The mechanism of neogambogic acid-induced apoptosis in human MCF-7 cells

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Neogambogic acid (NGA), an active ingredient in garcinia, can inhibit the growth of some solid tumors and result in an anticancer effect. We hypothesize that NGA may be responsible for the inhibition of proliferation of human breast cancer cell line MCF-7 cells. To investigate its anticancer mechanism in vitro, MCF-7 cells were treated with various concentrations of NGA. Results of MTT (methyl thiazolyl tetrazolum) assay showed that treatment with NGA significantly reduced the proliferation of MCF-7 cells in a dose-dependent manner. NGA could increase the expression of the apoptosis-related proteins FasL, caspase-3, caspase-8, caspase-9, and Bax and decrease the expression of anti-apoptotic protein Bcl-2 accompanied by the mitochondrial transmembrane damage. The antiproliferative effect of NGA on MCF-7 cells is due to the G0/G1 arrest, increased apoptosis and activation of Fas/FasL and cytochrome C pathway. These results provide an important insight into the cellular and molecular mechanisms through which NGA impairs the proliferation of breast cancer cells.

Keywords neogambogic acid; MCF-7 cells; cell apoptosis

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Introduction

Breast cancer is one of the most frequent cancers affecting women. Approximately 1,000,000 new cases occurred worldwide per year, with >370,000 deaths due to it [1]. Although chemotherapy is regarded as an effective therapeutic measure, patients are always subjected to a series of severe treatment-related side effects. Thus, searching for new alternatives to breast cancer is imperative and important.

Gamboge is the dry resin of gamboge trees found in garcinia or cambogia plants. Clinical studies have shown that garcinia had significant therapeutic effects on pancreatic adenocarcinoma and skin cancers [2,3]. Neogambogic acid (also called gambogenic acid, NGA) is one of the active ingredients in garcinia, first isolated and purified in 1984 [4,5]. The structure of NGA is shown in Fig. 1. Many studies have shown that NGA presents an anti-tumor potential in vitro [6]. For example, NGA could inhibit the proliferation and induce the apoptosis of Ehrlich ascites carcinom cells, S180-Lewis or La795 lung cancer cells, and CNE-1 human nasopharyngeal carcinoma cells in a dose-dependent manner [7–9]. NGA also inhibited the tumor growth of nude mice transplanted with A549 lung cancer cell in vivo [7,9]. Furthermore, NGA mediated apoptosis through inactivation of Akt, accompanied with mitochondrial oxidative stress in CNE-1 cells [10]. But whether NGA can block the growth of human breast cancer MCF-7 cells and the underlying mechanisms has not been reported.

To confirm the hypothesis, the MCF-7 cells were treated with NGA at different concentrations. Cell proliferation and cell apoptosis relative index were detected in this study. The results suggested that NGA could effectively induce apoptosis of the MCF-7 cells via endogenous and exogenous pathways.

Materials and Methods

Cells and reagents

The MCF-7 cell line was provided by Dr Jianwei Zhou (the Molecular Toxicology Laboratory, Nanjing Medical University). Cells were cultured in Dulbecco’s minimum essential medium (DMEM, high-glucose) (Hyclone, Logan, USA) supplemented with 10% calf serum (PAA, Ontario, Canada) at 37°C with 5% CO2. NGA (Batch 090327) was purchased from Shanghai Ronghe Medical Technology Co. (Shanghai, China) and was dissolved in DMSO (Sigma, St Louis, USA) to make a stock solution. Stock solution of 100 mg/ml was stored at 4°C. MTT (methyl thiazolyl tetrazolum), JC-1 cell apoptosis kit, and
FITC-Annexin V/PI kits were purchased from KeyGEN Biology Co. Ltd (Nanjing, China).

**MTT assay**

MCF-7 cells ($5 \times 10^4$) were seeded into 96-well plates (Corning, Ithaca, USA). Four hours later, 10 \( \mu \)l NGA in DMSO was added into the wells at various concentrations (0.5–24 \( \mu \)g/ml) and 0.1% DMSO was set as a negative control. After 72 h, 50 \( \mu \)l MTT was added and cells were incubated for another 4 h. Then, media was removed and 150 \( \mu \)l 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 thrice and the rate of cell inhibition was calculated using the following formula: inhibition rate $= [1 - (OD_{test}/OD_{negative\ control})] \times 100\%$.

**Annexin V-FITC staining**

MCF-7 cells seeded in 6-well plates were treated with different concentrations of NGA for 72 h. Then, $5 \times 10^4$ cells were collected, resuspended by adding 500 \( \mu \)l binding buffer (KeyGEN), followed by adding 5 \( \mu \)l Annexin V-FITC and 5 \( \mu \)l PI dye. After mixing at room temperature in the dark for 5–15 min, flow cytometry analysis was performed following the manufacturer’s instructions (KeyGEN).

**JC-1 test mitochondrial transmembrane potential**

MCF-7 cells ($5 \times 10^4$) were seeded in 6-well plates and incubated (37°C, 5% CO\(_2\)) for 24 h. After removing the media and washing twice with PBS, 1 ml complete DMEM medium containing different concentrations of NGA was added to each well. After 72 h of treatment, the cells were collected, washed twice with PBS, and resuspended in 500 \( \mu \)l JC-1 working solution for 15–20 min. Cells were centrifuged at room temperature, and the supernatant was removed. The cells were then resuspended in 500 \( \mu \)l 1 \( \times \) incubation buffer, and fluorescence changes were detected by flow cytometry (Ex/Em=488 nm/530 nm).

**Western blot analysis**

MCF-7 cells were treated with 0.5, 1.0, and 1.5 \( \mu \)g/ml NGA for 72 h, and then collected by centrifugation, washed twice with cold PBS, and resuspended in 200 \( \mu \)l of pre-cooled lysis buffer (KeyGEN). After incubation on ice for 30 min, cells were centrifuged at 4°C at 13,000 \( g \) for 10 min and the supernatant was stored at 4°C. The protein concentration was determined by the BCA method (Pierce, Rockford, USA), and 50 \( \mu \)g of protein was loaded onto 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes. Membranes were blocked in 5% non-fat milk for 1 h followed by incubation with primary antibodies of Fas/FasL (1:200), caspase-3, caspase-8, caspase-9, Bcl-2, and Bax (1:400) (Cell Signaling, Denver, USA), respectively, overnight at 4°C with shaking. After washing in TBST, membranes were incubated with peroxidase-linked IgG (immunoglobulin G) conjugates for 2 h at room temperature, washed again in TBST, and then detected by enhanced chemiluminescence reagent kit (Amersham Life Science, Cleveland, USA). The gray values were determined by a gel image analysis system (Bio-Rad, Hercules, USA) normalized with the \( \beta \)-actin. In order to confirm the caspase cascade activation effect caused by NGA, 100 \( \mu \)M z-devd-fmk (pan-caspase inhibitor) combined with 1.0 \( \mu \)g/ml NGA were added at the same time.

**Statistical analysis**

Data were represented as mean $\pm$ SD. The data were analyzed with single factor analysis of variance by SPSS 12.0 software. $P < 0.05$ was considered statistically significant.

**Results**

**Inhibition of MCF-7 cell proliferation by NGA**

MCF-7 cells proliferation was inhibited by NGA, as measured by the MTT assay (Fig. 2). After 72 h incubation, NGA inhibited the proliferation of MCF-7 cells
within 0.5–2 μg/ml in a dose-dependent manner. The IC50 was calculated to be 1.76 μg/ml.

The influence of NGA on cell apoptosis
Flow cytometry assay with Annexin V/PI double staining showed that NGA induced MCF-7 cell apoptosis. The rates of early apoptosis after 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μg/ml NGA treatment for 72 h were 8.9% ± 0.17%, 31% ± 0.20%, 42% ± 0.13%, 60% ± 0.21%, 57% ± 0.12%, and 68% ± 0.23%, respectively. The total rate of apoptosis was also increased gradually with NGA, in a dose-dependent manner (Fig. 3).

MCF-7 mitochondrial transmembrane potential changes
JC-1 test showed that NGA induced MCF-7 cell mitochondrial membrane potential damage. The rates of impaired mitochondrial membrane potential after treatment with 0.5, 1.0, and 1.5 μg/ml NGA were 40% ± 2.9%, 45% ± 3.7%, and 76% ± 5.1%, respectively (Fig. 4).

The effects of NGA on apoptotic protein expression
After treatment with NGA (0.5, 1.0, and 1.5 μg/ml) for 72 h, cell apoptotic protein expression was detected by western blot. Results showed that the expressions of FasL, Bax, and caspases-3, -8, -9, Bcl-2, and Bax were increased by treatment of NGA in a dose-dependent manner. However, Bcl-2 expression showed a decrease. Fas protein level did not change significantly (Fig. 5). Treatment with 100 μM z-devd-fmk (caspase inhibitor) combined with 1.0 μg/ml NGA for 72 h, the caspase-3 protein expression level were detected by western blotting.

Effects of NGA on the cell cycle
Untreated MCF-7 cells (control) showed 41.93% population in G0/G1 phase. After being treated with NGA for 72 h, the cells showed a progressively increased population in G0/G1 phase in a dose-dependent manner. The increase in G0/G1 phase cells after NGA treatment was accompanied by an increase in apoptotic population relative to the control (Table 1).
NGA, a component of a traditional Chinese drug cambogia, has been confirmed as the antitumor components. Cheng et al. [7] confirmed that NGA inhibited HCT-8, Bel-7402, BGC-823, A549, and A2780 cell proliferation, with an IC_{50} between 1.75 and 3 μM. In vivo experiments also demonstrated that NGA arrested A549-induced tumor growth in nude mice [9]. It has been reported that NGA-induced A549 cells apoptosis arrested the cells to G_{0}/G_{1} phase in vitro [11].

The present study showed that NGA exhibited an anti-proliferative effect on MCF-7 cells (Fig. 2). The anti-proliferative effect was related to the ability of NGA to provoke growth inhibition at the G_{0}/G_{1} phase transition of the cell cycle and enhancement of cell apoptosis. As shown in Fig. 2 and Table 1, MCF-7 cells treated with NGA remarkably accumulated in the G_{0}/G_{1} phase of the cell cycle in a dose-dependent manner up to ~160% by 72 h. In previous studies, it had been shown that NGA inhibited various tumor cells proliferation in a dose-dependent manner [12]. Our results demonstrated that MCF-7 cells treated with NGA arrest in the G_{0}/G_{1} phase.

Apoptosis of MCF-7 cells exposed to NGA were also detected after G_{0}/G_{1} phase arrest by cytofluorimetric analysis using PI and AnnexinV-FITC double staining. When cells were treated with NGA, the apoptotic population was increased in a dose-dependent manner, which was accompanied by an increase in G_{0}/G_{1} populations (Fig. 3, Table 1). Mechanisms for cell apoptosis may involve a multiplicity of interactions, such as death receptor, mitochondrial, and endoplasmic reticulum pathways [13,14]. However, the exact molecular mechanisms by which NGA induce MCF-7 cell apoptosis are not known.

Caspase-3 reported as a final apoptotic effect molecule, activated by caspases-4/-8/-9, could hydrolyze downstream-specific substrates poly (ADP-ribose) polymerase and induced cell apoptosis [15–17]. After treatment with NGA, the expression of caspase-3 was increased; however, this increase was blocked by caspase inhibitor, z-devd-fmk (Fig. 6), indicating that NGA might cause MCF-7 cell apoptosis through a typical apoptotic pathway.

Mitochondria play an important role in cell apoptosis. A variety of apoptotic proteins were transported to the mitochondria, which led to a decreased permeability and reduced membrane integrity resulting in a disappearance of hydrogen ions and mitochondrial membrane potential. A variety of apoptosis-inducing factors and pro-apoptotic proteins, such as cytochrome C and caspases-3/-8/-9, were released from mitochondrial and caused typical apoptosis [18]. In the present study, we tested the effect of NGA on MCF-7 cell’s mitochondria by JC-1. Ours results showed that mitochondrial membrane potential damage accumulated with an increased NGA concentration, consistent with increased expressions of caspase-3 and caspase-9 (Figs. 4 and 5). These results confirmed that NGA-induced apoptosis of MCF-7 cells mainly through the mitochondrial pathways.

Bel-2 is well known as a key anti-apoptotic protein [19], which interacts with the Bax/Bak, preventing the release of cytochrome C and activation of caspase to inhibit apoptosis [20]. FasL, a natural ligand of Fas, prompted the intracellular death domain to express Fas, induced the precursors of caspases-8 and -10 to activate caspase-3, and induced cells to undergo apoptosis [21,22]. This death receptor (Fas) pathway is an important role of apoptosis. In this study, it was observed that protein expressions of FasL, caspase-8, caspase-9, and caspase-3 were increased while the expression of Bcl-2 was decreased. These data indicated that NGA-induced apoptosis of MCF-7 cells through the death receptor (Fas) pathway and Bel-2 also played an important role.

Limiting proliferation of cancer cells represents one strategy to treat the cancer. To the best of our knowledge, this is the first report showing the antiproliferative effect and the signaling mechanism of NGA in breast cancer cell line MCF-7. A significantly increased population of cells in G_{0}/G_{1} phase, increased number of apoptotic cells and transmembrane potential, elevated expression of FasL, and decreased expression of Bel-2 indicated the probable

<table>
<thead>
<tr>
<th>Groups (NGA, μg/ml)</th>
<th>G_{1} phase</th>
<th>S phase</th>
<th>G_{2}/M phase</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>41.93 ± 0.85</td>
<td>9.88 ± 0.88</td>
<td>46.44 ± 1.07</td>
<td>0.6 ± 0.19</td>
</tr>
<tr>
<td>0.5</td>
<td>57.34 ± 1.79*</td>
<td>12.75 ± 1.49</td>
<td>28.66 ± 1.28*</td>
<td>1.16 ± 0.98*</td>
</tr>
<tr>
<td>1.0</td>
<td>65.62 ± 2.83*</td>
<td>12.93 ± 0.88</td>
<td>20.67 ± 2.61*</td>
<td>2.25 ± 1.0*</td>
</tr>
<tr>
<td>1.5</td>
<td>68.75 ± 2.61*</td>
<td>8.05 ± 1.19</td>
<td>22.10 ± 2.30*</td>
<td>18.66 ± 1.07*</td>
</tr>
</tbody>
</table>

Data (%) are expressed as mean ± SD from three independent experiments.

*P < 0.05 vs. the control (0 μg/ml).
mechanism of the antiproliferative effect of NGA. Our results provide an important insight into the cellular and molecular mechanisms by means of which NGA works as an anticancer drug.

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