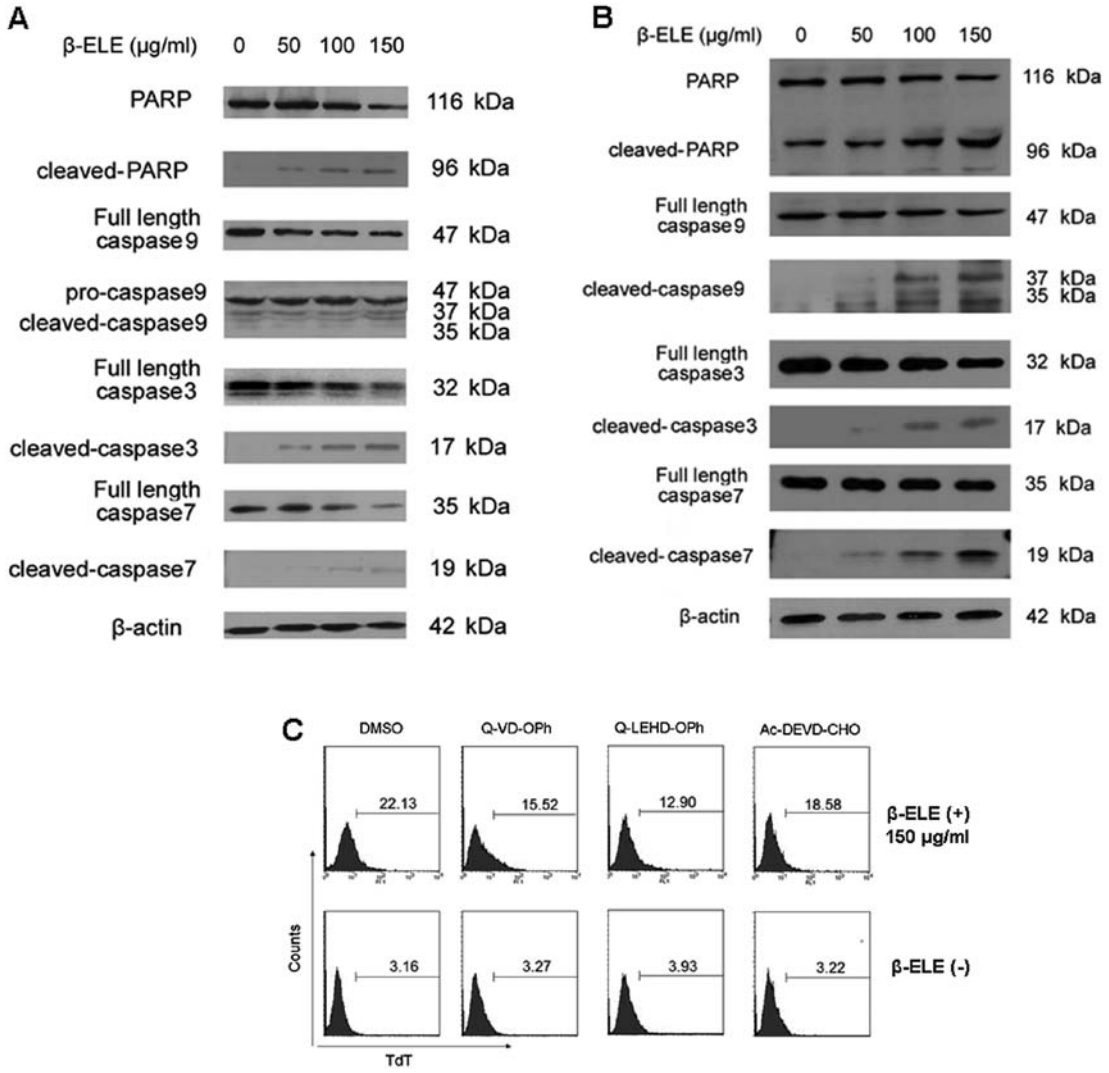

Figure 2. β-elemene promotes caspase activation in glioma cells. (A) U251 cells were treated with 0, 50, 100 and 150 μg/ml β-elemene for 24 h. The levels of pro- and cleaved-PARP and caspases were detected, respectively, by western blotting. β-actin was used as a loading control. (B) Similar results were detected in A172 cells. (C) U251 cells were pretreated with β-elemene for 2 h, and then cultured in the presence of the caspase inhibitors zVAD (20 μmol/l), QVD (20 μmol/l), Z-DEVD-FMK (100 μmol/l), or the vehicle for 6 h. Cell apoptosis was determined by TUNEL assay as described in Materials and methods. Representative histograms of three experiments are shown.

then compared the effect of β-elemene on the U251-HA and U251-sur cells. Transfection efficiency of survivin gene was proved by western blotting (Fig. 4A) and RT-PCR analysis showed that β-elemene treatment did not affect the exogenous HA-tagged survivin expression (Fig. 4B). As expected, the treatment caused a marked decrease in endogenous survivin in both U251-HA and U251-sur cells (Fig. 4B and C), and TUNEL assay showed apoptosis of U251 cells was induced by β-elemene. Remarkably, forced expression of survivin caused a reduction in apoptosis of U251-sur cells when compared with U251-HA cells (Fig. 5A), suggesting that apoptosis of U251 cells induced by β-elemene was overridden by forced expression of survivin.

Since survivin exerts an anti-apoptotic effect through caspase-9, -3 and -7-dependent pathway (25-27), we further evaluated the apoptosis-related proteins in U251-HA and U251-sur cells upon β-elemene stimulation. The cells were treated with 150 μg/ml β-elemene for 24 h and the levels of caspase-9, -3, -7 and PARP were detected by western blotting. Results showed that the forced expression of survivin in U251 cells significantly decreased β-elemene-induced activation of caspase-9, -3, -7, as well as the cleavage of PARP (Fig. 5B).

β-elemene inhibits the interaction of survivin and HBXIP. It has been proved that survivin-HBXIP complexes, but neither survivin nor HBXIP individually, have the capability to bind pro-caspase-9 and inhibit the activation of caspase-9 (23), we hypothesized that in human glioma cells, β-elemene can inhibit the interaction of survivin and HBXIP. To test this hypothesis, we examined if the binding of survivin to HBXIP was affected by β-elemene treatment. Firstly, the ORF of HBXIP gene was cloned into pCMV-Tag2B plasmid to construct pCMV-HBXIP plasmid which can express a FLAG-HBXIP fusion protein in mammalian cells. pMEG-HA-survivin and pCMV-HBXIP were then co-transfected into U251 cells and treated with β-elemene (100 μg/ml). Using co-immunoprecipitation method

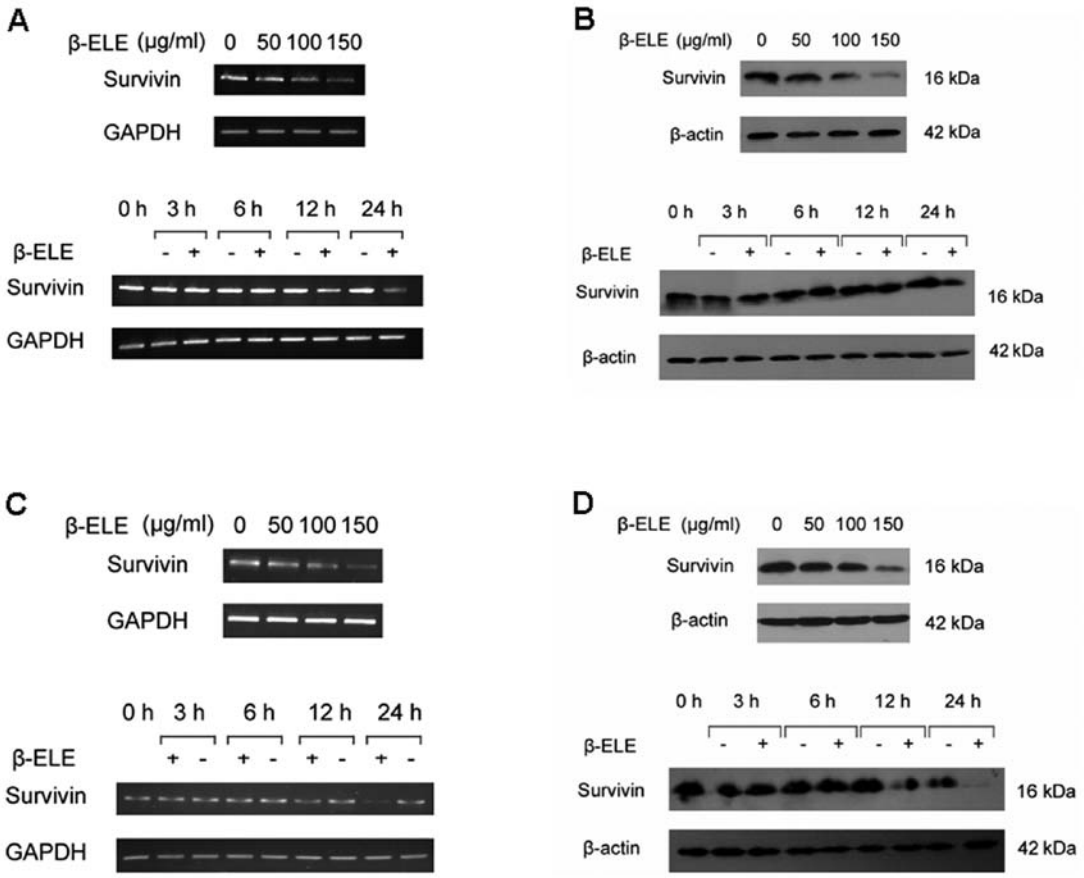

Figure 3. β-elemene reduces survivin levels in glioma cells. U251 cells were treated with 0, 50, 100 and 150 μg/ml β-elemene for 24 h (upper), or treated with 150 μg/ml β-elemene for the indicated time (lower). (A) RNA levels of survivin were detected by RT-PCR. GAPDH was used as a loading control. (B) Protein levels of survivin were detected by western blotting. β-actin was used as a loading control. (C and D) Similar results were obtained in A172 cells.

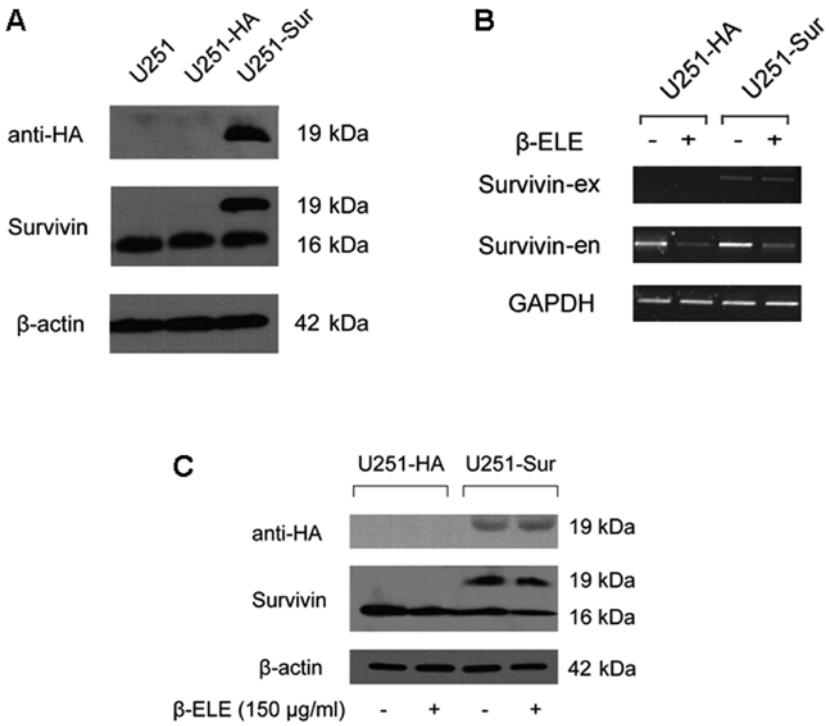

Figure 4. Establishment of survivin gene overexpression cell lines. (A) U251 cells were transfected with pMEG06-3HA-survivin (U251-sur) or pMEG06-3HA vector as a control (U251-HA). Stable cell lines were established by G418 selection. Levels of survivin were detected by western blotting using anti-survivin or anti-HA antibodies, respectively (B and C). U251-HA and U251-Sur cells were treated with 150 μg/ml β-elemene for 24 h. The mRNA transcription levels of exogenous and endogenous survivin gene were detected by RT-PCR using specific primers. GAPDH was used as a loading control (B). Protein levels were detected by western blotting using anti-survivin or anti-HA antibodies, respectively. β-actin was used as a loading control (C). The data are representative of three separate experiments.

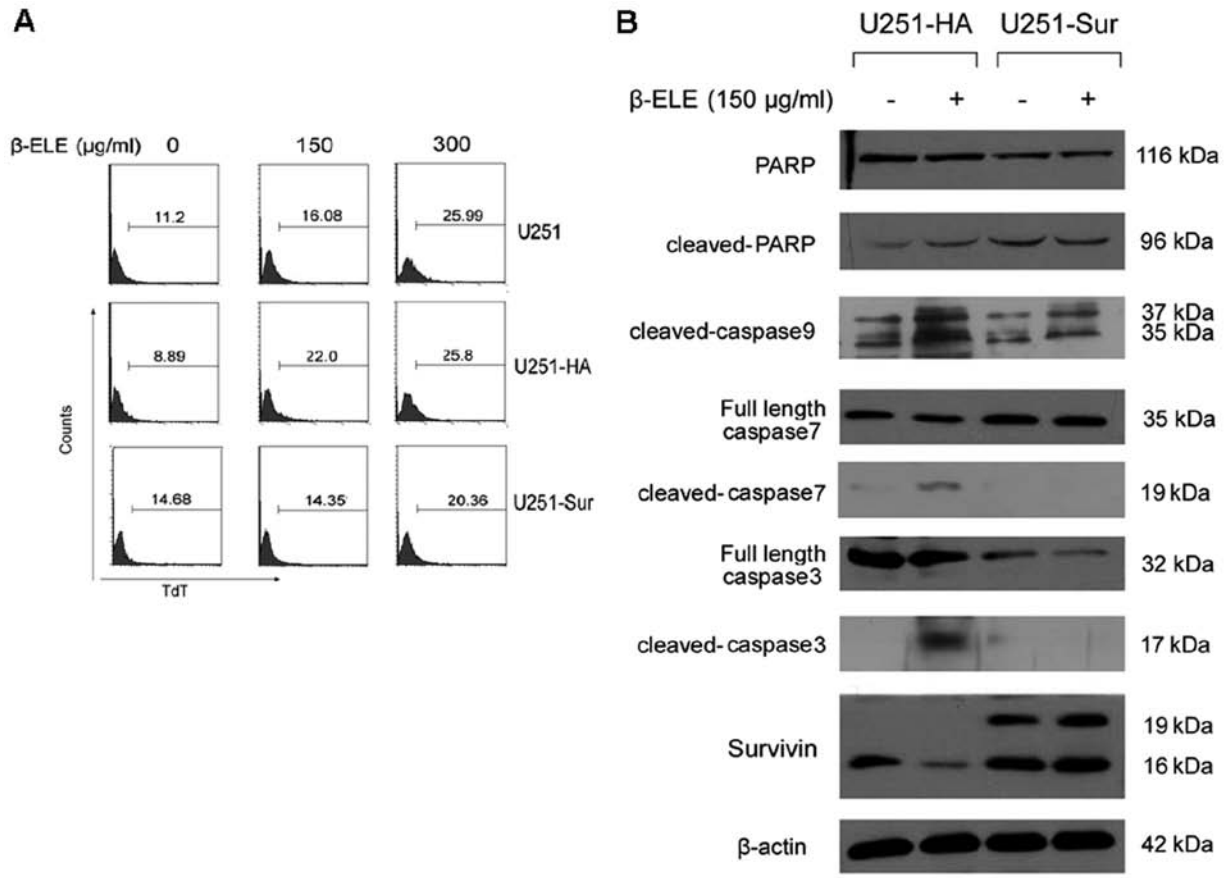

Figure 5. Overexpression of survivin gene inhibits β-elemene-induced glioma cell apoptosis. (A) U251, U251-HA and U251-Sur cells were treated with the indicated concentrations of β-elemene for 24 h. Cell apoptosis was determined by TUNEL assay as described in Materials and methods. (B) U251-HA and U251-Sur cells were treated with 150 μg/ml β-elemene for 24 h. The expression levels of caspase-3, -7, -9, PARP, and survivin were detected by western blotting using specific antibodies. β-actin was used as a loading control. The experiment was repeated three times with similar results.

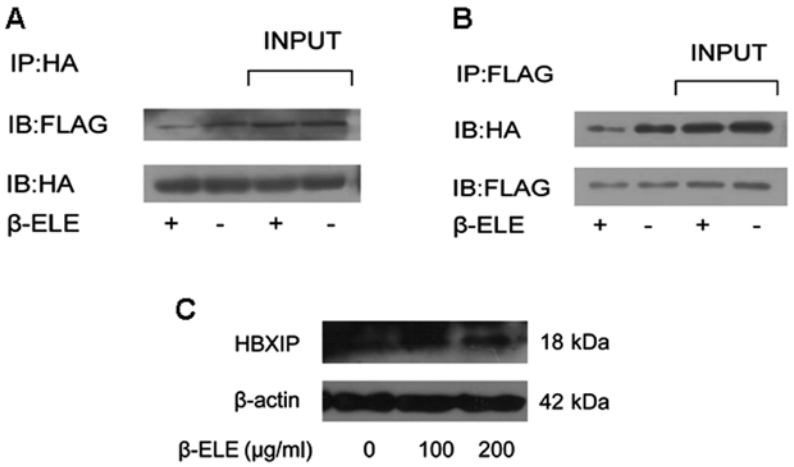

Figure 6. β-elemene inhibits the interaction between survivin and HBXIP. U251 cells cotransfected with pCMV-HBXIP plasmid and pMEG-survivin plasmid, were treated with β-elemene or the vehicle for 24 h. Cellular proteins were immunoprecipitated (IP) with anti-FLAG antibody and followed by immunoblotting (IB) with anti-HA antibody to detect HBXIP-bound survivin (A) or in the reverse order to detect survivin-bound HBXIP (B). (C) U251 cells were treated with different concentrations of β-elemene for 24 h and the levels of HBXIP were detected by western blotting. β-actin was used as a loading control. The results are representative of three separate experiments.

we clearly showed that survivin was bound to HBXIP protein in glioma cells. More importantly, the interaction between the two molecules was inhibited by β-elemene treatment (Fig. 6A and B). As survivin-HBXIP complex was assumed to be responsible for the retaining and retarding of caspase-9 activity, we suggested that β-elemene-induced impairment in survivin-HBXIP interaction might promote cell apoptosis. Besides, HBXIP level was not obviously affected by β-elemene as shown in Fig. 6C.

Discussion

β-elemene, an extract from the ginger plant *R. zeodaria*, is a novel anti-cancer herbal medicine with broad antitumor effects *in vitro* and *in vivo* (5,8,28,29). It has been approved by the Chinese FDA (the State Food and Drug Administration) for clinical treatment of malignant effusion and some solid tumors. However, the effects of β-elemene on glioma cancer cells have not been documented yet. In the present study, we provide the first evidence that β-elemene could promote apoptosis of human glioma cells and revealed a major pathway underlying this effect.

Apoptotic process is mediated through various pathways and regulated by multiple mechanisms. One of the pathways is mitochondria-dependent apoptotic route which is essentially dependent on the cleavage and activation of caspase-9 (30-32), Caspases are therefore considered as central executors of the apoptotic process, especially caspase-9, -3 and -7 (30,33,34). We showed that β-elemene induced apoptosis of human glioma U251 and A172 cells, as determined by TUNEL assay, annexin V staining and western blot analysis. The cell apoptosis was closely associated with activation of caspase-9, -3 and -7 and increased levels of cleaved PARP. Caspase inhibitors substantially attenuated the β-elemene-induced cell apoptosis. These data indicate that the effect of β-elemene on cell apoptosis was mediated via a mitochondrial-dependent apoptotic pathway.

One notable event associated with β-elemene treatment was marked inhibition of survivin expression in glioma cells. Survivin was found to be highly expressed in various human cancers, including bladder cancer (35), gastric cancer (36), hepatocellular carcinomas (37), colon/colorectal cancer (38), neuroblastomas (39), and human gliomas (40). Survivin, a number of IAP protein family, showed to be bound to caspase-3 and caspase-7 (41). Moreover, studies on transgenic mice revealed that survivin was able to inhibit the intrinsic, caspase-9-dependent apoptotic pathway (25). However, unlike XIAP, which can inhibit caspases after activation, survivin does not have the capability to directly interact with pro-caspase-9. HBXIP, originally identified as HBV-interacting protein, was unexpectedly found to act as an anti-apoptotic cofactor of survivin. HBXIP allowed survivin to bind and suppress the activation of pro-caspase-9, thus blocking the mitochondrial pathway for cell death (23). In the present study, we showed that the downregulation of survivin induce by β-elemene is responsible for glioma cell apoptosis, as death of cells can be rescued by forced expression of surviving shown in our test. More importantly, our results revealed that β-elemene inhibited the protein-protein interaction between survivin and HBXIP, which might facilitate the release and activation of caspase-9, enhancing human glioma cell apoptosis with β-elemene treatment. Our results merit further investigation into the characteristic and kinetics of survivin and HBXIP complex in tumorigenesis as well as in antitumor activity.

In conclusion, we demonstrated that β-elemene exerts an effective antitumor effect in human glioma cells by regulating survivin gene expression, and also by suppressing the interaction between survivin and HBXIP. These results establish β-elemene as a promising therapeutic agent in the treatment of patients with glioma.

Acknowledgments

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