Gambogenic Acid Induction of Apoptosis in a Breast Cancer Cell Line

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Abstract

Background: Gambogenic acid is a major active compound of gamboge which exudes from the Garcinia hanburyi tree. Gambogenic acid anti-cancer activity in vitro has been reported in several studies, including an A549 nude mouse model. However, the mechanisms of action remain unclear. Methods: We used nude mouse models to detect the effect of gambogenic acid on breast tumors, analyzing expression of apoptosis-related proteins in vivo by Western blotting. Effects on cell proliferation, apoptosis and apoptosis-related proteins in MDA-MB-231 cells were detected by MTT, flow cytometry and Western blotting. Inhibitors of caspase-3,-8,-9 were also used to detect effects on caspase family members. Results: We found that gambogenic acid suppressed breast tumor growth in vivo, in association with increased expression of Fas and cleaved caspase-3,-8,-9 and bax, as well as decrease in the anti-apoptotic protein bcl-2. Gambogenic acid inhibited cell proliferation and induced cell apoptosis in a concentration-dependent manner. Conclusion: Our observations suggested that Gambogenic acid suppressed breast cancer MDA-MB-231 cell growth by mediating apoptosis through death receptor and mitochondrial pathways in vivo and in vitro.

Keywords: Gambogenic acid - apoptosis - breast cancer - MDA-MB-231 cells

Introduction

Breast cancer is one of the most common malignancies and is the second leading cause of cancer death in women (American Cancer Society, 2011). The recurrence rate of breast cancer has significantly decreased due to early detection and adjuvant therapies. However, it still causes high mortality. The current therapies for breast cancer include surgery, radiation, chemotherapy, and hormonal and biological therapies (Lin et al., 2010). Although improved treatments have been achieved recently, many breast tumors are not eradicated effectively due to acquired resistance or relapse following an initial response, resulting in metastatic disease at later stages that leads to patient death (Lin et al., 2010). Chemotherapy is a standard treatment option for patients with cancers. However, resistance to chemotherapy is a major problem in the management of breast cancer (Perez 2009). Therefore, the development of novel effective agents that can suppress breast cancer growth is urgent and necessary to improve clinical outcome of patients.

Natural products are the most important sources of novel cancer therapeutics (Tan et al., 2011). Gamboge, the dry resin of Garcinia hanburyi Hook.f. (Guttiferae), has been used as a medicine for detoxification, homeostasis and anti-inflammation and as a parasiticide for thousands of years (Panthong et al., 2008). Gambogenic acid (GNA), one of the active ingredients in Garcinia, was first isolated and purified in 1984 (Lu et al., 1984; Zhou et al., 2008). The molecular structure of GNA is shown in Figure 1A. Many studies have reported that GNA has a broad-spectrum antitumor effect. Only one study demonstrated that GNA could effectively inhibit tumor growth in A549 xenograft nude mice models (Li et al., 2010). However, the detail mechanism of GNA suppressed tumor growth was not studied in vivo. All the other studies shown that GNA induced cell apoptosis through mitochondrial pathway in vitro: GNA induced mitochondrial-dependent apoptosis and referred to phosphor-Erk1/2 and phosphor-p38 MAPK in human hepatoma HepG2 cells (Yan et al., 2012); GNA induced time- and dose-dependent growth inhibition and apoptosis involving Akt pathway inactivation in U251 glioblastoma cells (Chen et al., 2012). But, in our previous study, we found that GNA could effectively induce apoptosis via not only the mitochondrial pathway but also the death receptor pathway in MCF-7 cells (Wang et al., 2011). In order to further analyze the potential mechanisms of GNA-induced apoptosis in vivo and in vitro, MDA-MB-231 cells, an aggressive breast cancer cell, were chosen for this study.
In this study, we found that GNA effectively suppressed tumor growth in MDA-MB-231 xenograft nude mouse breast cancer models. Furthermore, we investigated the effect of GNA on the apoptosis of MDA-MB-231 cells and its underlying molecular mechanisms in vivo and in vitro. Based on the experiments, we determined that GNA significantly induced the apoptosis of MDA-MB-231 cells via mitochondrial pathways and the Fas/FasL death receptor in vivo and in vitro.

**Materials and Methods**

**Cell culture and reagents**

The MDA-MB-231 cell line was purchased from the Chinese Academy of Science Shanghai Cytobiology Research institute. Cells were cultured in Leibovitz’s L-15 Medium with L-glutamine ( Gibco) supplemented with 10% fetal bovine serum (Invitrogen) and 1% ampicillin and streptomycin in an incubator with 5% CO₂ at 37°C. GNA (Batch 090327) was purchased from Shanghai Ronghe Medical Technology Co. and was dissolved in DMSO (Sigma) to make a stock solution. A stock solution of 100 mg/ml was stored at -20°C and then diluted as needed in complete culture medium immediately before used.

**Proliferation assay**

MDA-MB-231 cells (2×10⁵) were seeded into 96-well plates (Corning). After cells were treated with the indicated concentrations of GNA (terminal concentration <1%) for 24 h, 48 h and 72 h, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to the culture medium, and the cells were incubated for another 4 h at 37°C. The negative control was 0.1% DMSO. After the culture medium was removed, 150 ml DMSO was added, and the plates were placed on a shaking table at 150 rpm for 10 min. Optical density (OD) was measured at 490 nm. The experiment was repeated three times, and the rate of cell inhibition was calculated using the following formula: inhibition rate = (1 - (ODtest/ODnegative control))×100%. The IC50 was calculated using SPSS 19.0 software.

**Xenograft nude mouse models**

The 5-week-old to 6-week-old female BALB/c nude mice (purchased from the Shanghai Laboratory Animal Commission) weighing <20 g were divided into groups with 6 mice per group. All of the mice were injected subcutaneously with MDA-MB-231 cells (1×10⁶) in the scruff of neck. After the tumors were established (~0.5 cm³), the mice were treated with different concentrations (0-1 µg/ml) of GNA for 72 h. MDA-MB-231 cells were collected and washed twice with cold PBS before lysis. The cells were lysed using the mammalian protein extraction reagent RIPA (Beyotime) supplemented with a protease inhibitor cocktail (Roche) and PMSF (Roche). The proteins in the mouse tumor tissues were also extracted using the same protocol. The protein concentration was measured using the Bio-Rad protein assay kit. Protein (50 µg) was loaded into precast 4% stacking, 10% tris-glycine gels and separated by gel electrophoresis. After electrophoresis, the proteins were transferred onto 0.22 µm NC membranes (Sigma). The membranes were blocked with 5% non-fat milk for 1 h followed by incubation with primary antibodies for Fas/FasL (1:200) (Abcam), Bcl-2 and Bax (1:400) (BioWorld), and cleaved caspase-3, -8, -9 (1:500) (KeyGEN) overnight at 4°C with shaking. After washing with TBST, membranes were then blotted with the secondary antibodies (goat anti-rabbit; 1:5000 Bioworld) for 2h at room temperature and washed again with TBST. The blots were visualized using the enhanced chemiluminescence detection system (Amersham Life Science). The gray values were determined by a gel image analysis system (Bio-Rad) normalized with β-actin. To confirm the caspase cascade activation effect caused by GNA, 10 µM Az-DEVD-FMK (a caspase-3 inhibitor), 10 µM Az-IETD-FMK (a caspase-8 inhibitor) and 10 µM Az-LEHD-FMK (a caspase-9 inhibitor) (KeyGEN) combined with 0.6 µg/ml GNA were added at the same time.

**Statistical analysis**

Data were represented as the mean±SD. The data were analyzed with a single-factor analysis of variance by SPSS 19.0 software. P<0.05 was considered statistically significant.

**Results**

The inhibitory effect of GNA on MDA-MB-231 cells

The inhibitory effect of GNA on MDA-MB-231 cells proliferation was measured by the MTT assay (Figure 1B). GNA significantly inhibited the proliferation of MDA-MB-231 cells at 0.2-3 µg/ml in a dose-dependent manner. The IC50 was 0.78 µg/ml.

**GNA inhibits tumor growth in vivo**

To determine the effect of GNA on tumor growth at
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Figure 1. A: The Chemical Structure of Gambogenic Acid (GNA). B: The Inhibitory Effect of GNA on the Proliferation of Breast Cancer Cells. Cells in 96-well plates were treated with various concentrations of GNA for 72 h. MDA-MB-231 cells viability was assessed by the MTT assay. The values are the mean ± SD of at least three independent experiments.

Figure 2. GNA Inhibits Tumor Growth in Vivo. MDA-MB-231 nude mice were injected with different concentrations of GNA. A. Tumor size was measured using a sliding caliper every four days. B. Tumors were isolated from different groups with or without GNA treatment. The tumors in the GNA-treated groups were significantly smaller than those of the control group. C. The proteins were extracted from tumor tissues. Western blot analyzed the expressions of Fas, FasL, and cleaved caspase-3, -8, -9. The data are the means ±SD (*p<0.05; **p<0.01).

Figure 3. The Effect of GNA on MDA-MB-231 Cells Apoptosis. GNA induces the apoptosis of MDA-MB-231 cells. GNA was used to treat MDA-MB-231 cells at concentrations of 0, 0.4, 0.6, 0.8, 1.0 µg/ml. The cells were collected after 72 h, and the apoptosis rates were detected by flow cytometry.

Figure 4. The Expression of Apoptosis-related Proteins was Analyzed by Western Blot. A. MDA-MB-231 cells were treated with different concentrations of GNA for 72 h, and the expression levels of Fas, FasL, cleaved caspase-3, -8, -9, Bcl-2 and Bax were detected by western blot. B. After treatment with 10 µM Ac-DEVD-FMK (a caspase-3 inhibitor) combined with GNA for 72 h. The cleaved caspase-3 protein expression levels were analyzed by western blot. C. After treatment with 10 µM Ac-LEID-FMK (a caspase-8 inhibitor) combined with GNA for 72 h. The cleaved caspase-8 protein expression levels were analyzed by western blot. D. After treatment with 10 µM Ac-LIEd-FMK (a caspase-9 inhibitor) combined with GNA for 72 h. The cleaved caspase-9 protein expression levels were analyzed by western blot (*p<0.05; **p<0.01).

The effects of GNA on MDA-MB-231 cell apoptosis

Flow cytometry with annexin V/PI double staining was used to analyze whether GNA induced MDA-MB-231 cell apoptosis. The rates of apoptosis after treatment with 0, 0.4, 0.6, 0.8, and 1.0 µg/ml GNA for 72 h were 3.5%, 14.7%, 40.8%, 47.7%, and 76.6%, respectively. The total rate of apoptosis increased gradually with the GNA dose, and GNA significantly induced MDA-MB-231 cells apoptosis in a dose-dependent manner (Figure 3).

The effects of GNA on apoptosis-related protein expression

After treatment with GNA (0, 0.4, 0.6, 0.8, and 1.0 µg/ml) for 72 h, the expression of apoptosis-related proteins was detected by western blot. The results showed that the expressions of Fas, Bax, cleaved caspase-3, caspase-8, and caspase-9 were increased by GNA treatment in a dose-dependent manner. However, Bcl-2 expression was decreased, and FasL did not change significantly (Figure 4A).
After treatment with Az-DEVD-FMK 10 µM (a caspase-3 inhibitor), the expression of cleaved caspase-3 in MDA-MB-231 cells was significantly decreased (Figure 4B, lane 4). After treatment with Az-IETD-FMK 10 µM (a caspase-8 inhibitor), the expression of caspase-8 in MDA-MB-231 cells was significantly decreased (Figure 4C, lane 4). Similarly, after treatment with 10 µM Ac-LEHD-FMK (a caspase-9 inhibitor), the expression of caspase-9 in MDA-MB-231 cells was significantly decreased (Figure 4D, lane 4). All the data indicate that GNA induced the expression of caspase family members (Figure 4B, C, D).

Discussion

Anti-tumor research based on traditional Chinese medicine has a long history in clinical application. GNA has been confirmed as the anti-tumor component of the traditional Chinese drug cambogia (Lei et al., 2003). Accumulating evidence indicates that GNA has a potential anti-tumor effect in many tumor cells (Li et al., 2010; Wang et al., 2011; Chen et al., 2012; Yan et al., 2012), but only Li et al. demonstrated that GNA inhibited tumor growth in A549 xenograft nude mouse models (Li et al., 2010). In order to determine whether GNA plays an anti-tumor role in other tumors in vivo, MDA-MB-231 cell, an aggressive breast cancer cell line, was been chosen. We constructed MDA-MB-231 xenograft nude mouse breast tumor models and showed that GNA could inhibit breast tumor growth in a dose-dependent manner. Subsequently, we evaluated the anti-tumor effect of GNA on MDA-MB-231 cells and found that the apoptosis rate was significantly increased by GNA in a dose-dependent manner. However, the potential mechanism of GNA suppresses tumor growth in vivo was not studied in the past research.

Apoptosis, a strictly regulated form of programmed cell death, is the most common form of cell death in various cell types (Kerr et al., 1972; Jing et al., 2012). In the present study, we found that the expressions of cleaved caspase-3, -8, -9 were significantly up-regulated by GNA in vivo and in vitro. Meanwhile, the increases of caspase-3, caspase-8, and caspase-9 were blocked by caspase inhibitors in MDA-MB-231 cells. Our results demonstrated that GNA might inhibit breast cancer MDA-MB-231 cells apoptosis through the apoptotic pathway, which was consistent with the increased apoptosis rate.

Apoptosis involves three pathways: the death receptor, mitochondrial death and endoplasmic reticulum pathways (Niu et al., 2011; Wang et al., 2011). The intrinsic or mitochondrial apoptotic pathway is a major cell death pathway that includes death receptors triggering apoptosis from the cell surface, Bcl-2 proteins as the gatekeepers of the mitochondrial pathway, and caspase as the executioner enzymes (Spierings et al., 2005). Cells over-expressing Bcl-2 or deficient in Bax are resistant to apoptosis. Conversely, over-expressing Bax promotes cytochrome c release and activates apoptotic enzymes, leading to cell death (Kane et al., 1993; Zhai et al., 2008). Many studies have reported that GNA induces cell apoptosis through mitochondrial pathway: GNA induced mitochondrial-dependent apoptosis and referred to phosphor-Erk1/2 and phosphor-p38 MAPK in human hepatoma HepG2 cells (Yan et al., 2012); Yan et al. also showed that GNA mediated apoptosis through mitochondrial oxidative stress and inactivation of the Akt signaling pathway in human nasopharyngeal carcinoma CNE-1 cells (Yan et al., 2011). And, in our previous study, we also found that GNA induced breast cancer MCF-7 cells apoptosis through the mitochondrial pathway. Our data demonstrated that the expression of Bax was increased after treated with GNA in a dose-dependent manner, while Bcl-2 was decreased in breast cancer MDA-MB-231 cells. Interestingly, the phenomenon was not found in tumor tissues. So, to further explore the potential mechanism of GNA in vivo is desire.

In our previous study, we found that GNA induced apoptosis not only through the mitochondrial pathway but also through the Fas/FasL death receptor pathway in MCF-7 cells. The Fas death receptor is known as another important inducer of apoptosis. Fas, through binding to its ligand FasL, recruits and activates the precursor form of cysteine protease caspase, particularly procaspase-8 and procaspase-10, which in turn activate caspase-3 and the downstream apoptotic cascades (Kojima et al., 2006; Park et al., 2008). Whether GNA inhibited tumor growth through the Fas/FasL death receptor pathway in vivo and in various cell lines had been researched in this study. The proteins were extracted from tumor tissues. The results of western blot showed that the expressions of Fas and FasL were increased in GNA-treated groups in a dose-dependent manner. The results in cell levels were consistent with that in vivo.

Based on our results, we proposed that GNA inhibits breast tumor growth through Fas/FasL death receptor pathway and induces apoptosis both through mitochondria-dependent pathway and Fas/FasL death receptor pathway in breast cancer MDA-MB-231 cells. Unfortunately, we did not continue to investigate the detail signaling pathways. Li et al. have reported that GNA can inhibit A549 cell proliferation through apoptosis by inducing the up-regulation of the p38 MAPK cascade and cell cycle arrest (Li et al., 2010; Yan et al., 2012). Chen et al. found that GNA induced time- and dose-dependent growth inhibition and apoptosis Akt pathway inactivation in U251 glioblastoma cells (Chen et al., 2012). Many studies have shown that PI3K/Akt and MAPK pathways are involved in the regulation of growth and migration of breast cancer (Hu et al., 2012; Tomas et al., 2012). In the future study, we will investigate the signaling pathways in detail related to GNA-induced apoptosis in breast cancer and in other cancers.

In summary, our studies showed that GNA suppressed breast cancer growth via inducing apoptosis in vivo and in vitro. We examined changes in the expression levels of apoptosis-regulating proteins, including cleaved caspase-3, -8, -9, Bax, Bcl-2, Fas and FasL to explore the possible apoptosis pathways in vivo and in vitro. Our results first confirmed that GNA could effectively induce the apoptosis of breast cancer via the death receptor and mitochondrial pathways.

Therefore, we have elucidated the mechanisms of breast cancer apoptosis, provided important insight into
the pathogenesis of breast cancer and offered evidence for the treatment of breast cancer. There is reason to believe that GNA is a promising novel anti-cancer drug.

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References


