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Glabridin induces apoptosis and autophagy through JNK1/2 pathway in human hepatoma cells

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ABSTRACT

Background: Extensive research results support the use of herbal medicine or natural food to augment therapy for various cancers. Studies have associated glabridin with numerous biological activities, such as regulating energy metabolism and estrogenic, neuroprotective, antiosteoporotic, and skin-whitening activities.

Hypothesis/Purpose: However, how glabridin affects tumor cell autophagy has not been clearly determined.

Methods: Autophagy is a lysosomal degradation pathway essential for cell survival and tissue homeostasis. In this study, the roles of autophagy and related signaling pathways during glabridin-induced autophagy in human liver cancer cells were investigated. Additionally, the molecular mechanism of the anticancer effects of glabridin in human hepatoma cells was investigated.

Results: The results revealed that glabridin significantly inhibited cell proliferation in human hepatoma cells. Glabridin induced apoptosis dose-dependently in Huh7 cells through caspase-3, -8, and -9 activation and PARP cleavage. Furthermore, autophagy was detected as early as 12 h after exposure to a low dose of glabridin, as indicated by the up-regulated expression of LC3-II and beclin-1 proteins. The inhibition of JNK1/2 and p38 MAPK by specific inhibitors significantly reduced glabridin-induced activation of caspases-3, -8, and -9. Blocking autophagy sensitize the Huh7 cells to apoptosis.

Conclusion: This study demonstrated for the first time that autophagy occurs earlier than apoptosis does during glabridin-induced apoptosis in human liver cancer cell lines. Glabridin induces Huh7 cell death through apoptosis through the p38 MAPK and JNK1/2 pathways and is a potential chemopreventive agent against human hepatoma.

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Introduction

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver. HCC is now the third leading cause of cancer deaths worldwide, with over 500,000 people affected (Kessler et al. 2015). The incidence of hepatocellular carcinoma is highest in Asia and Africa. This type of cancer occurs more often in men than women. Patients with hepatitis B or C are at high risk of liver cancer, even

Abbreviations: hepatocellular carcinoma, HCC; dietary supplement, DS; focal adhesion kinase, FAK; transforming growth factor beta, TGF-β; nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB; activator protein 1, AP-1; 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide, MTT; bafilomycin A1, BafA1; 4'-6-diamidino-2-phenylindole, DAPI; Dulbecco's modified Eagle's medium, DMEM; fetal bovine serum, FBS; polyvinylidene difluoride, PVDF; acidic vesicular organelles, AVOs; poly (ADP-ribose) polymerase, PARP; microtubule-associated protein 1A/1B-light chain 3, LC3; mitogen-activated protein kinases, MAPK.

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if they do not develop cirrhosis (Leake 2014). HCC is not the same as metastatic liver cancer, which starts in another organ (such as the breast or colon) and spreads to the liver. Chemoprevention, using phytochemicals with potent antioxidant and anti-inflammatory properties, represents a fascinating strategy, which has been a subject of intense investigation in the recent years (Panossian 2014). Increasing attention has been paid to the use of products derived from natural plants against malignant invasive progression in the late stage of neoplastic diseases (Ivanov et al. 2007) and as potent chemopreventive drugs (da Rocha et al. 2001), particularly for relatively chemorefractory tumors, such as HCC (Chua and Choo 2011). Increasing attention has been focused on providing a scientific basis for using these agents as a preventive strategy for people with a high risk of cancer.

Isoflavones are secondary vegetable substances, produced from a branch of the general phenylpropanoid pathway that produces all flavonoid compounds in higher plants, which can act as estrogens in the body and have protective functions. Isoflavones have been identified as dietary components having an important role in reducing the incidence of breast and prostate cancers (Sarkar and Li 2003). Glabridin is an isoflavone and, as a key chemical and biological marker of *G. glabra*, is critical in the food, dietary supplement (DS), and cosmetics industries. Glabridin is a part of a larger family of plant-derived molecules, the natural phenols. Previous study revealed that glabridin can inhibit lung and breast cancer metastasis by inhibiting the Focal Adhesion Kinase (FAK)/rho signaling pathway (Hsu et al. 2011; Tsai et al. 2011). Glabridin-mediated anti-inflammatory action and attenuates colonic inflammation in mice with dextran sulfate sodium-induced colitis (Kwon et al. 2008). Glabridin has exhibited positive effects in down-regulating iNOS expression and activity under high glucose stress and inflammation (Yehuda et al. 2015). Glabridin inhibits the CSC-like properties of HCC cells through the miR-148a-mediated inhibition of the Transforming growth factor beta (TGF-β)/SMAD2 signal pathway (Jiang et al. 2014). Glabridin also inhibits migration and invasion by transcriptionally inhibiting matrix metalloproteinase 9 through the modulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1) activity in human liver cancer cells (Hsieh et al. 2014a). Glabridin attenuates the migratory and invasive capacity of breast cancer cells by activating microRNA-200c (Ye et al. 2014). Furthermore, glabridin mediates caspase activation and induces apoptosis in human promyelocytic leukemia cells (Huang et al. 2014). However, the effects of glabridin on human hepatoma autophagy have yet to be evaluated.

Autophagy is a major intracellular degradation mechanism that operates under stress conditions to promote survival during starvation or cause type II programmed cell death under specific conditions, such as the inhibition of apoptosis (Gozuacik and Kimchi 2007; Liu and Lenardo 2007; Yu et al. 2004). The autophagy process is initiated by the engulfing of large sections of a cytoplasm by a crescent-shaped phagophore that elongates into an autophagosome, which subsequently fuses with a lysosome, causing its contents to be degraded by lysosomal hydrolases (Chang et al. 2007; Kanzawa et al. 2003). Because autophagy is vital in regulating growth and maintaining homeostasis in multicellular organisms, defective autophagy contributes to the pathogenesis of several diseases, including myopathies, neurodegenerative diseases, and some cancers (Kelekar 2005). The study characterized the effects of glabridin and the underlying molecular mechanism of autophagy and apoptosis in glabridin-induced cytotoxicity. We investigated the cytotoxic effects of glabridin on hepatoma and its underlying mechanisms *in vitro*.

Materials and methods

Chemicals

Glabridin, ≥98% (HPLC), powder was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solution of Glabridin was made at 25, 50 and 100 mM concentration in DMSO and stored at −20 °C. The final concentration of DMSO for all treatments was consistently less than 0.1%. 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), bafilomycin A1 (BafA1), 4'-6-Diamidino-2-phenylindole (DAPI), wortmannin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). General caspase inhibitor Z-VAD-FMK was purchased from Promega (Madison, WI, USA). Specific inhibitors for caspase-3 (Z-DEVE-FMK), caspase-8 (Z-IETD-FMK) or caspase-9 (Z-LEHO-FMK) were purchased from BioVision (Mountain View, CA). Specific inhibitors for ERK1/2 (U0126), JNK1/2 (SP600125) or p38 (SB202190) were purchased from Calbiochem (San Diego, CA). Antibodies were purchased from Cell Signaling.

Cell culture

Huh7, HepG2 and Sk-Hep-1, human hepatoma cell line, obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan), were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 1 mM glutamine, 1% penicillin/streptomycin, 1.5 g/l sodium bicarbonate, and 1 mM sodium pyruvate (Sigma, St. Louis, Mo, USA).

In vitro *cytotoxicity assay*

The effect of glabridin on cell growth was assayed by the MTT method, as previously described (Yang et al. 2010). Briefly, cells were cultured in 24-well plates and stimulated with different concentrations of glabridin. After 24, 48 or 72 h of glabridin stimulation, MTT was added to each well (0.5 mg/ml final concentration) with a further incubation for 4 h. The viable cell number was directly proportional to the production of formazan following the solubilization with isopropanol. The color intensity was measured at 595 nm.

DAPI (2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride) staining

As described in detail previously (Hsieh et al. 2013) after being subjected to indicate treatment, cells were fixed with 4% paraformaldehyde for 20 min. Extensive PBS washing was conducted between each reaction to remove any residual solvent. Cells were subjected to DAPI staining for 10 min and then observed under fluorescence microscopy equipped with filters for UV.

Cell cycle analysis

Cells were first cultured in serum-free medium for starvation at 18 h and then exposed to glabridin for 24 h. Cells were fixed with 70% ethanol and incubated for 30 min in the dark at room temperature with propidium iodide (PI) buffer. The cell cycle distribution was analyzed for 3000 collected cells by a FACS Vantage flow cytometer that uses the CellQuest acquisition and analysis program (Becton Dickinson FACSCalibur).

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