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Ginsenoside Rh2 induces apoptosis and paraptosis-like cell death in colorectal cancer cells through activation of p53

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ABSTRACT

Ginsenosides are the main bioactive components in American ginseng, a commonly used herb. In this study, we showed that the ginsenoside Rh2 exhibited significantly more potent cell death activity than the ginsenoside Rg3 in HCT116 and SW480 colorectal cancer cells. Cell death induced by Rh2 is mediated in part by the caspase-dependent apoptosis and in part by the caspase-independent paraptosis, a type of cell death that is characterized by the accumulation of cytoplasmic vacuoles. Treatment of cells with Rh2 activated the p53 pathway and significantly increased the levels of the pro-apoptotic regulator, Bax, while decreasing the levels of anti-apoptosis regulator Bcl-2. Removal of p53 significantly blocked Rh2-induced cell death as well as vacuole formation, suggesting that both types of cell death induced by Rh2 are mediated by p53 activity. Furthermore, we show that Rh2 increased ROS levels and activated the NF-κB survival pathway. Blockage of ROS by NAC or catalase inhibited the activation of NF-κB signaling and enhanced Rh2-induced cell death, suggesting that the anti-cancer effect of Rh2 can be enhanced by antioxidants.

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1. Introduction

Colon cancer is the third leading cause of cancer-related deaths in the western world [1]. Current treatment of this cancer generally employs surgical resection combined with chemotherapy using cytotoxic drugs and radiation therapy. Because this therapy is only moderately successful, novel approaches to the treatment of colorectal cancer are required.

Natural products are potentially valuable source for the development of new anti-cancer drugs [2,3]. American ginseng (*Panax quinquefolius*) is a very popular herb in the United States, and its main effective components are ginsenosides that have been reported to have a wide variety of biological activities including immunomodulatory effects, anti-inflammatory and anti-tumor activity [4–6]. We recently reported that steamed American ginseng extract potently killed colorectal cancer cells and that Rg3 and Rh2, derivatives of protopanaxadiol (PPD), are the main ginsenosides in the extract [7,8]. Interestingly, it is reported that Rg3 can be metabolized by human intestinal bacteria to Rh2 and further to PPD [9]. In this report, we characterized the effects of Rg3 and Rh2 on the colorectal cancer cell lines, HCT116 and SW480.

Abbreviations: DCF, 2,7-dichlorofluorescein; H2DCFDA, 5-(and-6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate acetyl ester; NAC, N-acetyl-L-cysteine; PCD, programmed cell death; ROS, reactive oxygen species.

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Apoptosis is programmed cell death involving the activation of caspases through either a mitochondria-dependent cell intrinsic or mitochondria-independent cell extrinsic pathway [10,11]. In addition to apoptosis, several types of caspase independent programmed cell death have been identified including autophagy, paraptosis, mitotic catastrophe, and necroptosis [12,13]. Autophagy is characterized by the sequestration of bulk cytoplasm and/or organelles in double membrane autophagic vesicles and can be visualized by the localization of Atg8/LC3 to the membrane of pre-autophagosome [14]. Paraptosis is characterized by cytoplasmic vacuolization. It lacks apoptotic morphology and does not respond to caspases inhibitors. However, paraptosis does require new protein synthesis and MAP kinase activation [15–17]. Necroptosis is a form of programmed necrosis that is caspase-independent and have been reviewed recently (reviewed in [13]). It should be pointed out that a dying cell may exhibit characteristics of several death pathways. It is postulated that the dominant death phenotype is determined by the relative speed of the available death programs [12].

In this study, we show that Rh2 exerts significantly more potent colorectal cancer cell killing activities than Rg3. We show that Rh2-induced cell death is partially dependent on caspase-3 activation. Interestingly, we find that Rh2 induces a significant level of cytoplasmic vacuole formation, which is characteristic of paraptosis. Similar to our studies of the steamed ginseng extracts, we show that Rh2 induces ROS generation in colorectal cancer cells, which in turn activates the NF-κB signaling and partially counteracts the cancer cell killing activities of Rh2. Consistent with this, inhibition of ROS or the NF-κB pathway increases the toxicity of Rh2 to colorectal cancer cells. Furthermore, we show that p53 transcription activity is induced by Rh2 and that inactivation of p53 significantly decreases Rh2-induced vacuole formation and cell death.

2. Methods and materials

2.1. Chemicals and reagents

N-acetyl-L-cysteine (NAC) and PS1145 were obtained from Sigma. Rh2 and Rg3 were obtained from National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China; and were of biochemical reagent grade and at least 95% pure as determined by HPLC. NAC was dissolved in the growth medium. PS1145, a specific inhibitor of NF-κB pathway, was dissolved in DMSO as a 20 mM stock buffer. Luciferase assay kits were purchased from Promega. Anti-Bad and monoclonal anti-β-actin was obtained from Cell Signaling Technology. Anti-Bcl-2, anti-Bcl-XL and anti-Bax were obtained from Santa Cruz. Annexin V Kit was purchased from BD Biosciences, and 5-(and-6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate acetyl ester (H2DCFDA) was obtained from Invitrogen. Experiments were carried out at least three times to obtain the mean and the standard deviation.

2.2. Cell culture

Normal human colon epithelial cells, FHC, and human colorectal cancer cells HCT116 and SW480 were obtained from the American Type Culture Collection. HCT116 and SW480 cells were maintained in McCoy's 5A medium supplemented with 5% fetal bovine serum (FBS, Hyclone Laboratories), 50 IU of penicillin/streptomycin (Gemini Bio-Products) and 2 mmol/l of L-glutamine (Invitrogen) in a humidified atmosphere with 5% CO_2 at 37 °C. FHC cells were maintained in the ATCC suggested complete growth medium (DMEM:F12 medium with 25 mM HEPES supplemented with 10% FCS, 10 ng/ml cholera toxin, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, and 100 ng/ml hydrocortisone).

2.3. FACS analysis, Trypan Blue staining, and quantification of vacuolization

For the cell death assay, 25×10^4 cells/well were seeded into 6-well plates. Samples were prepared based on the instruction provided together with Annexin V Apoptosis Kit. Briefly, after treatment as indicated in Section 3, the adherent and detached cells were collected and washed twice with binding buffer containing 10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl, and then 1×10^5 cells were resuspended in 100 μl of binding buffer. 5 μl of Annexin V-FITC and 10 μl of propidium iodide (50 μg/ml, stocking concentration) were added to the cell suspension. After gently mixing, the cells were incubated for 15 min at room temperature, and then 400 μl of binding buffer was added to get the sample ready. Quantification of cell death was performed using a FACScan (BD Biosciences). Annexin V-positive and/or PI-positive cells were considered cell death.

For Trypan Blue Staining, after treatment as indicated in Section 3, the adherent and detached cells were collected and stained with Trypan Blue dye for 5 min at room temperature. Cell death is determined as the percent of cells that are stained.

For quantification of vacuolization, cells were treated as described in Section 3 and observed under microscope to determine the fraction of cells with obvious cytoplasmic vacuoles.

Intracellular ROS production was monitored by the permeable fluorescence dye, H2DCFDA. H2DCFDA can readily react with ROS to form the fluorescent product 2,7-dichlorofluorescein (DCF) [18]. The intracellular fluorescence intensity of DCF is proportional to the amount of ROS generated by the cells [19]. After the indicated treatment, the cells were incubated with 10 μM of H2DCFDA for 30 min and then cells were harvested and resuspended in PBS (10^6 cells/ml). The fluorescence intensity of intracellular DCF (excitation 488 nm, emission 530 nm) was measured using FACScan. All the data analyses were performed using FlowJo analysis software, version 6.0 (Tree Star).

2.4. Western blot and luciferase activity assay

For western blots, after desired treatments as specified in Section 3, cells were washed twice with PBS, lysed in

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