Glycyrrhizic acid induces human MDA-MB-231 breast cancer cell death and autophagy via the ROS-mitochondrial pathway

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Abstract. Glycyrrhizic acid (GA), the main component of licorice root extracts, has been shown to suppress cell proliferation and induce apoptosis in various types of cancers. However, the molecular mechanism of its anticancer activity remains poorly understood and warrants further investigation. MDA-MB-231 cells were treated with various concentrations of GA and the cytotoxic effects of GA were determined using the CCK-8 assay. Apoptosis and cell cycle status were detected by flow cytometry. Reactive oxygen species (ROS) levels and mitochondrial membrane potential (ΔΨm) were assessed using DCFDA, MitoSOX and JC-1 staining. Western blot analysis was used to quantify the expression of autophagy-related proteins. In the present study, induction of autophagic cell death was observed in GA-treated MDA-MB-231 cells. Downregulation of p62- and beclin 1-associated proteins occurred after GA treatment, and, the conversion of LC3 and increased ROS without significant changes in caspase-associated proteins and intracellular responses were detected. Furthermore, loss of mitochondria and its membrane potential in cells demonstrated that mitochondria were involved in the GA-regulated MDA-MB-231 cell death. The addition of a pan-caspase inhibitor (z-VAD-fmk) did not suppress the GA-induced apoptotic effect, and GA-induced apoptosis was not accompanied by processing of procaspase-8, -9 and -3. However, GA triggered the translocation of the apoptosis-inducing factor (AIF) from the mitochondria into the nucleus. In contrast, GA-induced LC3 conversion was significantly inhibited by 3 methyladenine (3MA), an inhibitor of PI3K-regulated autophagy. Therefore, these results suggest that enhancement of both AIF- and LC3-dependent GA-derived ROS generation plays an important role in the inhibition of the growth of MDA-MB-231 human breast cancer cells.

Introduction

Breast cancer is highly metastatic, leading to multiorgan dysfunction and a high mortality rate (1,2), which can be reduced by early detection and optimized therapeutic options (3-5). Triple-negative breast cancer (TNBC) is an aggressive subtype that does not respond to estrogen receptor (ER)-, progesterone receptor (PR)- and human epidermal growth factor receptor 2 (HER2)-targeted therapies, and TNBC patient prognoses are poor (6,7). Surgery, radiotherapy, chemotherapy and targeted therapy are the standard treatments for breast
cancer (8,9). However, the side-effects associated with these treatments may include critical alopecia, nephrotoxicity and hepatotoxicity (10,11). Drug resistance in cancers is also a serious impediment to treatment and can undermine therapeutic efficacy (12). Furthermore, target therapeutic agents, such as trastuzumab, lapatinib and the anti-angiogenic drug Avastin have demonstrated indeterminate benefits in clinical settings (13-15). Under these circumstances, development of new drugs is particularly compelling and imperative. Although great efforts have been made to design more powerful drugs for the treatment of breast cancer, the results remain unsatisfactory. In fact, some herbal drugs in alternative medicine have been suggested to be a better choice to improve the current therapeutic strategy.

Glycyrrhizic acid (GA) is the main component of licorice root extracts. GA contains 8 hydroxyl groups and is highly hydrophilic; therefore, it is water-soluble and is easily absorbed and metabolized by the digestive tract. GA constitutes ~2-24% of the dry weight of licorice root. Licorice root extracts are sweet, and GA is ~50 times sweeter than sucrose. Therefore, GA is widely used as an additive in candy and food; it is also used in medicine and in the beer manufacturing industry (16). GA was recently demonstrated to induce the apoptosis of human endometrial cancer cells, and potently inhibited the growth of lung cancer cells via caspase activation (17). Previous studies indicate that a mass of compounds induce autophagy for cell death by various mechanisms (18-21), e.g. glycyrrhetinic acid induces autophagy in leukemia and myeloma cells by suppression of the mTOR pathway (22). However, the potential therapeutic effects of GA on mechanisms associated with autophagy have not yet been explored in breast cancer.

Mounting evidence indicates that GA-induced cell apoptosis may be regulated by the caspase cascade in various types of cancers (23-26). However, few studies have specifically focused on GA-induced autophagy, particularly in breast cancer cells (27,28). In the present study, we investigated the effects of GA on mitochondrial cell death in a highly aggressive MDA-MB-231 breast cancer model to determine whether GA provides dual inhibition by enhancing apoptosis and autophagy caused by damaged mitochondria.

Materials and methods

Materials. GA, N-acetyl-cysteine (NAC), staurosporine (STS), hydrogen peroxide (H2O2), N-phenylmaleimide (NPM), 3 methyladenine (3MA), JC-1 and anti-β-actin (A5411) were purchased from Sigma Co. (St. Louis, MO, USA). Anti-AIF (113306), anti-BAX (109683), anti-BCL2 (100064), anti-PARP1 (100573), anti-porin (114187), anti-histone (122148) and anti-β-actin (A5411) were purchased from Enzo Life Sciences, Inc., Farmingdale, NY, USA. Cells (at 1x10^5 cells/well) were stained with DCFH-DA (2 µM) reagent and MitoSOX Red (Molecular Probes Inc., Eugene, OR, USA) at 37°C for 30 min for detection of the mitochondrial membrane potential and mitochondrial ROS, respectively. Cells were washed twice with PBS, re-suspended in a binding buffer and stained with 1 µg/ml of a PI solution (Sigma Co.) for 15 min at room temperature in the dark. After incubation, the cells were analyzed using flow cytometry.

Cell viability was analyzed using Cell Counting Kit-8 (CCK-8) that detects the metabolic activity of live-cell images. After treatment, cells (at 1x10^5 cells/well) were stained with Hoechst 33258 and propidium iodide (PI) double staining for live-cell images. In fact, some herbal drugs in alternative medicine have been suggested to be a better choice to improve the current therapeutic strategy.

Caspase activity. Caspase-8,-9 and -3 activities were measured by the colorimetric activity assay kits (Chemicon International, Temecula, CA, USA). The assay is based on cleavage of the chromogenic substrates, DEVD-pNA and LEHD-pNA, by caspase-3 and -9, respectively. Cells (at 1x10^4 cells/well) were lysed with the chilled lysis buffer on ice for 10 min and centrifuged for 5 min at 10,000 x g. Supernatant of each treatment was transferred to a fresh tube and equal protein concentration was adjusted to that of the positive control before incubating with the caspase inhibitor in a 96-well microplate for 10 min. Caspase substrate solution with specific peptide substrate was added to each well and the absorbance was recorded using an ELISA microplate reader at 450 nm.

Cell morphology staining. Cell morphology was studied by Hoechst 33258 and propidium iodide (PI) double staining for live-cell images. After treatment, cells (at 1x10^5 cells/well) were stained with Hoechst 33258 and PI solution at room temperature for 10 min. Cells were washed twice with phosphate-buffered saline (PBS) for analysis by an inverted fluorescence microscope (Carl Zeiss Axiolvert M200; Carl Zeiss, Jena, Germany).

Cell proliferation assay. Cell viability was analyzed using Cell Counting Kit-8 (CCK-8) that detects the metabolic activity of cells. At the end of the various treatments, 10 µl of the CCK-8 reagent was added to each well (at 1x10^6 cells/well), and the cells were then incubated at 37°C for 4 h. Absorbance was recorded using an ELISA microplate reader at 450 nm.

Cell death measurement. Cell survival was assessed by labeling cells with PI. Briefly, the cells (at 1x10^5 cells/well) were washed twice with PBS, re-suspended in a binding buffer and stained with 1 µg/ml of a PI solution (Sigma Co.) for 15 min at room temperature in the dark. After incubation, the cells were analyzed using flow cytometry.

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Cell culture. MDA-MB-231 human breast carcinoma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma Co.) supplemented with 10% fetal bovine serum (FBS) (v/v), 100 U/ml penicillin/streptomycin/amphotericin (Sigma Co.) and 0.1 M sodium bicarbonate. Cells were maintained in a humidified incubator at 37°C with 5% CO2 and passage by 0.25% trypsin-EDTA every 2-3 days.

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then added into the supernatant and incubated for 2 h at 37°C before measuring by ELISA reader at 405 nm.

**Cell death detection by Annexin V-FITC/PI double staining.** Analysis of the cell death of each condition was performed using an Alexa Fluor Annexin V/Dead Cell Apoptosis kit (Molecular Probes Inc.) according to the manufacturer's protocol. Briefly, cells (at 1x10^5 cells/well) were harvested after treatment. Cells were then washed twice with cold binding buffer, resuspended in binding buffer and stained with 5 µl each of Annexin V-FITC and PI solution for 15 min at room temperature in the dark. After incubation, 1 ml binding buffer was added, and cells were analyzed by flow cytometry.

**Statistical analysis.** The intensity of bands in western blot analyses or fluorescence was quantified using ImageJ software (NIH) and Zeiss Zen software. The intensity value for each protein was normalized against the intensity of the loading control for that same sample. The values after normalizing to the loading control in the control groups were set as 1.0. Data presented are the mean ± standard error of the mean (SEM) from at least 3 independent experiments and were analyzed using a Student's t-test with two-tailed distribution between groups as indicated in the graphs. All calculations were performed using GraphPad Prism 6.01 version.

**Results**

**Induction of cell death by GA in MDA-MB-231 cells.** The effect of GA on cell survival is shown in Fig. 1A. The cytotoxicity of the GA was measured by CCK-8 assay. As shown in Fig. 1A, GA exerted a significant cytotoxic effect at concentrations 2.5-20 µM at 24 h and the IC50 value was found to be 20 µM. These results indicated that the GA treatment significantly reduced cancer cell viability in a dose-dependent manner. To further evaluate the cytotoxic effect of GA-induced breast cancer cell death, cells were stained with DAPI after exposure to GA for 24 h. Significant change in blebbing, cell shrinkage, DNA condensation was observed in the MDA-MB-231 breast cancer cells; an increase in fluorescence intensity was observed by 2.5 D reconstruction image after treatment with GA (Fig. 1B). As shown in Fig. 1C, GA markedly increased the percentage of PI-positive cells by live-cell fluorescent. In addition, cell cycle analyses showed an increase in the subG1 phase population at 24 h in the GA treatment groups. The increased subG1 population of GA-treated breast cells at 24 h coincided with that of the cell viability assay (Fig. 1D).
Treatment with GA significantly elevates the generation of ROS. To investigate the effect of GA on intracellular ROS generation, MDA-MB-231 cells were treated with GA at different concentrations and stained with fluorescent probe DCFH-DA and MitoSOX, which detect hydroperoxide and mitochondrial ROS, respectively. Cells following GA treatment showed higher green and red fluorescence compared with that in the normal control group (Fig. 2A and B). A flow cytometric analysis was performed to quantify the effect of GA-induced intracellular and mitochondrial ROS generation, and the results are presented in Fig. 2A and B. A flow cytometric analysis was performed to quantify the effect of GA-induced intracellular and mitochondrial ROS generation, and the results are presented in Fig. 2A and B. GA induced a significant shift in green and red fluorescence, whereas following GA, enhanced total ROS and mitochondrial ROS production were well apparent. To investigate whether ROS were involved in the GA-induced cell death, we introduced NAC, an inhibitor that blocks ROS generation, before GA treatment. The results showed that NAC alone had no effect on the mitochondrial ROS. However, NAC treatment blocked the GA-induced mitochondrial ROS production in MDA-MB-231 cells (Fig. 2C). Moreover, there was increased in cell viability after treatment with GA and NAC (Fig. 2D).

GA induces caspase-independent cell death in MDA-MB-231 cells. Annexin V/PI double-staining was used to detect the PS out-flipping phenomenon, one of the clearest characteristics of apoptotic cell death. To examine whether inhibition of GA-induced cell proliferation was associated with apoptosis, cells were detected after 24 h of GA exposure by flow cytometry. GA reduced the number of viable MDA-MB-231 breast cancer cells from 87 to 60% in a dose-dependent manner. However, we found that the cell population shifted directly to PI+ quadrants without going through the PI-/Annexin V- transition. This observation suggests that cell death induced by GA differs from apoptosis in MDA-MB-231 cells, apparently relying mainly on necrosis or autophagy (Fig. 3A). Mitochondrial integrity or the loss of Δψm has been linked to initiation of apoptosis and multiple cell death, including autophagy and necrosis. To determine Δψm loss, breast cancer cells were stained with JC-1 when harvested and subjected to flow cytometric analysis. JC-1 concentrates in the mitochondria as red fluorescent aggregates at high membrane potentials and is converted to green monomers when Δψm is lost. Red fluorescence in MDA-MB-231 breast cancer cells after GA treatment was reduced from 99 to 64%, indicating mitochondrial membrane potential (MMP) depolarization in breast cancer cells (Fig. 3B). To determine whether GA-induced apoptosis, characterized mainly by activation of caspase-3-associated proteins leading to cleavage of PARP1, protein expression was measured by western blotting (Fig. 3C). Along with decreased levels of PARP1 and a significantly increased BAX/BCL2 ratio was observed after GA treatment (Fig. 3D). No changes in cleaved caspase-3, -9 and -8 expression and activity were found in MDA-MB-231 breast cancer cells (Fig. 3C and E). To corroborate that GA-induced cell death was independent of caspase, the cells were pre-treated with 1 mM pan-caspase inhibitor z-VAD-FMK for 2 h. No significant recovery in cell
viability was observed after inhibitor pre-treatment in the MDA-MB-231 cells, indicating that GA induced cell death in a caspase-independent manner (Fig. 3F).

**Roles of apoptosis-inducing factor (AIF) proteins in GA-induced MDA-MB-231 cell death.** To further investigate the cellular mechanism of GA-induced cell death, the protein levels of AIF were detected. AIF is a FAD-dependent oxidoreductase that has a vital role in oxidative phosphorylation and is independent of the caspase pathway. We investigated whether the translocation of AIF occurred in the GA-induced cell death of MDA-MB-231 cells. As shown in Fig. 4A and B, addition of GA resulted in a significant increase in overlap coefficient between nuclear AIF. AIF translocation occurred and subsequently appeared in the nuclear fraction of MDA-MB-231 cells treated with GA for 24 h. We also confirmed and quantified the cellular distribution of AIF. Immunoblotting revealed that AIF mostly resided in the mitochondria of the cancer cells (Fig. 4C). To investigate whether the AIF pathway is involved in the GA-induced cell death, we introduced NPM, an inhibitor that blocks AIF activation, before GA treatment. The results showed that NPM combined with GA had a significant increased on the expression of AIF and PARP1 (Fig. 4D). After the treatment of NPM, JC-1 gathered in the mitochondrial matrix and produced red fluorescence (Fig. 4E). As shown in the results of flow cytometry, GA caused a dramatic reduction in red fluorescence which indicated a loss of mitochondrial membrane potential ($\Delta\Psi_m$) and damage of mitochondria in the MDA-MB-231 cells (Fig. 4F). The ratio of red and green fluorescence represented the level of depolarization in mitochondria: NPM protected MDA-MB-231 cells against the damage caused by GA treatment (Fig. 4G). Moreover, there was increased cell viability after treatment with NPM, indicating that AIF may be the major factor involved in GA-induced cell death (Fig. 4H). Furthermore, NAC treatment also blocked the GA-induced AIF translocation to the nucleus, suggesting that ROS may be the regulator involved in this process (Fig. 4I).

**Autophagy and ROS are involved in GA-mediated cell death.** Autophagic protein LC-3 is a component of double-membrane vesicle formation into autophagosomes. To explore whether autophagy was involved in GA-induced cell death, the fluorescence expression was utilized. A large number of bubbles were observed in the GA-exposed cells after 24 h (Fig. 5A). Moreover, a number of autophagosomes or lysosomes containing segregated were observed in the GA-treated cells. Conversion from cytosolic LC3-I to LC3-II, the membrane-binding form, was analyzed by western blotting. The protein expression of LC3-II was significantly increased in the MDA-MB-231 breast cancer cells and p62 was downregulated, which is one of the selective substrates for autophagy. These results suggested that AIF and LC3-II may be involved in GA-induced autophagy (Fig. 5B and C). Autophagy is a major process of organelle and protein degradation. An increasing number of studies have shown that autophagy may be a new target for anticancer therapy. To further confirm
that autophagy was involved in GA-induced cell death, 2 mM 3 methylamphetamine (3MA), an autophagy blocker that inhibits PI3K class III protein, was added. After addition of 3MA, the increase in LC3 was significantly inhibited compared with the control cells (Fig. 5D). Considering all of the above results, we conclude that GA-induced cell death in MDA-MB-231 cells was mainly due to autophagy. Moreover, there was increased in cell viability after treatment with 3MA, indicating that autophagy may be the major factor involved in GA-induced cell death (Fig. 5E). We further determined the involvement of ROS in GA-induced autophagy, and protein expression of associated signaling molecules (Fig. 5F). NAC reversed the effects of GA on protein expression of LC3 and P62. However, treatment with AIF inhibitor NPM showed no significant effects (Fig. 5G). These results suggested that the anticancer effects of GA on breast cancer cells were mediated by ROS-dependent mitochondrial dysfunction with the involvement of AIF and LC3 signaling pathway.

**Discussion**

The present study demonstrated that glycyrrhizic acid (GA), the main component of licorice root extracts, is cytotoxic to human breast cancer cell line MDA-MB-231. This effect appears to involve promotion of apoptosis, autophagy and ROS generation via mitochondria. A recent study showed that GA caused a dose-dependent activation of NF-κB as well as ROS generation in HepG2 cells (29). In contrast, Thirugnanam et al reported that the GA-induced apoptosis in prostate cancer cells was dependent of caspase-3 activation (26). Additional evidence shows that DNA damage-inducing agents trigger caspase-independent apoptosis in various cancer cell lines (23,24). The present study using MDA-MB-231 cells showed that caspase activity was not required for GA-induced apoptosis. zVAD-fmk did not suppress GA-induced apoptosis. Moreover, GA did not induce caspase activation (Fig. 3C). In the present study, our results suggest that GA-induced
apoptosis in MDA-MB-231 cells is mediated by a caspase-independent mechanism.

Autophagy or autophagocytosis is a metabolic process involving degradation of cellular components by lysosomes that responds to metabolic stress (30,31). This tightly regulated process helps cells maintain balance and subsequent recycling of cellular organelles and proteins. It is a key mechanism by which starving cells allocate nutrients away from unnecessary processes (32). This mechanism is also associated with progression of certain diseases such as atherosclerosis, neurodegenerative disease and cancers. The two major processes involved are Atg-regulated and chaperone-mediated autophagy (33). Microphagy sequesters damaged organelles other than proteins in a double-membrane autophagosome. Autophagosomes derived from the elongation of small membrane structures are known as autophagosome precursors. The outer membrane of the autophagosome fuses with a lysosome in the cytoplasm to form an autolysosome, in which cell contents undergo acidic degradation (34,35).

As already mentioned earlier, PARP1 belongs to a family of nuclear enzymes, modulating DNA repair, transcriptional regulation, chromatin modification and genomic stability through polyADP-ribosylation (36,37). PARP1 activation leads to ATP depletion, thereby inducing necrosis and apoptosis (38). However, PARP1 can be degraded through multiple downstream proteins, including DNA-dependent protein kinase, ubiquitination and hydrolysis via the proteasome or by the caspase pathway (39). Notably, PARP1 activation interfaces with signalling pathways known to promote autophagy (40). It is well documented that GA-induced cancer cell apoptosis occurs through a mitochondrial pathway. We also observed that GA decreased the levels of ΔΨm in MDA-MB-231 cells (Fig. 3B) in a dose-dependent manner. Furthermore, upregulation of BAX, reduction of mitochondrial membrane potentials, enhancement of AIF and LC3 expression, and activation of autophagy were observed in the present study.

In turn, LC3 and AIF activation led to DNA damage, and eventually, induction of autophagic cell death. DNA damage was found in MDA-MB-231 cells after treatment with GA, and downregulation of PARP1 inhibited DNA synthesis and repair; thus, enhanced cytotoxicity through inhibition of DNA repair processes is another possible mechanism explaining the cytotoxic effect of GA. In addition, the greatly increased level of LC3 protein may have contributed to autophagic cell death in the MDA-MB-231 breast cancer cells and reduced cell survival even over long-term treatment. Moreover, autophagosomes were observed through LC3 staining, which may have been activated by excess ROS production in the GA-treated cells. To explore the molecular mechanism of autophagy in GA-induced cell death, an inhibitor of autophagy, 3MA, was added. 3MA partially reversed conversion of LC3, corroborating that autophagy was involved in the GA-induced cell death. These results confirmed the hypothesis that mitochondrial dysfunction occurred via facilitation of apoptosis and autophagy potentially regulated by increased ROS levels in GA-treated breast cancer cells. Based on the antitumor activity profiles of GA treatment in vitro and the absence of cytotoxicity, we believe that GA has strong therapeutic value for use against breast cancers.

In conclusion, the present study demonstrated that GA induced apoptotic and autophagic cell death, and inhibited cell growth in vitro in breast adenocarcinoma MDA-MB-231 cells. We conclude that GA-induced mitochondrial apoptosis and
autophagy were mainly caused by AIF and LC-3. Our findings suggest that GA warrants further investigation as a promising complementary therapeutic drug for the treatment of human breast cancer.

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References


