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PEITC induces apoptosis of Human Brain Glioblastoma GBM8401 Cells through the extrinsic- and intrinsic -signaling pathways

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ABSTRACT

Glioblastoma is the most common and most aggressive primary brain malignancy. The multimodality treatments for this tumor including surgery, radiotherapy, and chemotherapy, are still not completely satisfied. Phenethyl isothiocyanate (PEITC), one member of the isothiocyanate family, has been shown to induce apoptosis in many human cancer cells. In this study, we investigate the pro-apoptotic effects caused by PETIC in human brain glioblastoma multiforme GBM 8401 cells.

In our data, PEITC induced the cell morphological changes and decreased the cell viability of GBM8401 cells in a dose- and time-dependent manner. Moreover, the analysis of cell cycle distribution detected by flow cytometry showed that PEITC induced significantly sub-G1 phase (apoptotic population) in GBM 8401 cells. In addition, PEITC promoted the production of reactive oxygen species (ROS) and increase in [Ca2+]I, but decreased the mitochondrial membrane potential ($\Delta\Psi m$) in treated cells. PEITC also induced caspases activities in GBM 8401 cells. Results from Western blot analysis indicated that PEITC promoted Fas, FasL, FADD, TRAIL, caspase-8, -9, -3, increased the pro-apoptotic protein (Bax, Bid and Bak), and inhibited the anti-apoptotic proteins (Bcl-2 and Bcl-xl) in GBM 8401 cells. Furthermore, PEITC promoted the release of cytochrome c, AIF and Endo G. GADD153, GRP 78, XBP-1 and IRE-1α, Calpain I and II in GBM 8401 cells. PEITC also promoted the expression of associated protein with endoplasmic reticulum (ER) stress. PEITC induces apoptosis through the extrinsic (death receptor) pathway, dysfunction of mitochondria, ROS induced ER stress, intrinsic (mitochondrial) pathway in GBM 8401 cells. The possible molecular mechanisms and signaling pathways of the anti-cancer properties of PEITC for human brain glioblastoma cells were postulated.

1. Introduction

Glioblastoma is the most common and most aggressive primary brain malignancy. Even with maximum feasible surgical resection with radiotherapy and adjuvant temozolomide (TMZ), survival rates are at a median of 14.6 months from diagnosis in molecularly unselected patients (Stupp et al., 2005). Radiotherapy and TMZ provide better survival outcomes than radiotherapy alone to treat glioblastoma (Yang et al., 2014). Both extent of resection and residual volume are significantly associated with survival and recurrence (Chaichana et al., 2014). Gross total resection is associated with survival improvement, but it is not always possible because the preservation of neurological functions is necessary. The current multimodality treatments including surgery, radiotherapy, chemotherapy for this tumor are still not completely satisfying. Phenethyl isothiocyanate (PEITC) is one of the most extensively studied isothiocyanates (Moon et al., 2011). Its effective chemopreventive activity for various tumors and no obvious toxicity in animal models have been reported (Hecht, 1995). PEITC can induce cell cycle arrest and apoptotic cell death (Antosiewicz et al., 2008; Chen et al., 2002; Hu et al., 2003; Jakubikova

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et al., 2005; Kang and Wang, 2010; Telang et al., 2009; Tseng et al., 2004). PEITC selectively kills cancer cells, but not normal cells, by generating reactive oxygen species (ROS) to trigger signal transduction, leading to cell cycle arrest and/or apoptosis (Wu and Hua, 2007). Studies have disclosed the cell growth inhibitory effects of PEITC in human leukemia (Xu and Thornalley, 2000), lung cancer (von Weymarn et al., 2006), HeLa cervical cancer (Yu et al., 1998), HT-29 colon adenocarcinoma (Hu et al., 2003), pancreatic cancer (Nishikawa et al., 2004), and human prostate cancer PC-3 cells (Xu et al., 2005). In human glioma cells, PEITC inhibits the HIF-1α expression through inhibiting the Phosphoinositide 3-kinase (PI3K) and mitogen activated protein kinase (MAPK) signaling pathway, and reduces the hypoxia-induced secretion of vascular endothelial growth factor (VEGF) (Gupta et al., 2013); PEITC also promoted the actions of tumor necrosis factor-related apoptosis-induced ligand (TRAIL) through the upregulation of death receptor-5 (DR5) with ROS-induced-p53 and the downregulation of cell survival proteins (Lee et al., 2014). But the overview of mechanisms of PEITC-induced apoptosis of human brain glioblastoma cells has not been understood well.

In the present study, we investigated the effects of PEITC on human brain glioblastoma cells including the induction of cell cycle arrest and apoptosis through the ROS and dysfunction of mitochondria signaling transduction pathways in GBM8401 cells.

2. Materials and methods

2.1. Chemicals and reagents

PEITC, dimethyl sulfoxide (DMSO), propidium iodide (PI), Trypan blue and 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640, fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin and trypsin-EDTA were purchased from Gibco BRL/Invitrogen (Carlsbad, CA, USA). Primary antibodies (BAX, BCL-2, catalase, Mn-SOD, cytochrome c, caspase-2, -3, -4, -8 and -9, PARP, GRP78, GADD153 and β-actin) and second antibodies for Western blotting were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). They were diluted in PBS Tween-20 before use. Fluo-3/AM, $DiOC_6$ and 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Molecular Probes (Invitrogen, Eugere, OR, USA).

2.2. Cell culture

Human brain glioblastoma multiforme (GBM 8401) cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were plated onto 75 cm^2 tissue culture flasks in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine, and grown at 37 °C under a humidified 5% CO_2 and 95% air at one atmosphere. The medium was changed every 2 days (Wang et al., 2002).

2.3. Cell morphological changes and viability

GBM 8401 cells (2×10^5 cells/well) onto a 24-well plate were treated with 0, 4, 6, 8, 10, 12, and 14 μM PEITC, or 0, 500 μM TMZ and were incubated for 0, 24 and 48 h. Cells in each well were examined and we took representative photographs at 200× magnification by Nikon TE2000-U inverted microscope for morphological change examinations. After cells from each well were trypanized and collected by centrifugation at 1500 rpm for 5 min, washed twice with PBS, 5 μg/ml PI in PBS was added to the cells to determine the percentage of viable cells. Nonviable cells were stained by PI dye exclusion (indicative of an intact membrane) and displayed brighter fluorescence than the unstained (viable cells). Cells were counted by flow cytometric analysis with FACSCalibur utilizing Cell Quest software (Becton-Dickinson, San Jose, CA, USA) (Lu et al., 2010a).

2.4. Determination of cell cycle and apoptosis by flow cytometry

GBM 8401 cells (2×10^5 cells/well) were grown in a 24-well plate for 24 h then treated with 0, 4, 6 and 8 μM PEITC. Cells were isolated, washed with ice-cold PBS, and then fixed in 70% ethanol overnight, followed by the re-suspension in PBS containing 40 μg/ml PI and 0.1 mg/ml RNase and 0.1% Triton X-100 in dark room for 30 min at 25 °C. Cells were washed twice before cell cycle analyses were performed by using a flow cytometer (Beckton-Dickinson) equipped with an argon ion laser at 488 nm wavelength (Lu et al., 2010b). Fluorescence intensity of the sub-G1 cell fraction represented the apoptotic cell population. Each treatment was performed in triplicate for statistical evaluation.

2.5. Annexin V-FITC/PI double staining

Cell death induced by PEITC was analyzed by flow cytometry. Cell surface exposure of phosphatidylserine (PS) in apoptotic cells was measured by Annexin V-FITC Apoptosis Detection Kit (Biovision, USA). GBM 8401 cells (1×10^5 cells/well) were treated with 8 μM PEITC for 24 h and then stained with Annexin V-fluorescein isothiocyanate (FITC) and PI. Cells were washed with PBS after staining, then were re-suspended in binding buffer and immediately analyzed using a fluorescence-activated cell sorting (FACS) flow cytometer (Beckman Coulter, USA) (Ho et al., 2013). In fluorescence channels FL1 (488 nm excitation and 530 nm emission for Annexin V-FITC binding) and FL2 (488 nm excitation and red emission for PI), fluorescence from PEITC-treated and untreated cells was detected.

2.6. Detections of reactive oxygen species (ROS), Ca^{2+} and mitochondrial membrane potential ($\Delta\Psi m$) using flow cytometric assay

GBM 8401 cells (2×10^5 cells/well) onto 24-well plates were treated with 0, 8 μM PEITC or 0, 500 μM TMZ and incubated for 0, 24 and 48 h. Cells were harvested from each treatment by centrifugation, washed twice with PBS and re-suspended in 500 μL of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (10 μM) for ROS measurements, in 500 μL of Flou-3/AM (2.5 μg mL) for Ca^{2+} level examination and in 500 μL of DiOC6 (200 nmol L) for ΔΨm determination. All samples from control and PEITC treated groups were incubated at 37 °C for 30 min in the dark room at 25 °C and analyzed by flow cytometry (Ho et al., 2013; Lu et al., 2010b).

2.7. Measurements of caspase-3, -8 and -9 activities using flow cytometric assay

GBM 8401 cells (5×10^5 cells/well) onto 24-well plate were treated with 0, 8 μM PEITC or 0, 500 μM TMZ and incubated for 0, 24 and 48 h. Cells were harvested, washed twice with PBS then caspase-3, -8 and -9 substrates (CaspaLux8-L1D2,- CaspaLux 9-M1D2 and PhiPhiLux-G1D2) were added, respectively. Flow cytometric assay was applied to measure the activities of caspase-3, -8 and -9 (Ho et al., 2013).

2.8. Western blotting assay

GBM 8401 cells (1×10^6 cells/well) onto 12-well plate were treated with 6, 8 μM PEITC and incubated for 0, 24 and 48 h to detect the apoptosis-inducing proteins. Cells were collected and lysed in lysate buffer composed of 50 μM tris (pH 8.0), 150 μM NaCl, 5 μM ethylenediaminetetraacetic acid and 0.5% NP-40 with protease inhibitor solution (Roche, Mannheim, Germany). The protein concentration from each treatment was determined by using Bio-Rad protein assay kit. About 30 μg of protein from each sample was separated on a 10% sodium dodecyl sulfate-polyacrylamide electrophoretic gel (SDS-PAGE) and transferred to nitrocellulose