Honokiol exerts an anticancer effect in T98G human glioblastoma cells through the induction of apoptosis and the regulation of adhesion molecules

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Abstract. Glioblastoma is one of the most lethal and common malignant human brain tumors, with aggressive proliferation and highly invasive properties. Honokiol derived from Magnolia officinalis is able to cross the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB), suggesting a strong possibility that it could be an effective drug for the treatment of brain tumors, including glioblastoma. Thus, we investigated the effects of honokiol on the expression of adhesion molecules in TNF-α-stimulated endothelial cells, and cancer growth and invasion were determined in T98G human glioblastoma cells. Honokiol dose-dependently inhibited the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in human umbilical vein endothelial cells (HUVECs) stimulated with TNF-α for 6 h. Pretreatment with honokiol significantly reduced the adhesion of T98G cells to HUVECs. Moreover, honokiol inhibited the invasion of T98G cells, suggesting that honokiol has an anti-metastatic effect. In addition, honokiol increased the cytotoxicity of T98G cells in a dose- and time-dependent manner as assayed by MTT. TUNEL assay showed that honokiol significantly induced apoptosis in T98G cells at doses of 10 µM or more. The induction of apoptotic cell death was mediated by the downregulation of the anti-apoptotic protein Bcl-2 and the upregulation of the pro-apoptotic protein Bax. Taken together, the results of this study suggest that honokiol exerts an anticancer effect by preventing metastasis and inducing apoptotic cell death of brain tumor cells.

Introduction

Malignant gliomas account for approximately 70% of the 22,500 new cases of malignant primary brain tumor diagnosed in adults in the United States each year (1). Glioblastoma, the most common malignant primary central nervous system (CNS) glioma in adults, represents 51% of all CNS gliomas. Glioblastoma is comprised of poorly differentiated, heterogeneous neoplastic astrocytes that exhibit aggressive proliferation and highly invasive properties (2). It diffusely infiltrates various regions of the normal brain, making total surgical removal impossible; thus, patients diagnosed with glioblastoma have a poor prognosis, even in response to multidisciplinary treatment strategies including surgery, radiotherapy and chemotherapy (3). Moreover, in the case of chemotherapy, the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB) hampered the effects of both conventional and targeted therapies. Therefore, for drugs to act within the brain, drugs must cross the BBB and the BCSFB.

The root and stem bark of Magnolia officinalis has been used as a folk medicine by the Chinese people for the treatment of thrombotic stroke, gastrointestinal complaints, anxiety and nervous disturbance (4). Honokiol is a well-known bioactive constituent of the bark of Magnolia officinalis and has long been known to possess antioxidant (5), anxiolytic (6-9), and antidepressant activities (10), as well as to prevent and protect the brain from damage (11). Previous studies have shown that honokiol also demonstrated extensive antitumor efficacy in vitro and in vivo (12-15) and that treatment with honokiol was a potential strategy to overcome immunoresistance in glioma (16). According to a recent report, honokiol was able to cross the BBB and the BCSFB (17), suggesting a strong possibility that it could be an effective drug for the treatment of brain tumors, including glioblastoma.

Adhesion molecules play an important role in the inflammatory response and the interactions of cancer cells with the...
extracellular matrix (ECM). Cancer progression is a multi-step process in which some adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, play a pivotal role in the development of recurrent, invasive and distant metastasis. Cell adhesion molecules (CAMs) are expressed on a variety of cells, including vascular endothelial cells (ECs), lymphocytes, fibroblasts, hematopoietic cells and tumor cells (18-22), that have been activated by cytokines such as interleukin-1α (IL-1α), IL-6 or tumor necrosis factor-α (TNF-α) (23,24). TNF-α, in particular, induces upregulation of ICAM-1 and VCAM-1 in ECs (25-27). ICAM-1 and VCAM-1 have been shown to be involved in cell-cell and cell-ECM interactions and are mechanistically important for the extravasation of both monocytes during inflammation (28) and cancer cells during metastasis (29,30). The adhesion of circulating tumor cells to the microvascular endothelium of organs at distant sites is an important step in blood-borne metastasis.

It has been reported that honokiol possesses potent activities against CNS diseases and anti-angiogenic properties. However, to date few studies have reported on the effect of honokiol on cell death and invasion of glioblastoma. Therefore, the aim of the present study was to determine the effect of honokiol on invasion of T98G glioblastoma cells, cell death and the possible mechanisms involved.

Materials and methods

**Materials.** Honokiol (MW 266.33, C₁₉H₁₉O₄, Fig. 1) was purchased from Wako Chemical (Wako, Japan). Fetal bovine serum (FBS) was purchased from Gibco-BRL (Rockville, MD). Anti-ICAM-1 and anti-VCAM-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) western blotting detection reagent was purchased from Amersham (Buckinghamshire, UK). All other chemicals, including endothelial cell growth supplements (ECGS) and heparin, were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell culture.** Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (San Diego, CA), grown in medium 199 supplemented with 20% FBS, 2 mM L-glutamine, 5 U/ml heparin, 100 IU/ml penicillin, 10 μg/ml streptomycin and 50 μg/ml ECGS and incubated in a humidified 5% CO₂ incubator. HUVECs were plated at a density of 1x10⁴ cells/ml. Cells were gently washed with M199H. Fluorescent images were obtained using a high-resolution video camera (DXC-960MD; Sony) mounted on a BH-2 Olympus microscope (Melville, NY), and the immunoreactivity of these images was measured using SigmaGel 1.0 (Jandel Scientific; Germany). The analyses were repeated three times over the same region, and the results are the mean values of the three independent experiments.

**Adhesion assay.** HUVECs were seeded into two-well chamber slides 48 h before the experiments. T98G cells (3x10⁵) were incubated in an RPMI-1640 medium containing 2% FBS and 10 mg/ml of the fluorescent dye BCECF/AM (Boehringer, Mannheim, Germany) at 37°C for 30 min. Fluorescently labeled cells were pelleted and resuspended (7.5x10⁴ cells/ml) in medium 199 with 10 mM HEPES buffer (M199H). HUVECs were washed three times with M199H before the dye-loaded cells were added and incubated at 37°C. After 30 min, cell suspensions were withdrawn, and the HUVECs were gently washed with M199H. Fluorescent images were obtained using a high-resolution video camera (DXC-960MD; Sony) mounted on a BH-2 Olympus microscope (Melville, NY), and the immunoreactivity of these images was measured using SigmaGel 1.0 (Jandel Scientific; Germany). The analyses were repeated three times over the same region, and the results are the mean values of the three independent experiments.

**Matrigel invasion assay.** The Matrigel invasion assay was performed as previously described (31). Briefly, T98G cells treated with honokiol were collected, and 2x10⁴ cells/insert in serum-free media were added to the Matrigel-coated upper chambers (8 μm pore size, Falcon). RPMI media containing 10% FBS was added to the lower chambers, and the invasion chambers were incubated for 24 h in a 37°C cell culture incubator. The noninvasive cells that remained on the upper surface of the insert membranes were removed by scrubbing. The cells on the lower insert membranes were stained with DAPI, and the cells were counted under the light microscope.
Each sample was measured in triplicate, and each experiment was repeated three times.

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay. Cells at the exponential phase were seeded at 1x10^7 cells/well on a slide glass. The cells were treated with honokiol for 24 h at 37˚C, washed with PBS and fixed by the addition of methanol. Apoptotic cells were identified by a TUNEL assay of nucleosomal DNA fragments using a commercially available In Situ Cell Death Detection Kit (Roche, Penzberg, Germany), according to the manufacturer’s protocol, with a minor modification.

Statistical evaluations. Scanning densitometry was performed using an Image Master® VDS (Pharmacia Biotech Inc., San Francisco, CA). All data are expressed as the mean ± SD of results from the number (n) of experiments. Differences between data sets were assessed by Student’s t-test. P<0.05 indicated a statistically significant difference.

Results

Honokiol suppressed TNF-α-induced ICAM-1 and VCAM-1 expression in HUVECs. Adhesion molecules such as ICAM-1 and VCAM-1 have been shown to be involved in cell-cell and cell-ECM interactions and are mechanistically important for the extravasation of cancer cells during metastasis (1,29). The adhesion of circulating tumor cells to the microvascular endothelium of organs at distant sites is an important step in blood-borne metastasis. Accordingly, we first examined the effect of honokiol on ICAM-1 and VCAM-1 expression after TNF-α-stimulation of HUVECs. The cells were pretreated with varying doses of honokiol (1, 5, 10, 20 or 50 µM) for 24 h and then stimulated with TNF-α (10 ng/ml) for 6 h. After treatment, the protein was extracted from the cells, and (A) ICAM-1 and VCAM-1 protein levels were determined by western blot analysis. (B) Band intensities were assessed by scanning densitometry. Data are presented as the mean values ± SD of three independent experiments. Significance compared with TNF-α, *P<0.05, **P<0.01.

Honokiol inhibited the TNF-α-stimulated adhesion of T98G glioblastoma cells to ECs. Following the study of the effect of honokiol on ICAM-1 and VCAM-1 expression after TNF-α-stimulation of HUVECs. The cells were pretreated with varying doses of honokiol (1, 5, 10, 20 or 50 µM) for 24 h and then co-treated with TNF-α (10 ng/ml) for 6 h. The results showed that TNF-α increased both ICAM-1 and VCAM-1 expression. This increase was significantly suppressed by honokiol from 20 µM, or 5 µM, respectively, suggesting that honokiol regulates the TNF-α-induced expression of VCAM-1 more effectively than that of ICAM-1 (Fig. 2).

Honokiol effectively prevented T98G cell invasion. Cancer cell invasion is important during the formation of distant metas-
invasions. Therefore, an in vitro invasion assay was performed to assess whether honokiol could inhibit glioblastoma invasion. T98G cells not treated with honokiol exhibited significant migration across a transwell membrane; by contrast, treatment with 5 to 50 µM honokiol significantly inhibited cancer cell invasion (Fig. 4).

Honokiol decreased cell viability of T98G glioblastoma cells in a dose-dependent manner. Subsequently, we examined the cell viabilities of HUVECs and T98G cells in response to honokiol. When HUVECs and T98G cells were treated with varying doses of honokiol (1, 5, 10, 20 or 50 µM) for 24 h, honokiol significantly suppressed cell viability of T98G cells at doses of 10 µM or more; 50 µM of honokiol decreased cell viability of T98G cells by approximately 77% (Fig. 5A). Although honokiol also decreased the cell viability of HUVECs, honokiol-mediated cytotoxicity was not significant at doses lower than 20 µM, and the 50 µM dose was less toxic to HUVECs than to T98G cells (Fig. 5B).

Honokiol induced apoptotic cell death by increasing the Bax/Bcl-2 ratio. Fig. 5A shows that honokiol significantly induced T98G cell death from 10 µM, a lower dose than was toxic to HUVECs. To confirm that honokiol-induced cytotoxicity was due to the induction of apoptotic cell death in T98G cells, we performed a TUNEL assay and also assayed the levels of the anti-apoptotic protein, Bcl-2, and the pro-apoptotic protein, Bax, by western blot analysis. Cells were treated with honokiol (toxic doses; 10, 20 or 50 µM) for 24 h, and TUNEL-positive cells were determined as described in Materials and methods. As shown in Fig. 6, honokiol effectively increased the number of TUNEL-positive cells at 10, 20 and 50 µM, suggesting that honokiol-induced cytotoxicity was due to the induction of apoptotic cell death. Moreover, western blot analysis showed that honokiol significantly increased pro-apoptotic Bax protein levels and decreased anti-apoptotic Bcl-2 levels in T98G cells at doses of 10 µM or more (Fig. 7), corresponding to the honokiol-induced cell death that also occurs at doses of 10 µM or more. These results suggest that honokiol induces apoptotic cell death in glioblastoma cells through the upregulation of the Bax/Bcl-2 ratio.

Discussion

This study showed that honokiol, an active component isolated from the herb *Magnolia officinalis*, exerts anticancer effects in human glioblastoma T98G cells through the regulation of adhesion molecules and the induction of apoptotic cell death. In addition, we suggested that honokiol induces apoptotic cell death in glioblastoma cells through the upregulation of the Bax/Bcl-2 ratio and blocks glioblastoma cell invasion.
through regulation of adhesion molecules such as ICAM-1 and VCAM-1 (Fig. 8).

As mentioned in the Introduction, glioblastoma is one of the most lethal and common malignant brain tumors in humans. Due to its tendency to diffusely infiltrate various regions of the normal brain, complete surgical removal is impossible, leading to a poor prognosis. One chemotherapeutic strategy is to aim at altering the biological properties of the cancerous cells to encourage their apoptosis or to block their invasion into other regions. Apoptosis is a physiological mode of cell death that can be selectively triggered by cells in response to a stimulus. Therefore, the induction of apoptosis is a key target of anticancer drugs. Apoptotic machinery is composed of dozens or more anti-apoptotic and pro-apoptotic proteins. The balance of anti-apoptotic and pro-apoptotic proteins contributes to the balance of cell growth and cell death. Bax, a pro-apoptotic protein, is normally found as a monomer in the cytosol of non-apoptotic cells. In response to apoptotic stimuli, Bax oligomerizes and translocates to the outer mitochondrial membrane (32), where it induces mitochondrial membrane permeabilization (33) and cytochrome c release (34). Overexpression of the anti-apoptotic protein, Bcl-2, has been found to stabilize the outer membrane and prevent the release of cytochrome c following a variety of insults. In this study, honokiol dramatically increased the levels of the pro-apoptotic protein, Bax, and significantly

Figure 7. The effect of honokiol on the level of the anti-apoptotic protein, Bcl-2, or the pro-apoptotic protein, Bax. Cells were treated with various concentrations of honokiol (1, 5, 10, 20, or 50 µM) for 24 h, and (A) the levels of Bcl-2 and Bax were determined by western blot analysis. (B) Bar graph shows densitometric determination of the level of Bcl-2 and Bax. Significance compared with honokiol, *P<0.05, **P<0.01.
decreased the levels of anti-apoptotic protein, Bcl-2, in T98G glioblastoma cells, suggesting that the honokiol's apoptotic potential is directly related to its ability to alter the ratio of pro-apoptotic to anti-apoptotic proteins in targeted cells.

With regard to invasion of cancer cells, a great deal of evidence suggests that CAMs may be associated with invasion and metastasis in a variety of human malignancies. This study demonstrated that 10 ng/ml TNF-α significantly induced ICAM-1 and VCAM-1 expression in HUVECs and that this induction was dramatically inhibited by honokiol. Additionally, honokiol significantly reduced the TNF-α-mediated adhesion of cancer cells to ECs, which may be due to the inhibition of ICAM-1 and VCAM-1 expression. Interestingly, in this study, the lower doses (5 µM or lower) of honokiol more effectively inhibited the increase in VCAM-1 levels induced by TNF-α than the increase in ICAM-1 levels (Fig. 2). According to previous studies, VCAM-1 plays a more important role than ICAM-1 in cancer metastasis (35,36). Moreover, honokiol is less toxic to HUVECs than to T98G cells at the doses of 5 or 10 µM while honokiol inhibits VCAM-1 expression or is toxic to T98G cells at these doses. Thus, honokiol may have a beneficial effect in the treatment of cancer.

Although some drugs show promise in treating cancers, there is a limitation to their use against brain tumors due to the BBB and the BCSFB. The BBB and the BCSFB are composed of capillary endothelial cells connected by tight junctions. Their main function as physical and active barriers is to restrict and regulate the penetration of compounds into and out of the brain to maintain brain homeostasis. Recently, Wang et al (17) reported that honokiol crosses the BBB and the BCSFB and contributes to antitumor activity in the brain. For this reason, honokiol has been used as an herbal medicine to treat nervous disorders (7,10). Taken together, our study suggests that honokiol might be a potential therapeutic strategy against brain tumors such as glioblastoma.

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References


