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Microtubule depolymerization and phosphorylation of c-Jun N-terminal kinase-1 and p38 were involved in gambogic acid induced cell cycle arrest and apoptosis in human breast carcinoma MCF-7 cells

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ABSTRACT

Gambogic acid (GA), an ingredient isolated from Garcinia hanburyi, has potent anticancer activity both in vitro and in vivo. In the present study, we examined the effects of GA on intracellular microtubules and reconstituted microtubules in vitro. Immunofluorescence microscopy revealed that 2.5 μM GA caused microtubule cytoskeleton disruption and microtubule depolymerization in human breast carcinoma MCF-7 cells, thereby reducing the amount of polymer form of tubulin and increasing the amount of monomer form of tubulin. We further confirmed that GA could depolymerize microtubule associated protein (MAP)-free microtubules and MAP-rich microtubules in vitro. Thus we suggested that GA-induced G2/M phase cell cycle arrest may be attributed to its depolymerization of microtubules. We also revealed that phosphorylation levels of p38 and c-Jun N-terminal kinase-1 (JNK-1) were increased markedly by GA, resulting in apoptosis of MCF-7 cells. Taken together, our results suggested that GA depolymerized microtubules and elevated the phosphorylation levels of JNK1 and p38, which caused G2/M cell cycle arrest and apoptosis in MCF-7 cells.

Introduction

Gambogic acid (GA, $C_{38}H_{44}O_8$), an ingredient isolated from Garcinia hanburyi, is a kind of brownish-to-orange resin. It was used in Chinese traditional medicine for hundreds of years. Recently, GA was proved as a potent anticancer candidate. Many results revealed that GA could inhibit the growth of various types of cancer cells, suppress the growth of human tumors (e.g., lung carcinoma and hepatoma) and prevent tumor metastasis and angiogenesis (Liu et al., 2005; Lu et al., 2007; Wu et al., 2004; Zhao et al., 2004). Furthermore, we previously demonstrated that GA could inhibit the telomerase activity, reduce the expression of c-MYC, downregulate Bcl-2 level and induce apoptosis of tumor cells. Pandey et al. (2007) reported that GA inhibited NF-κB signaling pathway and potentiated apoptosis through its interaction with the transferrin receptor (Kasibhatla et al., 2005). Qin et al. (2007) suggested that GA inhibited the catalytic activity of human topoisomerase α by binding to ATPase domain. Our current study demonstrated that GA depolymerized microtubules and elevated the phosphorylation levels of c-Jun N-terminal kinase-1 (JNK-1) and p38, which caused G2/M cell cycle arrest and apoptosis in MCF-7 cells.

Microtubules, built by α/β-tubulin dimers, are major dynamic structural components of the cytoskeleton involved in a variety of cell functions (Mitchison and Kirschner, 1984). Microtubules contribute to the development and maintenance of cell shape, cell reproduction and division, cell signaling, intracellular transport and cell movement, especially in regulating the segregation of chromosomes during mitosis. The important role of tubulin in the cell division cycle, together with the fact that aberrant cell division is the hallmark of cancer has made tubulin and microtubules prime targets for cancer chemotherapy. Growing classes of microtubule-targeted agents have been used in clinical for cancer therapy. Microtubules are considered as key targets for developing anticancer drugs (Jordan and Wilson, 2004). Microtubule-targeted agents act cytotoxically by disrupting the microtubule dynamics and perturbing the formation and function of the mitotic spindle apparatus, thus arresting cell cycle in mitosis and leading to subsequent apoptotic cell death.

Many extracellular signals converge at a family of serine/threonine protein kinases called mitogen-activated protein kinases (MAPKs). Mammalian include extracellular signal-regulated kinases (ERK1/2), which are regulated mostly by growth factors and mitogenic stimuli,

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and stress-regulated kinases, p38, and c-Jun N-terminal kinase (JNK), involved in cellular response to various stress conditions (Raman et al., 2007). They participate in the signal transduction to the cell nucleus. MAPKs are activated by dual phosphorylation of Thr and Tyr within the motif Thr-Glu-Tyr (ERK) or Thr-Gly-Tyr (p38) or Thr-Pro-Tyr (JNK) in subdomain VIII of the protein kinases. This phosphorylation is mediated by a protein kinase cascade that consists of a MAPK kinase kinase (MKKK), which phosphorylates and activates one or more MAPK kinases (MKK) (Dhillon et al., 2007). Activation of extracellular stress-regulated kinase contributes to cell differentiation, proliferation and survival, whereas JNK and p38 are activated by proinflammatory cytokines and environmental stresses and induce apoptosis (Davis, 2000; Wilkinson and Millar, 1998). Oren et al. (1999) also showed that depolymerization of the cytoskeleton activated JNK and p38 mitogen-activated protein and induced c-Jun expression through a signaling pathway that depended on the activity of tyrosine kinase.

Materials and methods

Cell line and reagents

Human breast carcinoma MCF-7 cells were purchased from Cell Bank of Shanghai Institute of Biochemistry & Cell Biology. Cells were grown in RPMI-1640 medium (GIBCO) supplemented with 10% heat-inactivated calf serum (CS), 100 U/ml benzylpenicillin and 100 μg/ml streptomycin. And then the cells were cultured at 37 °C under a humidified atmosphere of 5% CO_2. GA was isolated and purified according to the established methods with slight modifications. A 10 mM solution of GA was prepared in dimethyl sulfoxide (DMSO), stored at −20 °C, and then diluted as needed in culture medium. The final DMSO concentration in each group did not exceed 0.1% throughout the study (In our study, all the control groups contained 0.1% DMSO). Taxol (Paclitaxel) was obtained from Sigma, and vincristine was obtained from Zhejiang Hisun pharmaceutical co., Ltd. The antibody to β-tubulin was obtained from Sigma–Aldrich Biotechnology Inc. Primary antibodies used in Western blotting for JNK2, JNK1/2 phosphorylated at Thr183/Tyr185, p38, p38 phosphorylated at Thr180/Tyr182 and β-actin were obtained from Santa Cruz Biotechnology Inc. IRDye™800-conjugated secondary antibodies were obtained from Rockland Inc.

Annexin-V/PI double-staining assay

MCF-7 cells were treated with GA (1 μM) for 24 h. Then they were harvested and resuspended with phosphate buffered saline (PBS) (Yang et al., 2007). Apoptotic cells were identified by double supravital staining with recombinant FITC (fluorescein isothiocyanate)-conjugated Annexin-V and PI, using the Annexin V-FITC Apoptosis Detection kit (BioVision) according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in a Becton–Dickinson FACSCalibur flow cytometer using CellQuest software. Cells unbound to FITC-annexin V and excluding propidium iodide were classified as annexin V-negative (AN-ve). Cells bound to FITC-annexin V [excitation wavelength (λ_{ex})=488 nm and emission wavelength (λ_{em})=520 nm] but excluding propidium iodide (λ_{ex}=540 nm and λ_{em}=630 nm) were termed as annexin V-positive (AN+ve). Cells permeant to propidium iodide (regardless of whether or not they bound to FITC-annexin V) were deemed necrotic (Taimor et al., 2000).

Cell cycle analysis by flow cytometry

Cells treated with DMSO or various concentrations of GA for different periods of time were harvested by trypsinization and washed once with PBS, and fixed in 70% ethanol at 4 °C overnight, and washed twice with PBS. Cells were then centrifuged and resuspended in 500 μl of propidium iodide (PI)/RNase staining buffer (BD Bioscience Pharmingen, CA) before DNA content was determined. DNA content and cell cycle were determined using a FACScan laser flow cytometer (FACSCalibur, Becton–Dickinson). The data were analyzed by the CellQuest software.

Immunofluorescence confocal microscopy

Cells were seeded onto glass coverslips in 6-well plates the day before treatment. The immunostaining method was modified from the previous description (Yoon et al., 2002). In brief, after the cells were incubated with GA for the indicated time period, they were washed twice with PBS for 5 min. Then the cells were fixed with paraformaldehyde (PFA) at room temperature for 20 min. When washed twice with PBS for 5 min, the cells on the coverslips were incubated with Triton X-100 at 4 °C for 20 min. Following the incubation, the cells were blocked with PBS containing 3% BSA for 1 h, and incubated with anti-β-tubulin antibody (1:1000) at 4 °C overnight. When washed with PBS, the cells were stained with FITC-conjugated anti-mouse IgG antibody (1:50) for 45 min at room temperature. And then the coverslips were stained with propidium iodide (PI) for 5 min. The images were captured with a ZEISS LSM 510 confocal spectral microscope.

Analysis of microtubule polymerization in vivo

A method was modified to quantitate tubulin polymerization, which was originally described by Minotti et al. (1991). MCF-7 cells grown to confluency were treated with DMSO, taxol, vincristine or GA for 6 h. Then the cells were washed twice with PBS and harvested by trypsinization. One hundred μl of hypotonic buffer (1 mM $MgCl_2$, 2 mM EGTA, 0.5% NP-40, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM orthovanadate and 20 mM Tris–HCl, pH 6.8) were added, and cells were lysed at 37 °C for 5 min in the dark. After a brief but vigorous vortex, the samples were centrifuged at 14,000 rpm for 10 min. The 100 μl supernatants containing soluble (cytosolic) tubulin were separated from the pellets containing polymerized (cytoskeletal) tubulin. The pellets were resuspended in 100 ml of hypotonic buffer. The soluble and polymerized tubulin were respectively mixed with 30 ml of SDS-polyacrylamide gel electrophoresis sample buffer (45% glycerol, 20% β-mercaptoethanol, 9.2% SDS, 0.04% bromphenol blue, and 0.3 M Tris–HCl, pH 6.8), and then heated at 95 °C for 10 min. Equal amount of each sample was analyzed by Western blotting and probed with the antibody against β-tubulin.

MAP-free tubulin polymerization assay in vitro

Five mg/mL purified bovine brain tubulin (Cytoskeleton Inc. Denver, CO.) was assembled in General Tubulin Buffer [80 mM PIPES (pH 6.9), 0.5 mM EGTA, 2 mM $MgCl_2$ and 1 mM GTP] plus 5% glycerol at 37 °C for 5 min. Various concentrations of GA solution were prepared in General Tubulin Buffer and preheated to 37 °C, then mixed with 50 μl tubulin. The mixture was transferred to the preheated 384-well plate, and incubated for an additional 13 min. The assembly of tubulin was measured every 30 s and monitored by light scattering at 340 nm using a Varioskan multimode microplate spectrophotometer (Thermo) and Skanlt software version 2.0 in a kinetic model (Huang et al., 2005).

MAP-rich tubulin polymerization assay in vitro

Two mg/mL bovine brain microtubule associated protein (MAP)-rich tubulin (Cytoskeleton Inc. Denver, CO.) was assembled in General Tubulin Buffer [80 mmol/L PIPES (pH 6.9), 0.5 mM EGTA, 2 mM $MgCl_2$ and 1 mM GTP] on 384-well plate at 37 °C for 5 min. Various concentrations of GA solution were prepared in General Tubulin Buffer and preheated to 37 °C, then mixed with 30 μl MAP-rich tubulin. The mixture was incubated for an additional 17 min. The assembly of tubulin was measured every 30 s for 12 min and monitored by light scattering at