Oleuropein inhibits the proliferation and invasion of glioma cells via suppression of the AKT signaling pathway

MING LIU1, JIAN WANG1,2, BIN HUANG1, ANJING CHEN1 and XINGANG LI1

1Department of Neurosurgery, Qilu Hospital of Shandong University and Brain Science Research Institute, Shandong University, Jinan, Shandong 250012, P.R. China; 2Department of Biomedicine, University of Bergen, 5009 Bergen, Norway

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Abstract. Oleuropein, the main phenolic compound of secoiridoids, has been proven to have inhibitory effects on various types of cancers. However, the antitumor effects and related mechanisms in glioma remain unclear. In the present study, U251 and A172 cells were used to assess the effects of oleuropein. Using cell viability assay, we found that oleuropein greatly inhibited the viability of the U251 and A172 cells. Additionally, flow cytometric apoptosis assay indicated that oleuropein induced the apoptosis of the two cell lines. Consistently, the inhibitory effects of oleuropein on migration and invasion were also observed in vitro. In regards to the mechanism, we found that oleuropein significantly decreased phosphorylation of AKT (p-AKT), accompanied by upregulation of Bax and downregulation of Bcl-2. We also found that there was a decrease in the expression levels of matrix metalloproteinase-2 (MMP-2) and MMP-9 after treatment with oleuropein. Furthermore, a specific phosphatidylinositol 3 kinase (PI3K) inhibitor, LY294002, enhanced the pro-apoptotic and anti-invasive effects induced by oleuropein, which suggested that oleuropein suppressed the growth and invasion of glioma cells via inhibition of AKT activity. Taken together, our results indicated that treatment with oleuropein may be an effective therapy for malignant glioma through suppression of the AKT signaling pathway.

Introduction

Malignant glioma, with high mortality and morbidity, accounts for ~10% of tumors in the central nervous system (1). Glioblastoma (GBM) is the most aggressive subtype, characterized by high proliferation, infiltration and invasion (2). Even combined with radiation and chemotherapy after surgical resection, the average lifespan expectancy of patients with GBM is still less than 14 months (3). Therefore, new treatments are needed to improve strategies for controlling glioma.

Oleuropein the main phenolic secoiridoid from the olive tree, has several positive functions for human health. Previous studies have reported that oleuropein possesses antioxidant, anti-inflammatory, antimicrobial, antiviral and neuroprotective activity (4-10). However, it has also revealed some promising antitumor function in various cancer cell lines including melanoma B16 cells, human lymphoblastic cell line Jurkat and human colon adenocarcinoma Caco-2 cells (11-13). Notably, recent studies have indicated that oleuropein inhibits growth and induces apoptosis in human breast cancer MCF-7 cells and prostate cancer cell line LNCaP (14,15). However, there are few studies concerning the pro-apoptotic and anti-invasive effects of oleuropein on glioma cells. In the present study, we aimed to ascertain whether oleuropein exhibits an antitumor effect on human glioma cells.

The Bcl-2 family, including Bcl-2 and Bax, is the central focus in the research of glioma cell apoptosis and Bcl-2 inhibits cell apoptosis while Bax promotes cell apoptosis (16). At the same time, the occurrence of glioma development is closely related with powerful invasion. Numerous studies have found that gliomas mostly spread through the white matter and the extracellular matrix. Damage of the extracellular matrix and cell surface adhesion molecules is the molecular basis of invasion. The matrix metalloproteinase (MMP) family, particularly MMP-2 and MMP-9, can degrade many types of extracellular matrix components, destruct the glioma cell histology barrier and play a key role in the process of invasion and metastasis of glioma cells (17).

In the present study, we investigated the effect of oleuropein on glioma cell apoptosis and invasion and explored which signaling pathway is involved in this process. U251 and A172 cells were utilized to investigate the effects of oleuropein. Our data established that oleuropein suppressed glioma cell proliferation by inducing apoptosis in vitro. We also discovered that oleuropein significantly inhibited the migration and invasion of glioma cells. Furthermore, exploration of the underlying
mechanisms indicated that oleuropein regulated the expression levels of Bax, Bcl-2 and MMPs by inhibiting AKT activation in the glioma cells. LY294002, a specific AKT inhibitor, enhanced the pro-apoptotic and anti-invasive effects of oleuropein. In conclusion, these novel findings demonstrated that the AKT pathway plays a key role in the antitumor effects of oleuropein in glioma cells by regulating the expression of Bax, Bcl-2, MMP-2 and MMP-9. Thus, oleuropein is a promising chemotherapeutic drug for targeting the AKT pathway.

Materials and methods

Cell lines, cell culture and reagents. The human malignant glioma cell lines U251 and A172 were obtained from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (CBTCCCAS; Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, USA) with 10% fetal bovine serum (FBS) at 37°C in a 5% CO2 humidified incubator. Oleuropein was purchased from Push Bio-technology, Ltd. (Chengdu, China) and was dissolved according to the manufacturer's instructions. Antibodies against p-AKT, AKT, p-p38, p38, p-ERK, ERK, p-JNK, JNK, GAPDH and HRP-conjugated goat anti-rabbit and anti-mouse IgG were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against MMP-2 and MMP-9 were obtained from ImmunoWay (Newark, DE, USA). Antibodies against p53, caspase-3, caspase-9, Bax, Bcl-2 were obtained from Abcam (Cambridge, UK). LY294002 was purchased from Sigma (St. Louis, MO, USA).

Cell viability assay. Cell viability was detected using the Cell Counting Kit-8 (CCK-8; Guangzhou, China) assay. U251 and A172 were seeded in 150 µl of medium were seeded into 96-well plates. After 12 h of incubation, the cells were treated with medium containing different concentrations of oleuropein and control cells received culture medium. After a 24-h incubation, the cells were subsequently treated with 20 µl of CCK-8 and incubated according to the manufacturer's instructions. The absorbance values were determined at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

In vitro scratch assay. The U251 and A172 cells were seeded in 24-well plates. After incubation for 24 h, each well was manually scratched with a 200-µl pipette tip, washed lightly with phosphate-buffered saline (PBS) and incubated with oleuropein (0, 200 and 400 µM) for 24 h. After 24 h of monitoring, the images were captured under a microscope. The distance between two cell edges was statistically analyzed.

Transwell assay. U251 (1x10^5) and A172 (1x10^5) cells were respectively collected in 200 µl serum-free medium and were added to the upper chambers. DMEM (800 µl) containing 20% FBS and oleuropein (0, 200 and 400 µM) were then added to the lower chamber. After 24 h, the cells that migrated to the lower surface were fixed with methanol and stained with eosin. The number of cells that migrated to the lower side was counted in five random fields under a light microscope. The cell number was counted and statistically analyzed. For Western blotting, U251 and A172 cells were treated with 0, 200 and 400 µM oleuropein, respectively, for 24 h. Then, the cells were lysed in RIPA buffer containing 1% protease inhibitors. Equal amounts of proteins from each sample were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% non-fat milk for 2 h at room temperature and incubated with the primary antibodies overnight at 4°C. Then, the membranes were probed with the appropriate secondary antibodies for 1 h at room temperature. Finally, the bands were developed using Western Chemiluminescent HRP Substrate and imaged by Image Station 4000MM Pro (Carestream Health Inc., Woodbrige, MA, USA).

Statistical analysis. The results are presented as the mean ± SD. Statistical differences between groups were analyzed by one-way ANOVA and further Tukey's test using the statistical software of GraphPad Prism 5. All experiments were repeated at least three times. Statistical significance is indicated in the figures as follows: *P<0.05, **P<0.01 and ***P<0.001.

Results

**Oleuropein inhibits the viability of glioma cells.** U251 and A172 cells were exposed to different concentrations (0, 100, 200 and 400 µM) of oleuropein for 24 h, and their viability was analyzed by the CCK-8 assay. The results showed a stable decrease in cell viability after treatment with oleuropein (Fig. 1A and B). Treatment with 100 µM oleuropein had a modest effect, while 400 µM oleuropein exhibited an obvious inhibitory effect. The viability of the U251 and A172 cells was reduced to 53.17 and 52.78% after treatment with oleuropein (400 µM) for 24 h, respectively.

**Oleuropein suppresses the migration and invasion of glioma cells.** We implemented the scratch assay to investigate the effect of oleuropein on the migration of U251 and A172 cells. Migration of the U251 cells was inhibited by oleuropein (Fig. 2A and B). The width of the control group accounted for only 22.72% of the original width while that of the cells treated with 200 and 400 µM accounted for 45.45 and 63.64% of the original width, respectively. A similar effect was also observed in the A172 cells (Fig. 2C and D). After treatment with 0, 200 and 400 µM oleuropein, the relative width increased from 38.46 to 73.07%.
To test the influence of oleuropein on cell migration and invasion, U251 and A172 cells treated with oleuropein were used to perform the Transwell assay. Our results indicated that oleuropein significantly restrained the migration and invasion of both cell lines. The inhibitory effect of oleuropein on cell mobility was dose-dependent, with significant decreases observed at concentrations of 200 and 400 µM. Data are presented as the means ± SD of three independent experiments; *P<0.05, **P<0.01, ***P<0.001 compared with the control group.
of the U251 cells (Fig. 2E and F), and the number of cells successfully migrating to the lower side in the 400 µM group only accounted for ~20%. Furthermore, we observed the same effect on the A172 cells (Fig. 2G and H).

**Oleuropein induces apoptosis in U251 and A172 cells.** To further detect the apoptotic cells induced by oleuropein, we used Annexin V-conjugated FITC and PI staining to analyze the percentage of apoptotic cells. The lower right (LR) and upper right (UR) quadrants of the histograms show the percentages of early and late apoptotic cells, respectively. The total percentage of apoptotic cells (UR + LR) increased from 1.17 (0 µM oleuropein) to 8.92% (200 µM oleuropein), and 15.46% (400 µM oleuropein) in the U251 cells, respectively (Fig. 3A and C), while the total percentage of apoptotic cells was increased from 0.86 (0 µM oleuropein) to 6.18% (200 µM oleuropein), and 17.10% (400 µM oleuropein) in the A172 cells (Fig. 3B and D). These results revealed that the treatment of U251 and A172 cells with 200 and 400 µM oleuropein for 24 h promoted apoptosis in a dose-dependent manner.

**Effects of oleuropein on the expression of cell apoptosis-related proteins.** Numerous studies have reported that pro-apoptotic protein Bax is associated with increased apoptosis, while the anti-apoptotic protein Bcl-2 is associated with the inhibition of apoptosis in target cells (18,19). To investigate the change in Bax and Bcl-2 in the U251 and A172 cells after treatment with oleuropein, western blotting was used to detect the expression of Bax and Bcl-2. The expression of Bax was increased while the expression of Bcl-2 was decreased after treatment with oleuropein (Fig. 4A). Additionally, the expression of cleaved caspase-9 and -3 were increased (Fig. 4A). Thus, the tumor inhibitory effect of oleuropein on the U251 and A172 cells may be attributed to apoptosis which was mainly regulated by changes in the levels of Bax and Bcl-2.

**Effects of oleuropein on MMP expression and the AKT pathway.** MMPs, particularly MMP-2 and MMP-9, play important roles in breaking down basement membranes and contribute to cancer invasion and metastasis (20). The expression levels of MMP-2 and MMP-9 were significantly decreased after oleuropein treatment (Fig. 4B). However, we found that after
treatment with oleuropein for 24 h, the phosphorylation level of AKT was effectively suppressed in both the U251 and A172 cells, and there were no changes in the expression levels of phosphorylated P38, ERK and JNK (Fig. 4C). Combined with the influence of oleuropein on apoptosis-related proteins, we speculated that AKT signaling may play an important role in the oleuropein-induced pro-apoptotic and anti-invasive effects.

Inhibition of AKT signaling enhances the pro-apoptotic and anti-invasive effects of oleuropein on U251 and A172 cells. LY294002, a pan-PI3K inhibitor, strongly inhibits AKT activation. U251 and A172 cells were cultured in the presence of either oleuropein (200 µM) or/and LY294002 (50 µM) for 24 h. The protein levels of p-AKT, AKT, Bax, Bcl-2, MMP-2 and MMP-9 were detected by western blotting. The results revealed that either LY294002 or oleuropein could significantly restrain the expression of AKT, MMP-2, MMP-9 and Bcl-2, and strongly promote the expression of Bax. However, combined treatment with LY294002 played a more significant role in the pro-apoptotic and anti-invasive effects (Fig. 5A and B).

Next, after treatment with oleuropein (200 µM) and/or LY294002 (50 µM) for 24 h, we used MTT assay to observe the proliferation of the U251 and A172 cells and conducted a Transwell assay to investigate the invasive abilities of the two cell lines. The results indicated that LY294002 cooperated with oleuropein to inhibit proliferation (Fig. 5C and D) and invasion (Fig. 5E-H) of the U251 and A172 cells. In addition, the results revealed that LY294002 can potentiate the pro-apoptotic and anti-invasive effects of oleuropein, which suggests that blocking the AKT signaling pathway may improve the anticancer effects of oleuropein.

Discussion

Glioma, particularly glioblastoma (GBM) is the leading cause of brain tumor-related death (21). Accumulating evidence has confirmed the anticancer effects of oleuropein against multiple malignant tumors, but the antitumor effects and related mechanisms of oleuropein in glioma are still unclear. In the present study, we demonstrated that oleuropein induced apoptosis and inhibited the invasion of glioma cells by regulating the expression of Bax, Bcl-2, MMP-2 and MMP-9 via suppression of the AKT signaling pathway.

Apoptosis plays vital roles in cell growth with the elimination of aberrant cells (22). Deregulation of apoptosis contributes to pathological conditions and many types of cancers (23,24). The Bcl-2 family consists of several proteins that execute pro- and anti-apoptotic functions and is tightly connected with cancer cell apoptosis, including glioma (25,26). The Bcl-2 family regulates the release of death factors including Smac/Diablo, apoptosis-inducing factor (AIF) and endonuclease G, which trigger either caspase-dependent or-independent apoptosis (27-30). Bcl-2 is a member of this family and acts as an anti-apoptotic protein, which is processed on the outer mitochondrial membrane and regulates cytochrome c release. Subsequently, activation of caspase-3 causes a series of events related to apoptosis (31-34). In contrast, Bax is a pro-apoptotic protein, which is processed on the outer mitochondrial membrane and regulates cytochrome c release. Subsequently, activation of caspase-3 causes a series of events related to apoptosis (35-37). Therefore, Bcl-2 and Bax are key factors in apoptosis. In the present study, we examined the changes in Bcl-2 and Bax expression in the U251 and A172 cells following treatment with oleuropein, and found that Bax expression increased and Bcl-2 expression decreased in vitro. However, expression of their downstream substrates, caspase-9 and -3, were obviously increased. The results indicated that the oleuropein-induced upregulation of Bax expression and downregulation of Bcl-2 expression may cooperate with each other to induce the apoptosis of U251 and A172 cells. We also assessed the expression of P53, which also plays important roles in cell apoptosis, but no significant change was found and we speculated that...
oleuropein-induced apoptosis of U251 and A172 may have little relation with p53.

The process of invasion and metastasis of glioma is complex and multiple factors may cooperatively be involved. MMPs are considered to be crucial proteinases in tumor invasion and metastasis (38,39). To date, it is widely accepted that degradation of the extracellular matrix and alteration of cell-cell extracellular matrix interactions mainly contribute to the process of invasion and metastasis of tumor cells (20). MMP-2 and MMP-9 proteins are two major members of the MMP family and they are widely involved in cancer metastasis. However, various studies have indicated that patients with increased expression of MMP-2 and MMP-9 proteins have a poor survival (40). MMPs also exert influences on the metastasis and invasion of glioma cells. Various studies have shown that upregulation of MMPs promotes the invasion of glioma stem cells (41). However, downregulation of the expression of MMP-2 and MMP-9 could reduce the invasion of glioma cells (42). Given the effects of MMPs on metastasis and invasion in glioma cells, we tested the changes in MMP-2 and MMP-9 after treatment with oleuropein and found that the expression of MMP-2 and MMP-9 decreased significantly after oleuropein administration in both cell lines in a dose-dependent manner. Combined with the Transwell assays, we considered that oleuropein may suppress metastasis and invasion of glioma cells by decreasing the expression of MMP-2.
and MMP-9. Given the influence of oleuropein on apoptosis-related proteins and MMPs, we speculated that some signaling pathway(s) may play an important role in oleuropein-induced inhibition of invasive and promotion of apoptosis.

The synthesis of Bax, Bcl-2, MMP-2 and MMP-9 is regulated by multiple signaling cascades. The AKT signaling pathway participates in many important cellular processes, including tumor cell proliferation, metastasis and invasion (43), and this pathway can also exert influences on the Bcl-2 family and MMPs (44,45). Recent studies indicate that the AKT signaling pathway is overactive in many cancers, and inhibition of this pathway is considered as a novel target for cancer therapy (46). One study demonstrated that oleuropein is implicated as a negative regulator of proliferation of glioma cells (47). However, the underlying mechanisms through which oleuropein exerts its antitumor effects are not clear. In the present study, to further explore the possible mechanism(s) of oleuropein in the inhibition of glioma cell growth, we assessed the levels of phosphorylation of AKT, P38, ERK, JNK in U251 and A172 cells after treatment with oleuropein. We found that after treatment of oleuropein for 24 h, the phosphorylation level of AKT was effectively restrained in both the U251 and A172 cells and there were no significant changes in the activity of phosphorylation of P38, ERK and JNK signaling pathways, which suggested that oleuropein inhibited the activation of AKT. To investigate whether the pro-apoptotic and anti-invasive effects induced by oleuropein are mainly associated with the AKT pathway. LY294002, a PI3K inhibitor, was used. We found that LY294002 alone increased the expression level of Bax and decreased the expression levels of Bcl-2, MMP-2 and MMP-9 while combined use with oleuropein markedly potentiated the pro-apoptotic and anti-invasive effects of oleuropein on the U251 and A172 cells, which suggests that blocking the AKT signaling pathway may improve the anticancer effect of oleuropein on glioma cells. Our data also suggest that AKT may be at the upstream of the pro-apoptotic and anti-invasive process induced by oleuropein (Fig. 6). We speculate that oleuropein may suppress the activation of AKT. Then, expression levels of MMP-2 and MMP-9 are downregulated which inhibits the invasion of glioma. In contrast, the proportion of Bax and Bcl-2 is changed which exerts an effect on the apoptosis of glioma cells.

In conclusion, the present study demonstrated the inhibitory effect of oleuropein on the apoptosis and invasion of glioma cells. The upregulation of Bax and downregulation of Bcl-2 induced by oleuropein were enhanced by suppression of AKT activity. Additionally, the decrease in MMP-2 and MMP-9 induced by oleuropein also attributed to inhibition of the AKT signaling pathway. These findings revealed that the AKT signaling pathway contributes to the anticancer effects of oleuropein on glioma cells and oleuropein may be a new potential therapeutic agent for glioma.

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