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Ganoderic acid Me induces apoptosis through mitochondria dysfunctions in human colon carcinoma cells

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ABSTRACT

Ganoderma lucidum, a traditional Chinese medicinal mushroom, has been used in Asia for several thousand years for the prevention and treatment of a variety of diseases including cancer. In our previous work, ganoderic acid Me (GA-Me), as one of bioactive triterpene compounds purified from *Ganoderma lucidum*, induced p53-mediated sub-G_1 arrest in human colon cells [1]. The apoptotic mechanism of GA-Me induction on tumor cells is yet unclear and was studied in this work. GA-Me was found to possess remarkable cytotoxicity on HCT-116 human colon carcinoma cells in a dose-dependent manner. Interestingly, the expression of anti-tumor protein p53 in GA-Me treated tumor cells was increased in a time dependent manner. Among the pro-apoptotic proteins, Bax was up-regulated, whereas the expression of Bcl-2 was not significantly changed, thus the ratio of Bcl-2/Bax was decreased. Furthermore, GA-Me reduced mitochondria transmembrane potential, released cytochrome *c* and increased caspase 3 activity during the induced apoptotic process. Our findings show that the anti-cancer bioactivity of GA-Me was mediated by induced apoptosis, resulting from mitochondrial dysfunctions. Our study also suggests that GA-Me may be a novel promising agent for the treatment of human colon carcinoma cells.

1. Introduction

Ganoderma lucidum (Fr.) KARST (Polyporaceae), a traditional Chinese medicinal mushroom, has been used as traditional medicine for the prevention and treatment of a variety of diseases including cancers in Asia for several thousand years [1–5]. In recent years, it is well documented that the extracts from *G. lucidum* inhibited proliferation, induced cell cycle arrest, apoptosis and anti-invasion/anti-metastasis in human and mouse carcinoma cell lines or mice [1–15]. However, use of purified triterpene is necessary to reveal the actual mechanism of responsible compounds and to further screen and rationally design structurally similar lead compounds. Until now, over a hundred ganoderma triterpenes have been identified from *G. lucidum*, but only a few of them were demonstrated to exhibit anti-tumor activity [1–13].

Recently, ganoderic acid Me (GA-Me) was purified from the fermented mycelia of *G. lucidum* by our group. It was found that GA-Me suppressed cancer cell invasion [2], inhibited tumor growth and lung metastasis by increasing the immune function through enhancement of the expression of T-helper type 1 (Th1) cytokines (IL-2 and IFN-γ) [4]. In our previous studies, GA-Me induced p53-mediated sub-G_1 arrest in human tumor cells [1]. Therefore, we hypothesize that GA-Me could induce cancer cell apoptosis. However, the apoptotic mechanism of GA-Me is still unknown.

In this study, we investigated the mechanism of GA-Me induced cancer cell apoptosis. Our data suggest that GA-Me activated p53 and up-regulated Bax expression decreases the mitochondria membrane potential, releases cytochrome *c* to the cytosol and stimulates the activity of caspase 3 in treated HCT-116 cancer cells in a time dependent manner. Our investigations indicate that the protein p53 and the mitochondrion are the important targets in the intrinsic apoptotic pathway. Based on these results, GA-Me may be considered as a potentially useful chemotherapeutic lead compound to human colon cancer chemotherapy.

2. Materials and methods

2.1. Materials

Trypsin and 3-(4,5-dimethylthiazo-l-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from GIBCO Industries, Inc., Los Angeles, CA, USA. McCoy's 5A Medium Modified and Cytochrome *c* were obtained from Sigma, St. Louis, MO, USA. New bovine serum (NBS) and antibiotics (penicillin and streptomycin) were purchased from Sino American Biotechnology Co. (Shanghai, China). Propidium iodide (PI) was purchased from Amresco (OH, USA). Annexin V–propidium iodide (PI) kit was purchased from Bipec Biopharma Corporation (Mass, USA). Caspase-Glo 3/7 assay kit was purchased from Promega (WI, USA). General Caspase Inhibitor,

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Z-VAD-FMK was purchased from BD Biosciences (California, USA). Antibodies for actin, p53, Bax and Bcl-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylidene fluoride (PVDF) membrane, phenylmethylsulfonyl fluoride, sodium orthovanadate, leupeptin, aprotinin, β-mercaptoethanol, Hoechst 33258, and dithiothreitol (DTT) were purchased from Amresco (Solon, OH, USA). Goat anti-rabbit IgG-conjugated to horseradish peroxidase (HRP) and rabbit anti-goat IgG-conjugated to HRP were purchased from Biovision (Mountain View, CA, USA), BM Chemiluminescence Blotting Substrate (POD) substrate reagents were purchased from Roche Diagnostics (Yerevan, USA). Doxorubicin (Dox) was purchased from Shanghai Hua Lian MediPharma Ltd. (Shanghai, China).

GA-Me was purified with semi-preparative liquid chromatography in our lab with its purity over 99% [1,2]. Stock solution of GA-Me was prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C. Further dilution was made with RPMI 1640/McCoy's 5A medium just before use, and the final concentration of DMSO was less than 0.1%.

2.2. Cell culture

HCT-116 colon carcinoma cell line [16] was cultured in McCoy's 5A Medium (Modified) with 10% (v/v) dialyzed heat-inactivated new bovine serum (NBS), at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Cell proliferation assay

For the proliferation assay, viability of cells was evaluated by MTT reduction method. Briefly, 1×10^5/ml, 0.1 ml cells were plated in 96-well tissue culture plates. After 4 h incubation, cells were treated with different concentrations of GA-Me or the solvent DMSO for 24 h. After treatment, cells were stained with 10 μl MTT dye (5 mg/ml in PBS) for another 4 h and then the medium was removed. The MTT formazan formed by metabolically viable cells was dissolved in lyses buffer (20%SDS in 50%N,N-dimethylformamide) for another 15 min. Optical density at 570/630 nm was used as a measure of cell viability on a microplate reader model Multiskan Mk3 (Thermo). Effects of the drugs on inhibition of cell growth were calculated and cells treated with DMSO at the same concentrations carried by the drugs were taken as controls [1–5].

2.4. Microscopic observation of cellular morphology and nuclear fragmentation

The HCT-116 cells at 2×10^5 cells/ml were inoculated in 24-well plates and treated with different concentrations of GA-Me (18.1–90.4 μM) for 48 h. Dox (18.4 μM) was used as a positive control. The cells were fixed in 4% paraformaldehyde in 4 °C refrigerator for 30 min, and then stained with 10 μg/ml Hoechst 33258, a DNA-specific fluorescent dye, for 10 min at 37 °C. The stained cells were observed using standard excitation filters in random microscopic fields at 100× magnification under a Leica AF7000 fluorescence microscope (Leica Microsystems Inc., AG, Wetzlar, Germany) [17]. The apoptotic cells or apoptotic bodies were determined from 3 random fields per slide, from 3 independent experiments.

2.5. Apoptosis evaluation by flow cytometer

Apoptosis was detected by Annexin V–FITC binding assay. Annexin V, a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine (PS) was used to detect the early stage of apoptosis [5,18]. Normal, apoptotic and necrotic cells were distinguished by using the Annexin V–propidium iodide (PI) kit according to the manufacturer's instructions (BIPEC, USA). Briefly, HCT-116 cells were treated with different concentrations of GA-Me or Dox and cells were harvested and processed 24 h after treatment. The cells were washed in ice-cold PBS and resuspended in binding buffer. 5 μl of Annexin V–FITC staining solution was added and the cells were incubated in the dark for 10 min. Then 10 μl of propidium iodide (PI) was added to each sample and the cells were incubated in the dark for another 5 min before flow cytometric analysis. Data analysis was performed with standard Cell Quest software (BD FACScan™, CA, USA). The percentages of viable and dead cells were determined with about 1×10^4 cells/sample.

2.6. Flow cytometric evaluation of mitochondrial transmembrane potential ($\Delta\psi_m$) change

Mitochondrial transmembrane potential ($\Delta\psi_m$) was estimated using the fluorescent cationic dye rhodamine 123 (Rh123) [19]. Briefly, the HCT-116 cells were treated with 18.1, 36.3 and 90.4 μM GA-Me for 24 h, and then were harvested and incubated for 15 min at 37 °C with 5 μM Rh123 in the dark. Cells were then washed twice with PBS and analyzed immediately by flow cytometry (BD FACScan, USA). Loss in Rh123 staining indicates an association to the disruption of mitochondrial membrane integrity.

2.7. Determination of cytochrome c release

The release of cytochrome *c* was detected with HPLC and ultraviolet detector. After apoptosis induction treatment, the HCT-116 cells were treated with 90.4 μM GA-Me for different time points, and then 2×10^6 cells were rinsed three times with PBS, scraped from the dish and then lysed with lysis buffer. Initial spectroscopic measurements were made on a Shimadzu spectrophotometer (Shimadzu, Japan). Chromatography of cytochrome *c* was performed using a 5 μm C18 reverse-phase column (250 mm × 4.6 mm) by Shimadzu HPLC system with UV detector (Shimadzu, Japan). The detection wavelength was 393 nm. A gradient from 20% to 60% of acetonitrile in water with trifluoroacetic acid (0.1% v/v) over 20 min with a flow rate of 1.0 ml/min was used [5,20].

2.8. Measurement of activities of caspase 3

HCT-116 cancer cells were seeded into a 96-well plate (10,000 cells/well), and the HCT-116 cells were treated with 90.4 μM GA-Me for different time points. Caspase activity was measured using the Caspase-Glo 3/7 assay kit according to the manufacturer's protocol. (Promega, WI, USA). In 96-well plates, the 50-μl sample was mixed gently for 30 s with 50 μl of Caspase-Glo 3/7 reagent and incubated for 2 h at room temperature. The lysis buffer with the reagent served as a blank. Luminescence of the samples was measured using a GENios Pro plate reader (Tecan, Research Triangle Park, NC, USA). Luminescent intensity values were normalized to the sample protein concentration [5,17].

2.9. Analysis of protein expression by western blotting

HCT-116 cells (5×10^5) were placed in culture dishes (10 cm), and treated with or without (only DMSO) GA-Me for 24 h using different concentrations. Total populations of cells, including adherent and suspension cells were washed with PBS twice and then resuspended with lysis buffer (10 mM Tris–HCl (pH 7.5) containing 50 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM DTT, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 5 mM leupeptin, and 10 μg/ml aprotinin) for 30 min in ice. The lysates were centrifuged to remove insoluble materials, and normalized according to their protein content. Cell lysates cleared of debris and nuclei was considered the cellular total protein. One volume of 2× Laemmli buffer containing 20%, β-mercaptoethanol was added into one volume protein sample, and then the sample was boiled for 5 min and quickly frozen on dry ice. Samples were stored at −80 °C.

The equal amounts of proteins (40–60 μg) were separated by SDS-polyacrylamide 10% gels eletrophoresis and blotted onto a PVDF membrane. After blocking for 2 h at room temperature using TBST buffer (TBS buffer plus 0.1% (v/v) Tween-20) containing 3% (m/v) BSA, the filter was then applied sequentially with anti-p53 (diluted 1:1000), anti-Bax (diluted 1:750), anti-Bcl-2 (diluted 1:500; Santa Cruz Biotechnology), and appropriate secondary antibody conjugated horseradish peroxidase (diluted 1:5000). The blots were visualized with BM Chemiluminescence Blotting Substrate (POD) reagent. Total cellular protein was determined using the Bradford method [2,3].

2.10. Statistical analysis

Statistical analysis was performed using Student's *t*-test to evaluate the significance of differences between two groups and one-way or two-way ANOVA between three and more than three groups. In all graphs, * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$ between the untreated and treated cells. All data are expressed as the mean ± standard deviation (SD, for each group $n \geq 3$). One-way ANOVA followed by Dunnett's multiple comparison test was also used for statistical analysis using Origin Pro 8 software (Origin Lab Inc.).

3. Results

3.1. Effect of GA-Me on HCT-116 cell growth

As shown in Fig. 1, the viability of the HCT-116 cells was suppressed by GA-Me. It indicates that the HCT-116 human colon cells were sensitive to GA-Me as the inhibition ratio was about 75% at 90.4 μM of GA-Me at 24 h. The growth was inhibited in a dose-dependent manner using the indicated concentrations. In our previous report, the IC_{50} was estimated to be 36.9 μM [1]. The above results indicate that GA-Me affected the viability of HCT-116 cells and inhibited the cancer cell growth.

3.2. Effect of GA-Me on cancer cell apoptosis

To assess whether GA-Me induces apoptosis, the HCT-116 cancer cells were treated by GA-Me and Dox (positive control) and then nuclear staining with Hoechst 33258 and Annexin V–PI staining assay were conducted.

Determination of apoptosis in GA-Me-treated human colon cells was performed using fluorescence microscopy of Hoechst