Inhibition of multiple myeloma cell proliferation by ginsenoside Rg3 via reduction in the secretion of IGF-1

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Abstract. Ginsenoside Rg3 (Rg3) is one of the primary constituents isolated from ginseng, and has been found to exhibit cytotoxic effects against cancer cells. The present study aimed to investigate the effects of Rg3 on human multiple myeloma cell proliferation and apoptosis, and to examine its underlying molecular mechanisms. Cell viability was detected using a Cell Counting kit-8 assay, and cell cycle arrest and cell apoptosis were analyzed using flow cytometry. In addition, the expression levels of cell cycle-associated markers and apoptosis-associated proteins, and the release of cytochrome C were determined using western blot analysis. The effects of Rg3 on the insulin-like growth factor (IGF)-1/AKT/mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase signaling pathways were also investigated using western blot analysis. The results showed that Rg3 inhibited cell viability in U266, RPMI8226 and SKO-007 cells in a time- and dose-dependent manner, and caused cell cycle arrest in the G1 phase by regulating the cyclin-dependent kinase pathway. Furthermore, Rg3 induced multiple myeloma cell apoptosis, and was involved in B cell lymphoma-2 (Bcl2)/Bcl2-associated X protein imbalance, caspase activation and the release of cytochrome C from the mitochondria into the cytoplasm. Mechanistically, it was found that the inhibitory effects of Rg3 on multiple myeloma cell proliferation were essential for secretion of IGF-1 and inactivation of the Akt/mTOR pathway. Collectively, these findings demonstrated that Rg3 effectively inhibited cell proliferation and induced apoptosis of multiple myeloma cells. These data broaden the clinical investigation of Rg3 in the treatment of multiple myeloma, associated with the inactivation of IGF-1/AKT/mTOR signaling.

Introduction

Multiple myeloma is a clonal malignancy of plasma cells characterized by bone destruction, monoclonal proteins, hypercalcemia, excess bone marrow plasma, renal damage and immunodeficiency (1,2). The incidence of multiple myeloma varies globally, from 1/100,000 individuals in China to ~4/100,000 individuals in developed countries (3). Patients with multiple myeloma will often develop recurrence or an increased susceptibility to fungal, viral and bacterial infections, which are the major cause of multiple myeloma-associated mortality (4,5). The survival rates of patients with multiple myeloma can now exceed 10 years as a result of therapy comprising hematopoietic stem cell transplantation in combination with novel chemotherapeutic agents, including thalidomide, lenalidomide and bortezomib (6-8). However, chemotherapy often produces drug resistance and high levels of toxicity, therefore, developing a more effective agent remains a priority in the treatment of multiple myeloma.

Over previous decades, several natural products derived from plants have shown promising structures for the development of novel agents for use in cancer treatment. Ginsenosides, a traditional Chinese medicine, have been reported to exhibit antitumor properties (9,10). Ginsenoside Rg3 (Rg3), a monomer derived from heat-processed ginseng, has been found to have potent antitumor effects (11,12). Although Rg3 has been reported to inhibit cancer cell proliferation and induce cell death in melanoma (13), breast cancer (14), acute leukemia (15), glioma (16) and hepatocellular carcinoma (17), the activity of Rg3 against cell growth in multiple myeloma and its functional targets remain to be fully elucidated.

The objectives of the present study were to investigate the activity of Rg3 in inhibiting the growth of multiple myeloma cell lines, and to elucidate the underlying mechanisms. The results demonstrated the antiproliferative effect of Rg3 against multiple myeloma cells. In addition, the mechanism underlying the action of Rg3 was correlated with the inhibition of secretion of insulin-like growth factor (IGF)-1 and inactivation of the AKT/mammalian target of rapamycin (mTOR) signaling pathway. The present study indicates that Rg3 may be a potential clinical therapeutic agent for multiple myeloma.

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Materials and reagents. Ginsenoside Rg3 (Rg3) was purchased from Yatai Pharmaceuticals Co., Ltd (Jilin, China) with 98% purity, assayed using high-performance liquid chromatography (HPLC; Fig. 1A). The powder was dissolved in dimethyl sulfoxide (DMSO) in a stock concentration of 100 mg/ml. The final concentration of DMSO in the culture medium was ≤0.1%. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and phosphate-buffered saline (PBS) were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Antibodies obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) were as follows, all used at 1:500 dilution: Mouse anti-cyclin D1 monoclonal antibody (cat. no. sc-8396), mouse anti-p27 monoclonal antibody (cat. no. sc-393380), mouse anti-phosphorylated (phospho)-extracellular signal regulated kinase (Erk)1/2 monoclonal antibody (cat. no. sc-7383), rabbit anti-Erk1/2 polyclonal antibody (cat. no. sc-298238), mouse anti-phospho-c-Jun N-terminal kinase (JNK) monoclonal antibody (cat. no. sc-6254), mouse anti-JNK monoclonal antibody (cat. no. sc-7345), rabbit anti-phospho-p38 polyclonal antibody (cat. no. sc-17852-R), mouse anti-phospho-38 monoclonal antibody (cat. no. sc-81621) and rabbit anti-IGF-1 polyclonal antibody (cat. no. sc-9013). Antibodies obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA) were as follows, all used at 1:1000 dilution: Mouse anti-B cell lymphoma-2 (Bcl-2) monoclonal antibody (cat. no. 15071), rabbit anti-Bcl-2-associated X protein (Bax) polyclonal antibody (cat. no. 5023), mouse anti-cytochrome C monoclonal antibody (cat. no. 12963), rabbit anti-cytochrome C oxidase (Cox) IV polyclonal antibody (cat. no. 4850), mouse anti-caspase-9 monoclonal antibody (cat. no. 9508), mouse anti-caspase-8 monoclonal antibody (cat. no. 9746), rabbit anti-caspase-3 polyclonal antibody (cat. no. 9665), rabbit anti-phospho-AKT polyclonal antibody (cat. no. 5012), mouse anti-AKT monoclonal antibody (cat. no. 2920), rabbit anti-phospho-mTOR polyclonal antibody (cat. no. 2976) and mouse anti-mTOR monoclonal antibody (cat. no. 2983). IGF-1, rabbit polyclonal anti-retinoblastoma (Rb; cat. no. SAB4502589), mouse monoclonal anti-phospho-Rb (cat. no. R6878) and rabbit polyclonal anti-GAPDH (cat. no. G8795) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used at 1:1000 dilution. The Cell Counting kit-8 assay (CCK-8), radioimmunoprecipitation assay (RIPA) lysis buffer, bicinchoninic acid (BCA) kit, enhanced chemiluminescence (ECL) system and Fluorescein isothiocyanate (FITC)-Annexin V Apoptosis Detection kit were obtained from Beyotime Institute of Biotechnology (Jiangsu, China).

Cell culture. The U266, RPMI8226 and SKO-007 human multiple myeloma cell lines, were obtained from America Type Culture Collection (Rockville, MD, USA) and maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in a 5% CO₂ atmosphere.

Cell viability assay. Multiple myeloma cells were seeded in 96-well plates at a density of 5x10⁴ cells/well overnight and then treated with Rg3 at different concentrations (0, 20, 40, 60, 80 and 100 µg/ml), and for different durations (6, 12, 24 and 48 h), as indicated. Subsequently, fresh medium containing 10 µl CCK-8 reagent was added, followed by incubation at 37°C for 2 h. The absorbance of each well was measured at 450 nm on a plate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Western blot analysis. The cells were washed with cold PBS and lysed with lysis buffer. The protein concentration was determined using the BCA kit. Equal quantities (40 µg) of protein were separated on 12% SDS-PAGE gels (GenScript, Piscataway, NJ, USA) and then transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h, and then probed with appropriate primary antibodies overnight at 4°C, followed by blotting with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (cat. no. A0216) or HRP-labeled goat anti-rabbit IgG (cat. no. A0208) secondary antibodies (1:1,000; Beyotime Institute of Biotechnology) at room temperature for 1 h. The target bands were detected using the ECL system, and the band intensity was determined using ImageJ software (version 1.41; NIH, Bethesda, MD, USA).

Cell cycle analysis. The cells were harvested by centrifugation and processed for cell cycle analysis, as described previously (3). Briefly, the cells were digested with 0.25 g/l trypsin and harvested by centrifugation at 1,000 x g for 10 min at room temperature. Following incubation with 70% ethanol at -20°C for 15 min, the cells were stained with 20 mg/ml propidium iodide (PI) and incubated for 30 min at room temperature. The cells were analyzed for DNA content using FACScanibur flow cytometry (BD Biosciences, San Jose, CA, USA). The percentages of cells containing different DNA contents were quantified using CellQuest software (version 5.1; BD Biosciences).

Apoptosis detection. The cellular apoptotic ratios were detected with the FITC-Annexin V Apoptosis Detection kit using flow cytometry. Briefly, the cells were trypsinized and harvested by centrifugation. The cell pellets were re-suspended in a binding buffer of Annexin V-FITC and PI at room temperature in the dark for 15 min. The apoptotic cells were counted using flow cytometry (BD Biosciences), with the percentage of apoptotic cells expressed as the FITC/PI ratio.

Isolation of mitochondria. The isolation of mitochondrial and cytoplasmic proteins was performed using a Mitochondria Isolation kit (Thermo Fisher Scientific Inc.), according to the manufacturer's protocol. The cytosolic and mitochondrial fractions were analyzed using western blot analysis. Cox IV was used as an internal control for the mitochondrial fraction.

ELISA assay. The concentrations of IGF-1 were determined using a Human IGF-1 Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's protocol. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Tek Instruments, Inc.).

Statistical analysis. All data are presented as the mean ± standard error of the mean, and the n value indicates the number of independent experiments. Data were statistically analyzed using Student's t-test on GraphPad Prism 5.0 software.
Results

Effects of Rg3 on the viability of multiple myeloma cell lines. The uncontrolled cell proliferation of cancer cells is vital in the progression of cancer, therefore, the present study evaluated the effects of Rg3 on the viability of multiple myeloma cells. As shown in Fig. 1B, treatment with Rg3 inhibited the viability of the U266 cells in a time- and dose-dependent manner. The inhibition of cell viability in the U266 cells following treatment with Rg3 for 48 h reached the maximal level at 80 µg/ml, and the half maximal inhibitory concentration (IC50) value was 47.5 mg/l. In addition to U266, two other multiple myeloma cell lines, RPMI8226 and SKO-007, were also included in the present study to examine these effects. Rg3 had a more potent effect in inhibiting the viability of the RPMI8226 (IC50=36.8 µg/ml) and SKO-007 (IC50=31.5 µg/ml) cells, compared with the U266 cells (Fig. 1C and D).

Rg3 arrested the cell cycle of multiple myeloma cells. Flow cytometric cell cycle analysis was performed using PI staining to determine the effect of Rg3 on cell cycle progression. Treatment of the U266 cells with 20, 40 and 80 µg/ml Rg3 increased the percentage of the cell population in the G1 phase and decreased the percentage in the S phase. However, Rg3 had no effect on the G2/M phase (Fig. 2A and B). The effect of Rg3 on cell cycle progression was also examined in the SKO-007 cells (Fig. 2C and D). Similarly, the cell cycle of the SKO-007 cells was arrested in the G1 phase by Rg3, indicating that Rg3 may inhibit multiple myeloma cell proliferation through suppressing the cell cycle transition from the G1 to the S phase. The cell cycle transition between the G1 and S phase is strictly regulated by the balance of cyclins and cyclin-dependent kinase inhibitors (18,19). Therefore, to further investigate the mechanisms underlying how Rg3 arrested G1/S transition, the expression levels of proteins regulating cell cycle progression in U266 cells, including cyclin D1, p27 and phospho-Rb, were determined. The results of the western blot analysis showed that Rg3 decreased the protein expression of cyclin D1 and phosphorylation of Rb, and increased the expression of p27 in a dose-dependent manner (Fig. 2E and F).

Rg3 induces multiple myeloma cells apoptosis via the mitochondria-dependent pathway. To examine the effect of Rg3 on cell survival, its effects on cell apoptosis were determined. Annexin V-FITC/PI staining followed by flow cytometric analysis revealed that treatment of the U266 cells with 20, 40 and 80 µg/ml Rg3 for 48 h significantly increased the percentage of apoptotic cells in the in U266 cell population (Fig. 3A and B). Similar results were observed in
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Bcl-2 and Bax are anti-apoptotic and pro-apoptotic proteins, and the ratio of Bcl-2/Bax appears to be a determinant of cell survival and death. To understand the mechanism by which Rg3 induces cell apoptosis, the expression levels of Bcl-2 and Bax, and the ratio of Bcl-2 to Bax were measured. The results

Figure 2. Rg3 arrests cell cycle at the G1/S transition. Multiple myeloma cells were incubated with different concentrations of Rg3 (20, 40 and 80 µg/ml) for 48 h. Cell cycle was analyzed using flow cytometry. (A) Representative images of cell cycle distribution in the U266 cells, with (B) statistical analysis of the percentages of cells in the G0/G1, S and G2/M phases. (C) Cell cycle distribution of the SKO-007 cells with (D) statistical analysis of the percentages of cells in the G0/G1, S and G2/M phases. Expression levels of cycle-associated proteins were examined using western blot analysis in U266 cells. (E) Representative images of the western blot are shown, with the results of (F) densitometric analysis. Data are expressed as the mean ± standard error of the mean (n=6). 0 µg/ml indicates the dimethyl sulfoxide vehicle, *P<0.05 and **P<0.01, vs. 0 µg/ml group. Rg3, ginsenoside Rg3; Rb, retinoblastoma; p-, phosphorylated.
of the western blot analysis showed that Rg3 treatment markedly decreased the protein expression of Bcl-2 and increased the protein expression of Bax in the U266 cells, resulting in a further decrease in the Bcl-2/Bax ratio (Fig. 3C and D). The
mitochondria-dependent signaling pathway is critical for cell apoptosis. The release of cytochrome C from the mitochondria into the cytoplasm triggers the downstream apoptotic signal, consequently resulting in cell apoptosis (20). Therefore, the present study examined the release of cytochrome C. As expected, Rg3 treatment caused a significant decrease in the protein expression of cytochrome C in the mitochondria, and an increase in the cytoplasm (Fig. 3E and F). Cytochrome C release sequentially activates downstream apoptosis-associated proteins, including caspsases. Western blot analysis demonstrated that Rg3 increased the protein expression levels of cleaved caspase-9, caspase-8 and caspase-3 (Fig. 3G and H). Collectively, these data suggested that mitochondrial dysfunction may underlie, at least partially, the enhanced effect of Rg3 on myeloma cell apoptosis.

**AKT/mTOR, but not MAP kinase, signaling is involved in the action of Rg3 on multiple myeloma cell proliferation and survival.** The phosphorylation of AKT and downstream mTOR has been reported to be involved in the progression of several types of malignancy, including multiple myeloma (3,21). To clarify the signal transduction pathways by which Rg3 exerts its antitumor effects, the present study first examined the activation of the AKT/mTOR pathway. As shown in Fig. 4A and B, the phosphorylation of AKT and its downstream protein, mTOR, was attenuated by Rg3 treatment in a dose-dependent manner. MAP kinase signaling is also a regulator of cell cycle progression and tumorigenesis (22,23). However, Rg3 treatment did not alter the phosphorylation of Erk1/2, JNK or p38 (Fig. 4C and D). These data excluded the possibility that MAP kinase signaling was involved in the effects of Rg3 on multiple myeloma cell proliferation and survival.

Rg3 attenuates AKT/mTOR activation via inhibiting the secretion of IGF-1. IGF-1 is an important pathway of AKT/mTOR (24). Therefore, the present study investigated whether Rg3 affects this pathway. The results of the western blot analysis showed that Rg3 had no effect on the protein expression of IGF-1 (Fig. 5A). However, the secretion of IGF-1 was markedly decreased following Rg3 treatment (Fig. 5B).
In addition, treatment with IGF-1 reversed the Rg3-induced inactivation of the AKT/mTOR pathway, as evidenced by the significant restoration in AKT and mTOR phosphorylation (Fig. 5C and D). The present study further examined whether IGF-1 was involved in the effects of Rg3 on cell proliferation and survival. The results of the CCK-8 assay revealed that the reduction in cell viability induced following Rg3 treatment was gradually inhibited by IGF-1 in a dose-dependent manner (Fig. 5E). As expected, Rg3-induced cell apoptosis was almost eliminated by the addition of IGF-1 (Fig. 5F).
Discussion

Several experimental and clinical studies have been performed on the effects of Rg3 on cancer. These studies have identified Rg3 as being effective in the treatment of certain types of cancer. Shen Yi capsule has been approved by the China Food and Drug Administration, with Rg3 as the active pharmaceutical ingredient, for clinical use in cancer treatment (25). However, the specific functions of Rg3 in the treatment of multiple myeloma remain to be fully elucidated. The present study provided evidence of the anti-multiple myeloma activity of Rg3 in cultured cells. The data revealed that Rg3 inhibited the proliferation of the U266, RPMI8226 and SKO-007 human multiple myeloma cell lines, and induced cellular apoptosis, which were consistent with previous studies (26,27).

The data obtained in the present study revealed that the anti-proliferative effect of Rg3 in multiple myeloma cells was due to inhibition of the cell cycle transition between the G1 and S phases. Cell cycle progression is strictly regulated by cyclin-dependent kinases 4 and 6, which are activated by cyclin D1, but attenuated by p27 (28). In the present study, it was found that the expression of cyclin D1 and the phosphorylation of cyclin-dependent kinases 4 and 6 can lead to the phosphorylation of Rb and consequently promote the transition between the G1 and S phase (3). In the present study, it was found that the expression of cyclin D1 and the phosphorylation of Rb were attenuated by Rg3, whereas the expression of p27 was elevated. The apoptotic process is usually associated with the imbalance between the levels of Bcl-2 and Bax (29). During apoptotic stimulation, the increased expression of Bax enhances membrane permeability, which results in the release of cytochrome C from the mitochondria into cytoplasm, and activates a family of proteases, including caspase-9, caspase-8 and caspase-3, driving the cell toward apoptosis (30,31). The present study showed that Rg3 treatment induced the release of mitochondrial cytochrome C into the cytoplasm, increased the protein expression levels of cleaved caspase-8, caspase-8 and caspase-3, and decreased the Bcl-2/Bax ratio. These data indicated that Rg3-induced apoptosis in multiple myeloma cells was mitochondria-dependent.

A previous study demonstrated that Rg3 induced U266 human multiple myeloma cell apoptosis through activation of the Bax protein (26). In addition, Song et al (27) reported that inhibition of the secretion of vascular endothelial growth factor may contribute to the anti-proliferative effects of Rg3. However, apart from these reports, there is no more information regarding the mechanisms underlying the functions of Rg3 in inhibiting the growth of multiple myeloma cells. Deregulation of the Akt/mTOR and MAPK pathway is a common event in human cancer, and is crucial for tumor cell proliferation, cell cycle transition and apoptosis (32,33). Notably, the results of the present study provided the first evidence, to the best of our knowledge, that Rg3 affected cell proliferation and survival, predominantly via Akt/mTOR pathway, and less via the MAPK pathway. The detailed mechanisms to explain why Rg3 is linked less with MAPK and more with Akt/mTOR remain to be elucidated, and further investigations are required. In addition, the abnormal secretion of IGF-1 has been documented to be a tumorigenic factor (21). In the present study, it was found that Rg3 inhibited the secretion of IGF-1, but did not alter its expression. IGF-1 is essential for the inhibitory effect of Rg3 on activation of the AKT/mTOR pathway, suggesting that Rg3 mediated cell proliferation and survival through IGF-1/AKT/mTOR signaling.

In conclusion, the present study demonstrated that Rg3 acted on the IGF-1/AKT/mTOR signal transduction pathway to inhibit multiple myeloma cell proliferation. These results provided evidence to support further investigation for the development of Rg3 as a clinical drug candidate in the treatment of multiple myeloma.

References


