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Antitumor activity of ginsenoside Rd in gastric cancer via up-regulation of Caspase-3 and Caspase-9

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Ginsenoside Rd (GS-Rd), isolated from the Chinese traditional herbal medicine *Panax ginseng*, is used for the treatment of cardiovascular diseases, inflammation, different body pains, and trauma. Caspase-3 and Caspase-9 belong to cysteine aspartic acid specific protease (Caspase) family that plays an important role in apoptosis progression of cancers. In the present study, we investigated the anti-tumor effect of GS-Rd by MTT assay, colony formation assessment, flow cytometry, and Western blotting. Our results revealed that ginsenoside Rd significantly inhibits human gastric cancer (GC) growth and cell proliferation. Flow cytometer analysis showed that the GS-Rd could significantly induce apoptosis and arrest the G0/G1 phase in GC cells. Further, GS-Rd was found to increase the ratio of Bax/Bcl-2 and the expression of Caspase-3 and Caspase-9, respectively, and to decrease the expression of Cyclin D1. Taken together, our study suggests that GS-Rd significantly inhibits GC cell proliferation, induces cell apoptosis through increase the expression of Caspase-3, Caspase-9, and the ratio of Bax/Bcl-2. GS-Rd also induces cell cycle arrest at G0/G1 phase by down-regulation Cyclin D1. Thus, GS-Rd could serve as a lead to develop novel therapeutic agents to against human gastric cancer.

1. Introduction

Based on GLOBOCAN estimates, about 18.1 million new cancer cases and 9.6 million deaths occurred in 2018 worldwide (Bray et al. 2018). Gastric cancer (GC) is the third leading cause of cancer death globally with an estimated 1,000,000 new cases and 783,000 deaths in 2018, making it the fifth most frequently diagnosed cancer (Bray et al. 2018; Okuno et al. 2019). The development of GC is a complex, multistep process involving multiple epigenetic and genetic changes to cell-cycle regulators, oncogenes, apoptosis protein, and signaling molecules, etc (Cervantes et al. 2007; Rocken and Warneke 2012; Yakirevich and Resnick 2013). Many studies have revealed that the high mortality rate of gastric cancer is related to the lack of an effective therapy (Wang et al. 2018). Many conventional therapy options have been developed for the treatment of gastric cancer, including surgery, chemo- and radiation therapy and combination treatment. The most effective medical treatment is surgical removal of the tumor in the early stages (Su et al. 2014). Therefore, the study of GC mechanisms is necessary to identifying the diagnostic makers for the early detection and targeted treatment of GC.

Ginsenoside, extracted from the Chinese traditional herbal medicine *Panax ginseng*, has been known to exhibit numerous pharmacological efficacies including anticancer, anti-inflammatory, antidiabetic, antiaging, promote immune response and liver functions and protective effects against Alzheimer's disease (Wang et al. 2016; Mohanan et al. 2018). More than 150 ginsenosides have been identified; ginsenoside Rd (GS-Rd) has attracted increasing attention (Christensen 2009; Zhang et al. 2017). GS-Rd mainly displays a remarkable neuroprotective and cardioprotective action, and can also attenuate myocardial ischemia-reperfusion injury (Ye et al. 2009; Zhang et al. 2014; Zeng et al. 2015; Xie et al. 2016). Furthermore, increasing evidence indicates that GS-Rd has significant effects of anti-proliferation/pro-apoptosis on diverse human cancer, such as breast, liver, and cervical cancer *in vitro* (Yang et al. 2006; Lee et al. 2009; Kim 2013). GS-Rd also inhibits mouse mammary carcinoma 4T1 cells *in vivo* (Wang et al. 2016). The aim of this study was to investigate the anti-tumor role of GS-Rd in GC, and determine whether GS-Rd can inhibit GC cells growth, induce GC cells apoptosis and cycle arrest *in vitro*.

2. Investigations and results

2.1. GS-Rd inhibits GC cell proliferation and colony formation in vitro

The effect of GS-Rd on GC cell proliferation was analyzed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The results showed that the proliferation rate of SGC-7901 and MKN-45 cells was significantly inhibited ($P < 0.05$) in a time- and dose-dependent manner (Fig. 1). IC_{50} values for SGC-7901 and MKN-45 cells were 86.96±0.23 μg/mL and 71.70±2.16 μg/mL at 48 h. SGC-7901 and MKN-45 cells colony formation were observed. The colony-forming efficiencies were significantly reduced by GS-Rd in both SGC-7901 and MKN-45 cells (Fig. 2). These findings suggest that GS-Rd suppresses the proliferation of GC cells *in vitro*.

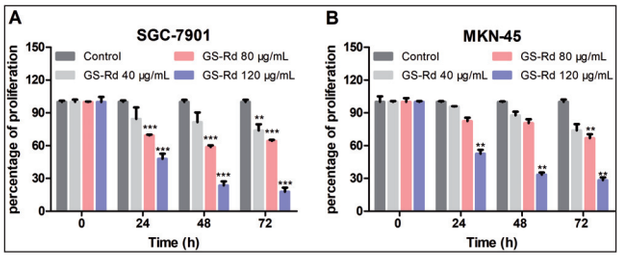

Fig. 1: Ginsenoside Rd (GS-Rd) inhibits human gastric cancer (GC) cell proliferation. (A) SGC-7901 proliferation was analyzed by MTT after treatment with GS-Rd. (B) MKN-45 proliferation was analyzed by MTT after treatment with GS-Rd.

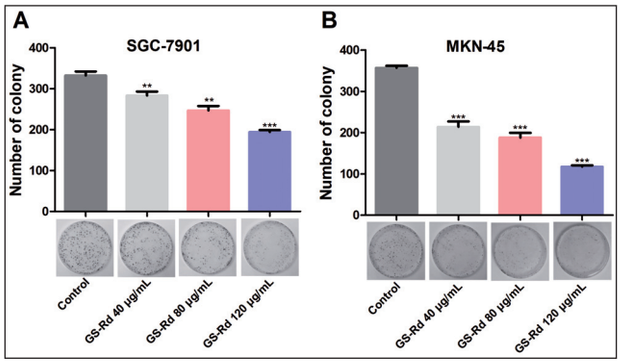

Fig. 2: Representative results of colony formation of control, 40 μg/mL, 80 μg/mL, and 120 μg/mL SGC-7901 and MKN-45 cells. (A) The effects of GS-Rd on colony formation of SGC-7901. (B) The effects of GS-Rd on colony formation of MKN-45.

2.2. GS-Rd promotes cell apoptosis in vitro

Apoptosis is one of the programmed cell death (PCD) pathways. To further examine whether GS-Rd may induce apoptosis, we performed AnnexinV-FITC/PI staining assays and flow cytometry analysis. Our results demonstrated that GS-Rd induced cell apoptosis in both SGC-7901 and MKN-45 cells (Fig. 3), and cell apoptosis was higher in the treatment groups than control group ($P < 0.05$).

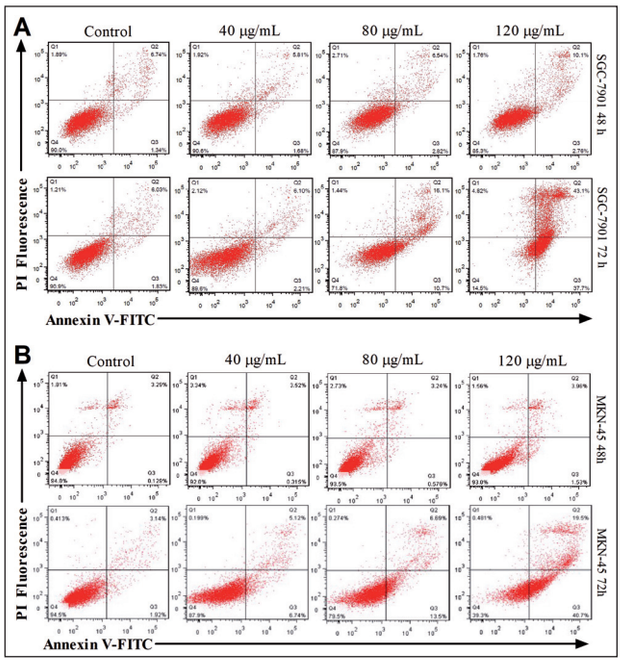

Fig. 3: The impact of GS-Rd on the cell apoptosis. The cells were harvested and processed for apoptosis assay using the Annexin V/PI staining. (A) GS-Rd induced apoptosis of SGC-7901. (B) GS-Rd induced apoptosis of MKN-45.

2.3. GS-Rd induces cell cycle arrest at G0/G1 phase

To explore the mechanism by which GS-Rd inhibits GC cell proliferation, cell cycle analysis was also performed to examine whether SGC-7901 and MKN-45 cells were arrested in a specific phase of the cell cycle. The results in Fig. 4 indicated that GC cells had statistically more G0/G1 phase cells in the GS-Rd treatment groups as compared to control cells ($P < 0.05$).

2.4. GS-Rd induces the up-regulation of Caspase in GC cells

Because the cysteine aspartic acid specific protease (Caspase) family is included in the regulatory network of cell apoptosis, we further measured the expression levels of Caspase-3 and Caspase-9

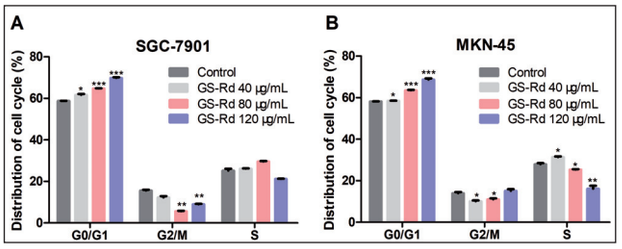

Fig. 4: The impact of GS-Rd on the cell cycle. Bar graphs show that cell cycle arrest at G0/G1 phase in GC cells. (A) The representative FACS plots displayed differences in cell cycle phase of SGC-7901 treated with GS-Rd or vehicle (PBS) for 48. (B) The representative FACS plots displayed differences in cell cycle phase of MKN-45 treated with GS-Rd or vehicle (PBS) for 48.

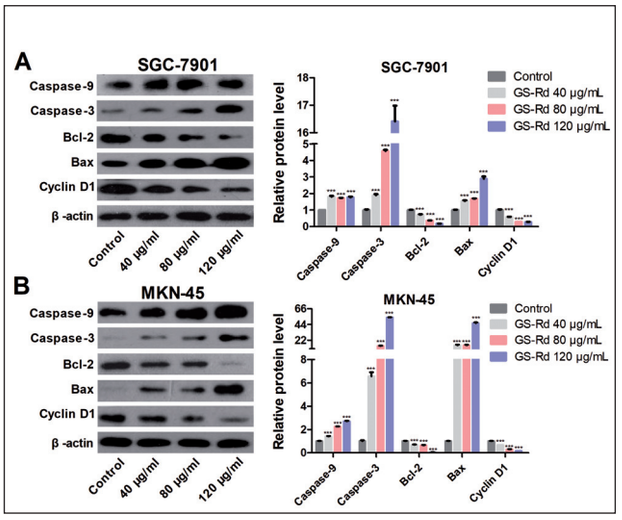

Fig. 5: The expression of Bax, Bcl-2, Cyclin D1, Caspase-3 and Caspase-9 were detected. (A) Protein expression of SGC-7901 was assessed by Western blot analysis after treatment with GS-Rd 48 h. (B) Protein expression of MKN-45 was assessed by Western blot analysis after treatment with GS-Rd 48 h.

in SGC-7901 and MKN-45 cells through Western blot. From the results in Fig. 5, we could infer that GS-Rd increased the expression of Caspase-3 and Caspase-9, while it inhibited that of Cyclin D1, and the ratio of Bax/Bcl-2 rose with dose increasing.

3. Discussion

GC is difficult to control clinically; thus, it is important to develop novel and effective agents for GC patients. The precise molecular underlying gastric tumorigenesis remains poorly understood, and improved knowledge will certainly bring new treatment options. GS-Rd, an extract obtained from *Panax ginseng*, has shown antitumor activity in various types of human cancers (Yang et al. 2006; Lee et al. 2009; Kim 2013). However, the number of studies investigating its role in GC is very limited.

Resisting proliferation is an important cutting-in point for development of anticancer drugs, so we determined SGC-7901 and MKN-45 cells viability using MTT assay at different times and concentrations. Our results shown that GS-Rd has a significant time- and dose-dependent manner on two gastric cancer cells, and most pronounced inhibition of GS-Rd accumulation occurring at concentration of 120 μg/mL. We also observed the influence of GS-Rd to colony formation and found that the colony-forming efficiencies were significantly reduced.

There are two important pathways that include apoptosis and programmed necrosis (Wallach et al. 2016). Apoptosis is characterized by a series of distinct biochemical and morphological changes, including increase in reactive oxygen species (ROS) level, activation of Caspases, cell shrinkage, chromatin condensation (Mao et al. 2014; Su et al. 2015). Mitochondrial dysfunction is one of the most significant events, loss of mitochondrial transmembrane potential

(MTP) elicits the release of cytochrome c from mitochondria to cytosol, thereby activating the caspase-cascade system (Green and Reed 1998; Thornberry and Lazebnik 1998). Apoptotic cells are featured by cell shrinking and fragmenting into apoptotic bodies and are phagocytized before undergoing membrane damage, leading to non-lysis cell death. The necrotic cells swell and release inflammatory factors, leading to lysis cell death (Wallach et al. 2016; Shi et al. 2017). In the present study, we applied Annexin V/PI double staining to investigate effects of GS-Rd on the apoptosis of human GC cells at 48 h and 72 h. Annexin V is a member of the annexin family of intracellular proteins that binds to phosphatidylserine (PS) in a calcium-dependent manner. Propidium iodide is a fluorescent dye that binds to DNA. Annexin V/PI double staining was used to distinguish between the necrotic and apoptosis cells. Flow cytometry analysis indicated that GS-Rd induced two GC cells apoptosis at different concentration, high-dose group (120 μg/mL) significant difference model group. Our findings also showed that late apoptosis cells were greatly increased, and percentages of apoptosis SGC-7901 and MKN-45 were 52.40±1.50 ($P < 0.01$) and 55.25±1.35 ($P < 0.001$).

Combined with cell proliferation assay results, it can be assumed that GS-Rd inhibits cell proliferation mainly caused by apoptosis. Many studies have revealed that apoptosis can be induced by outside (extrinsic) factors, such as TRAIL and FAS ligand, which lead to activation of Caspase-8, which triggers activation of executioner Caspases-3 and Caspases-7 leading to cell death (Julien and Wells 2017). Apoptosis can also be induced by internal (intrinsic) factors, such as DNA damage, leading to activation of caspase-9 through mitochondrial cytochrome c release, Apaf1 oligomerization and ultimately activation of Caspases-3 and Caspase-7 (Julien and Wells 2017). So we measured the expression levels of Caspase-3 and Caspase-9 in SGC-7901 and MKN-45 cells through Western blotting after GS-Rd treatment for 48 h. From our results, we could find that GS-Rd increases Caspase-3 and Caspase-9 expression. Bcl-2 family also plays a significant role in apoptosis and regulates the mitochondrial cell death pathway. In particular, the stoichiometries of Bax (pro-apoptosis member) and Bcl-2 (anti-apoptosis member) are critical for cytochrome C release and following downstream Caspase activation (Su et al. 2014; Teng et al. 2016). Therefore, we continued to detect the expression of Bcl-2 family in SGC-7901 and MKN-45 cells through Western blot after GS-Rd treatment 48 h. We found that the ratio of Bax/Bcl-2 rose with dose increasing.

Cell cycle arrest is another crucial mechanism underlying the regulation of tumor growth. Cell cycle progression comprises a series of events that take place during cell proliferation, including the G0, G1, S, G2 and M phase of the cell cycle (Meng et al. 2019). In the present study, cell cycle was observed by flow cytometry after GS-Rd treatment 48 h. Our date revealed that exposure of GC cells to GS-Rd arrested tumor cells at the G0/G1 phase of the cell cycle. Cyclins are a family of proteins whose name derives from their cyclic expression and quantitatively fluctuating pattern throughout the cell cycle. Cyclin synthesis and activation of their corresponding cyclin dependent kinases (CDKs) in the different phases of the cell cycle sequentially coordinate DNA replication and cell division (Ramos-Garcia et al. 2017). Cyclin D1 plays a key role in cell biology, including cell proliferation and growth regulation, mitochondrial activity modulation, DNA repair, and cell migration control (Ramos-Garcia et al. 2017). Cyclin D1 promotes cell cycle progression through the restriction point (R) during G1 phase, an event essential in G1/S transition. We further detected the expression of Cyclin D1 in SGC-7901 and MKN-45 cells, and the results have shown that GS-Rd could downregulate Cyclin D1. This indicates that GS-Rd induces cell cycle arrest at the G0/G1 phase by decreasing the expression of Cyclin D1, and verified the results of flow cytometry.

In conclusion, GS-Rd effectively inhibited SGC-7901 and MKN-45 cells proliferation may through upregulation Bax and downregulation Bcl-2 cause ratio of Bcl-2/Bax decrease, which may lead to reduction of mitochondrial transmembrane potential. And then, following downstream Caspase was activated to induce cells apoptosis. Meanwhile, our data demonstrate that the antitumor effects of GS-Rd in SGC-7901 and MKN-45 cells are also mediated by suppressing Cyclin D1 expression to induce GC cells cycle arrest at the G0/G1 phase. Based on the findings of the present study, GS-Rd may be a promising novel agent for the treatment of GC cells. However, the effectiveness of GS-Rd must be further assessed in a clinical trial.

4. Experimental

4.1. Cell lines and culture condition

Human GC cells, MKN-45 and SGC-7901 (obtained from the Type Culture Collection of the Chinese Academy of Science, Shanghai, China) were used and routinely maintained in RPMI-1640 medium (purchased from Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin sodium and 100 mg/mL streptomycin at 37 °C in 5% CO_2 humidified incubators.

4.2. Cell proliferation and colony formation assay

Cell proliferation was analyzed by MTT assay. SGC-7901 and MKN-45 cells were plated at 2×10^3 cells/well in 96-well plates and incubated for 24 h, and were then treatment with various concentrations (40 μg/mL, 80 μg/mL or 120 μg/mL) of GS-Rd. The day after treatment, MTT was performed for 4 h, and the absorbance at 570 nm was measured at 24 h, 48 h or 72 h. For colony formation assay, SGC-7901 and MKN-45 cells (400 cells/well) were seeded in 60 mm dishes and incubated for 24 h. RPMI medium with FBS was replaced with 5 mL fresh medium, containing GS-Rd (40 μg/mL, 80 μg/mL or 120 μg/mL) or PBS (control) and incubated at 37 °C for 15 days. Dishes were gently washed with PBS twice and fixed with fixative (7:1 ratio of methanol to glacial acetic acid) for 30 min, and then stained with 0.1% crystal violet for 30 min.

4.3. Flow cytometric analysis of apoptosis

Cell apoptosis was detected according to the instructions provided by the kits. Briefly, SGC-7901 and MKN-45 cells were seeded in 6-well plates (1×10^5 cells/well) and grown for 24 h, then incubated with GS-Rd (40 μg/mL, 80 μg/mL or 120 μg/mL) or vehicle (PBS) for 48 or 72 h. The cells were harvested using 0.25% trypsin without ethylene diaminete traacetic acid (EDTA), washed twice with cold PBS and then resuspended in 300 μL cold binding buffer, both 3 μL Annexin V-FITC and 2.25 μL Propidium Iodide (PI) were added to stain at room temperature in dark. The percentage of apoptotic cells was analyzed by flow cytometry.

4.4. Flow cytometric cell cycle distribution assay

For cell cycle analysis, SGC-7901 and MKN-45 cells (5×10^4 cells/well) were seeded in 60 mm culture dishes and grown for 24 h, then incubated with GS-Rd (40 μg/mL, 80 μg/mL or 120 μg/mL) or vehicle (PBS) for 48 h. The cells were harvested using 0.25% trypsin without EDTA, washed twice with cold PBS and then fixed in 700 μL 75% ethyl alcohol at -20 °C overnight. The next day, cells were washed twice with PBS and PI was added, and cell cycle was observed by flow cytometry.

4.5. Western blotting

SGC-7901 and MKN-45 cells (1×105 cells/well) were seeded in 10 cm culture dishes and grown for 24 h, then treatment with 0 μg/mL (control), 40 μg/mL, 80 μg/mL or 120 μg/mL GS-Rd for 48 h. Cells lysed in RIPA buffer containing PMSF for western blotting. Protein was separated through 12% sodium dodecyl sulfate polyacrylamide gels electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk and incubated with the primary antibodies: Bax (1:1000; Cell Signaling Technology, USA), Bcl-2 (1:1000; Cell Signaling Technology, USA), Caspase-3 (1:1000; Cell Signaling Technology, USA), Caspase-9 (1:1000; Cell Signaling Technology, USA), Cyclin D1 (1:1000; Cell Signaling Technology, USA), or β-actin (1:1000; Cell Signaling Technology, USA), overnight. After washing, blots were incubated with secondary antibodies. The protein concentrations of Caspase-9 and Caspase-3 in the Human GC cells of each group were detected. The protein concentrations of Bax and Bcl-2 were also determined.

4.6. Statistical analysis.

All dates are expressed as means±standard deviation (SD). Statistically significant differences are represented by * in which $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Conflicts of interest: None declared.

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