Effects of ginsenoside Rh2 on growth and migration of pancreatic cancer cells

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AIM: To investigate the effects of ginsenoside Rh2 on the human pancreatic cancer cell line Bxpc-3.

METHODS: The human pancreatic cancer cell line Bxpc-3 was cultured in vitro and treated with or without ginsenoside Rh2. Growth rates for Bxpc-3 cells were assessed by methyl thiazolyl tetrazolium (MTT) and colony formation assays. Cell cycle changes were analyzed by flow cytometry and Hoechst 33258 fluorescence staining. A scratch assay and a Matrigel invasion assay were used to detect cell migration and invasion. Expression of Bax, Bcl-2, survivin, cyclin D1, matrix metalloproteinase (MMP)-2, MMP-9, cleaved caspase-3, caspase-8, and caspase-9 mRNAs were determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Bax, Bcl-2, survivin, cyclin D1, cleaved caspase-3, caspase-8 and caspase-9 protein levels were examined by western blotting. Expression of MMP-2 and MMP-9 proteins in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA).

RESULTS: Rh2 significantly inhibited Bxpc-3 cell proliferation in a dose- and time-dependent manner, as evaluated by the MTT (P < 0.05) and colony formation assays (P < 0.05). Compared to the control group, Rh2 significantly increased the percentage of Bxpc-3 cells in the G0/G1 phase from 43.32% ± 2.17% to 71.32% ± 1.16%, which was accompanied by a decrease in S phase (from 50.86% ± 1.29% to 28.48% ± 1.18%) and G2/M phase (from 5.81% ± 1.19% to 0.20% ± 0.05%) in a dose-dependent manner (P < 0.05), suggesting that Rh2 arrested cell cycle progression at the G0/G1 phase, as measured by flow cytometry. Compared to the control group, cells treated with Rh2 showed significantly higher apoptosis ratios in a dose-dependent manner (percentage of early apoptotic cells: from 5.29% ± 2.28% to 38.90% ± 3.42% (F = 56.20, P < 0.05); percentage of late apoptotic cells: from 4.58% ± 1.42% to 36.32% ± 2.73% (F = 86.70, P < 0.05)). Rh2 inhibited Bxpc-3 cell migration and invasion, as detected by scratch wound healing assay and Matrigel invasion assay [percentages of scratch wound healing for 12 h, 24 h and 48 h (control vs experimental group): 37.3% ± 4.8% vs 18.30% ± 1.65%, 58.7% ± 3.5% vs 38.00% ± 4.09% and 93.83% ± 4.65% vs 65.50% ± 4.09%, respectively; t = 6.489, t = 6.656 and t = 7.926, respectively, P < 0.05; the number of cells invading at various concentrations (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L): 81.10 ± 9.55, 46.40 ± 6.95, 24.70 ± 6.88 and 8.70 ± 3.34, respectively (F = 502.713, P < 0.05)]. RT-PCR, western blotting or ELISA showed that mRNA and protein expression of Bax, cleaved caspase-3 and caspase-9 were upregulated (P < 0.05), while mRNA and protein expression of Bcl-2, survivin, cyclin D1, MMP-2 and MMP-9 were downregulated (P < 0.05).

CONCLUSION: Ginsenoside Rh2 inhibits proliferation, migration and invasion and induces apoptosis of the human pancreatic cancer cell line Bxpc-3.
INTRODUCTION

Pancreatic cancer (PC) is a disease with a high mortality rate and the 1-year survival rate is < 10%[1]. The successful surgical resection rate for PC is not high and current chemotherapy is not effective. Thus, there is an urgent need to develop novel treatment modalities. Ginseng is a traditional herbal medicine that is well known for its wide spectrum of pharmacological effects[2-4]. Ginsenoside is the main effective component of ginseng and has been widely used in oriental countries for thousands of years[5,6]. Recently, some experiments have demonstrated that ginsenoside has a wide variety of biological activities including immunomodulatory effects and anti-inflammatory and antitumor activity[5,7]. Ginsenoside Rh2 is a pure compound extracted from ginsenosides. Recently, researchers have found that ginsenoside Rh2 could inhibit growth of many kinds of cancer cells, including breast cancer, prostate cancer, hepatoma, gastric cancer and colon carcinoma[8-13]. Rh2 may play an antitumor role through the following mechanisms: (1) regulating tumor cells through the signaling pathway system, including the signaling pathway of protein kinase C, insulin-like growth factors, caspase family and Bcl-2 family; (2) affecting the activity of cell telomerase; (3) blocking anabolism and metabolism of important components in tumor cells; and (4) reversing abnormal differentiation and resistance of tumor cells.

However, to date, little is known about the role of ginsenoside Rh2 in PC. The purpose of this study was to investigate the effects of ginsenoside Rh2 on proliferation, apoptosis and migration of the human pancreatic cancer cell line Bxpc-3, and to explore the potential mechanisms of the effects.

MATERIALS AND METHODS

Materials

The human pancreatic cancer cell line Bxpc-3 was obtained from the Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. RPMI 1640 medium and fetal bovine serum were purchased from Gibco BRL (Gaithersburg, MD, United States). Ginsenoside Rh2 was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The drug was dissolved in dimethyl sulfoxide (DMSO) with a stock concentration of 40 mmol/L. The following materials were used: monoclonal antibodies to Bel-2, Bax, survivin, clyin D1, cleaved caspase-3, caspase-8 and caspase-9 (Santa Cruz Biotechnology, Santa Cruz, CA, United States), enzyme-linked immunosorbert assay (ELISA) kits for matrix metalloproteinase (MMP)-2 and MMP-9 (Boster Bioengineering Co., Wuhan, China), Annexin V-FITC Apoptosis Detection Kit and Cell Cycle Detection Kit (KeyGEN Biotech Co., Nanjin, Jiangsu, China), Hoechst 33258 staining kit (Beyotime Biotechnology Co., Jiangsu, China), Matrigel (BD Biosciences, United States) and 24-well invasion chambers (Corning-Costar, New York, United States).

Cell culture

Bxpc-3 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a 5% CO2 atmosphere.

Methyl thiazolyl tetrazolium assay

Cells were seeded on 96-well plates in triplicate. Following 24 h culture at 37 °C, the medium was replaced with fresh medium containing increasing concentrations of ginsenoside Rh2 (0 μmol/L, 10 μmol/L, 20 μmol/L, 35 μmol/L, 45 μmol/L, 55 μmol/L and 60 μmol/L) in a final volume of 200 μL. Cells were incubated at 37 °C for 24 h, 48 h and 72 h. Methyl thiazolyl tetrazolium (MTT) [20 μL, 2 mg/mL in phosphate-buffered saline (PBS)] was added and cells were incubated for a further 4 h. The medium was removed and 150 μL DMSO was added to each well. It was shaken mechanically for 10 min and the optical density was measured at 570 nm. The experiment was repeated three times.

Colony formation assay

Cells were plated in six-well plates at a density of 100 cells/well for 48 h, prior to the addition of various concentrations of Rh2 (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L). After 48 h treatment, the drug-containing medium was removed and replaced with complete growth medium. The medium was changed every 3 d for 14 d until visible colonies formed. Colonies were simultaneously fixed and stained with 0.5% crystal violet in methanol, and were manually counted. Individually stained colonies in each well were counted and the colony formation fraction was calculated as following: colony number/(number of cells seeded × plating efficiency), where plating efficiency was equivalent to the colony number divided by the number of cells seeded in the drug-free medium. The experiment was repeated three times.

Flow cytometry

Bxpc-3 cells seeded in six-well plates were treated with different concentration (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L) Rh2 for 48 h at a cell density of 1.5
× 10^5 cells/mL. Cells were processed using the following assay. (1) Cells were resuspended by adding 500 μL binding buffer, followed by adding 5 μL annexin V-FITC and 5 μL propidium iodide (PI) dye. After mixing at room temperature in the dark for 5-15 min, flow cytometry analysis was performed. Annexin V-FITC-positive and PI-negative cells were considered as apoptotic cells; (2) Cells were resuspended by adding 100 μL RNase A H2O and incubated in water at 37 °C for 30 min. After adding 400 μL PI and mixing at 4 °C for 30 min in the dark, flow cytometry analysis was performed. The G0/G1, S and G2/M stages were compared. The experiment was repeated three times.

Reverse transcriptase-polymerase chain reaction
Bxpc-3 cells (1.5 × 10^6/mL) were seeded in six-well plates. After 48 h culture, cells were treated with Rh2 (0 μmol/L and 45 μmol/L) for 48 h. Total RNA was extracted using TRIzol reagent. cDNA synthesis was performed using an RNA PCR kit. Samples were separated on 20 g/L agarose gels. The band intensity was determined by a gel image analysis system (Bio-Rad, Hercules, CA, United States) and normalized with β-actin. The PCR primers and regimen were as follows: 5'-AGGATCGAGCAGGGGC-GAATT-3', 5'-GCTCCCGGAAGAATCCAAAT-3' for Bax (345 bp); 5'-GATGGCAATGACACGACA-3', 5'-CGAGGGATACGACACAGGAT-3' for Bel-2 (346 bp); 5'-TGCCAGGATGATACTTCTT-3', 5'-ATCAAGGACAGGTTTGTTT-3' for cyclin D1 (316 bp); 5'-CTCTCTCAGCTATTACCCG-3', 5'-CAAAACACCACATCAA-3' for survivin (206 bp); 5'-CGTGCCCATCATCAGTTCC-3', 5'-GCAAAGCAGAATTCCAA-3' for MMP-2 (350 bp); 5'-CTTCCCGGAGGCTGACAC-3', 5'-CCAAGCTGCTTCCAA-3' for MMP-9 (423 bp); and 5'-TgaCTGCACTCGCAGA-3', 5'-CTGGAAAGTGGGACCGAGG-3' for β-actin (205 bp). The PCR conditions were denaturation at 94 °C for 5 min, annealing at 54 °C (Bax), 58 °C (Bel-2) and 60 °C (cyclin D1, survivin, MMP-2, MMP-9) for 30 s × 35 cycles, and extension at 72 °C for 7 min.

Western blotting
Bxpc-3 cells were treated with 0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L Rh2 for 48 h, and collected by centrifugation, washed twice with cold PBS, and resuspended in 200 μL protein lysate. Cells were centrifuged at 4 °C at 12 000 g for 5 min and the supernatant was stored at -20 °C. The protein concentration was determined by the bicinchoninic acid method (Beyotime Biotechnology Co., Jiangsu, China), and 40 μg protein was loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes at 4 °C for 4 h. Membranes were blocked in 5% non-fat milk for 1 h followed by incubation with primary antibodies of Bax, Bel-2, survivin, cyclin D1, cleaved caspase-3, caspase-8 and caspase-9, overnight at 4 °C with shaking. After washing in Tris-buffered saline with Tween 20 (TBST), membranes were incubated with peroxidase-linked IgG conjugates for 2 h at room temperature, washed again in TBST, and detected by an enhanced chemiluminescence reagent kit (Beyotime Biotechnology). The band intensity was determined by a gel image analysis system (Bio-Rad) and normalized with β-actin.

Hoechst 33258 assay for apoptosis
Bxpc-3 cells were treated with 0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L Rh2 for 48 h and rinsed with PBS twice, followed by incubation with 10 μg/mL Hoechst 33258 reagent at 37 °C in the dark for 15 min. Cells were fixed in 0.5 mL 4% paraform for 10 min and rinsed with PBS twice. The stained cells were examined and immediately photographed under a fluorescence microscope (Olympus, Shinjuku-ku, Tokyo, Japan) at an excitation wavelength of 330-380 nm. Apoptotic cells were identified on the basis of morphological changes in their nuclear assembly by observing chromatin condensation and fragment staining with Hoechst 33258. In each group, 10 microscopic fields were randomly selected and counted.

Invasion assay
Transwell chambers (Corning-Costar) were used to examine the ability of cells to invade through a Matrigel-coated filter following the manufacturer's instructions. RPMI 1640 medium was added to the upper chambers and allowed to hydrate for 2 h at 37 °C with 5% CO2. Next, 5 × 10^4 Bxpc-3 cells treated with various concentrations of Rh2 (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L) were added to the upper chamber and grown in medium containing 2% fetal bovine serum on 8.0 μm porous polycarbonate membranes, which were coated with diluted Matrigel basement membrane matrix. The lower chambers were filled with RPMI 1640 medium containing 10% fetal bovine serum. After 24 h incubation, the cells remaining on the upper surface of the filter were removed using cotton tips, and the cells that invaded to the underside of the membrane were fixed with 4% paraform and stained with crystal violet. Cells in 10 random fields of view at 400× magnification were counted and expressed as the average number of cells/field of view.

Migration assay
We scribed five parallel lines on the bottom of six-well plates using a marker pen and seeded cells at a density of 4.0 × 10^5 cells per well in triplicate for 48 h. A perpendicular scratch wound was generated by scratching with a pipette tip. After rinsing with PBS to remove the detached cells, medium containing different concentrations of Rh2 (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L) was added. Photographic images were taken from each well at 0 h, 12 h, 24 h and 48 h. The distance that cells migrated through the area created by scratching was determined by measuring the wound width at the above times and subtracting it from the wound width at the start. The values
obtained were then expressed as the rate of wound healing. The experiment was repeated three times.

**Statistical analysis**

The data were analyzed with single factor analysis of variance and a Student’s t test using SPSS 13.0 software. Data were represented as mean ± SD. P < 0.05 was considered statistically significant.

**RESULTS**

**Rh2 Inhibiting Bxpc-3 cell viability**

The inhibitory effect of Rh2 on the growth of Bxpc-3 cells was assessed by MTT and colony formation assays. The MTT assay showed that the various concentrations of Rh2 inhibited the viability of Bxpc-3 cells in a dose- and time-dependent manner. The viable Bxpc-3 cells were assessed by MTT and colony formation assays. The inhibitory effect of Rh2 on the growth of Bxpc-3 cells was observed in both a dose- and time-dependent manner; C: A colony formation assay was used to examine the growth of Bxpc-3 cells. 

**Rh2 altering Bxpc-3 cell cycle distribution**

The cell cycle distribution of Bxpc-3 cells treated with various concentrations of Rh2 (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L) for 48 h is shown in Figure 2A and 2B. The various concentrations of Rh2 altered cell cycle progression. Rh2 significantly increased the percentage of Bxpc-3 cells in the G0/G1 phase in a dose-dependent manner (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L: 43.32% ± 2.17%, 56.76% ± 1.33%, 67.40% ± 1.12% and 71.32% ± 1.16%, respectively, F = 208.37, P < 0.001), accompanied by a decrease in the percentage in S phase (50.86% ± 1.29%, 38.29% ± 1.40%, 32.08% ± 0.96% and 28.48% ± 1.18%, respectively, F = 32.45, P < 0.001) and G2/M phase (5.81% ± 1.19%, 4.95% ± 0.81%, 1.32% ± 0.83% and 0.20% ± 0.05%, respectively, F = 214.80, P < 0.001). This suggested that the cell cycle was arrested at the G0/G1 phase by Rh2. Western blotting and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of molecular marker, cyclin D1, which was related to G1 phase arrest, showed significant downregulation [Figure 2C and D, ratio of cyclin D1/β-actin: 0.885 ± 0.083 and 0.687 ± 0.074, respectively, F = 251.18, P < 0.05; Figure 2E and F, ratio of cyclin D1/β-actin: 0.885 ± 0.083 and 0.687 ± 0.096, respectively, F = 3.818, P < 0.05]. The western blotting and RT-PCR data were consistent with the G1 arrest phenomenon observed in flow cytometry analysis.

**Rh2 inducing Bxpc-3 cell apoptosis**

Rh2-induced apoptotic cell death was found using Annexin V-FITC/PI double stained flow cytometry. Annexin V-FITC-positive and PI-negative cells, which were considered as apoptotic cells, increased in a dose-dependent manner compared to the control group (Figure 1B).
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Figure 2 Rh2 induces G2/M arrest in Bxpc-3 cells treated for 48 h. A: Cell cycle distribution was assessed by flow cytometry. The results showed that the cell cycle was arrested at the G2/M phase when treated with Rh2. C, D: Western blotting was used to examine protein expression of cyclin D1; E, F: Reverse transcription-polymerase chain reaction was used to examine the expression of cyclin D1 mRNA. The results indicated that Rh2 downregulated protein and mRNA expression of cyclin D1 in Bxpc-3 cells. *P < 0.05 vs 0 μmol/L group. GADPH: Glyceraldehyde-3-phosphate dehydrogenase.

3A and B). The percentages of early apoptotic cells (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L) were 5.29% ± 2.28%, 12.15% ± 3.58%, 23.88% ± 4.07% and 38.9% ± 3.42%, respectively (F = 56.20, P < 0.05). The percentages of late apoptotic cells (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L) were 0.15% ± 0.5%, 4.15% ± 0.7%, 6.09% ± 0.9% and 7.07% ± 1.1%, respectively (F = 86.70, P < 0.05).

We also detected morphological changes in apoptotic cells by Hoechst 33258 staining (Figure 3C and D). In the untreated Bxpc-3 cells, the nuclei were stained weak homogeneous blue, while in the group treated with Rh2, bright chromatin condensation and nuclear fragmentation were observed. Furthermore, the rates of bright chromatin condensation and nuclear fragmentation increased in a dose-dependent manner. The percentages of apoptotic cells (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L) were 0.40% ± 0.05%, 16.4% ± 2.7%, 39.20% ± 2.28% and 50.4% ± 2.7%, respectively (F = 502.71, P < 0.05).

In order to investigate the mechanisms for Rh2 inducing Bxpc-3 cell apoptosis, western blotting and RT-PCR analysis of related apoptotic proteins and mRNA were used, including Bax, Bcl-2, survivin, cleaved caspase-3, caspase-8 and caspase-9. The results revealed significant downregulation of Bcl-2 and survivin, and upregulation of Bax, cleaved caspase-3 and caspase-9 (P < 0.05), but no change in cleaved caspase-8 (P > 0.05; Figure 4).

The ratios of Bax/GADPH (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L) were the following: 0.815 ± 0.147, 1.169 ± 0.127, 2.226 ± 0.398 and 12.580 ± 2.592, respectively (F = 109.651, P < 0.05). The ratios of Bcl-2/GADPH were 0.257 ± 0.095, 0.460 ± 0.097, 1.439 ± 0.111 and 1.805 ± 0.076, respectively (F = 367.81, P < 0.05). The ratios of survivin/GADPH were 1.096 ± 0.105, 1.457 ± 0.079, 1.900 ± 0.097 and 2.420 ±
0.238, respectively \( (F = 93.925, P < 0.05) \). The ratios of cleaved caspase-8/GADPH were 0.464 ± 0.095, 0.469 ± 0.106, 0.507 ± 0.112 and 0.468 ± 0.066, respectively \( (F = 0.255, P = 0.857) \). The ratios of Bax, Bcl-2, and survivin/β-actin \( (0 \mu\text{mol/L and } 45 \mu\text{mol/L}) \) were 1.148 ± 0.007 \( \text{vs} \) 1.361 ± 0.098 \( (t = -4.34, P < 0.05) \), 1.482 ± 0.120 \( \text{vs} \) 1.149 ± 0.143 \( (t = 4.358, P < 0.05) \) and 1.053 ± 0.144 \( \text{vs} \) 0.654 ± 0.120, respectively \( (t = 5.235, P < 0.05) \).

**Rh2 inhibiting Bxpc-3 cells invasion and migration**

We first examined the effect of Rh2 on the migration of Bxpc-3 cells. For the migration assay, a scratch wound healing assay was used. In the scratch wound healing assay, treatment with Rh2 of 45 \( \mu\text{mol/L} \) for 12 h, 24 h and 48 h significantly inhibited the migration of Bxpc-3 cells compared to the control group (Figure 5A and B). Compared to the control group, the rates of scratch wound healing for 12 h, 24 h and 48 h were the following (control \( \text{vs} \) trial group): 37.3% ± 4.8% \( \text{vs} \) 18.3% ± 1.65% \( (t = 6.489, P < 0.05) \), 58.7% ± 3.5% \( \text{vs} \) 38.00% ± 4.09% \( (t = 6.656, P < 0.05) \) and 93.83% ± 4.65% \( \text{vs} \) 65.50% ± 4.09% \( (t = 7.926, P < 0.05) \), respectively.
We next examined the effect of Rh2 on Bxpc-3 cell invasion using the Matrigel invasion assay. Compared to the control group, Rh2 inhibited cell invasion in a concentration-dependent manner. Even the lowest concentration of Rh2 (35 μmol/L) significantly inhibited cell invasion (Figure 5C and D). The numbers of cells invading through Matrigel and filters into the lower surface in the control group and the group treated with Rh2 in various concentrations were as follows (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L): 81.10 ± 9.55, 46.40 ± 6.95, 24.70 ± 6.88 and 8.70 ± 3.34, respectively ($F = 502.713, P < 0.05$).

We also used RT-PCR and ELISA to detect the expression of factors related to migration: that is, MMP-2 and MMP-9. Compared to the control group, Rh2 downregulated expression of MMP-2 and MMP-9 (Figure 6). The expression levels of MMP-2/β-actin (0 μmol/L and 45 μmol/L) were 0.644 ± 0.074 vs 0.424 ± 0.063 ($t = 5.543, P < 0.05$). The expression levels of MMP-9/β-actin (0 μmol/L and 45 μmol/L) were 0.995 ± 0.105 vs 0.408 ± 0.105 ($t = 9.679, P < 0.05$).

Protein expression levels of MMP-2 (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L) were 126.128 ± 9.132, 86.681 ± 8.134, 62.033 ± 6.979 and 37.672 ± 6.671, respectively ($F = 140.802, P < 0.05$). Protein expression levels of MMP-9 (0 μmol/L, 35 μmol/L, 45 μmol/L and 55 μmol/L) were 127.652 ± 6.792, 94.235 ± 7.427, 67.704 ± 6.731 and 44.195 ± 6.705, respectively ($F = 161.173, P < 0.05$).

**DISCUSSION**

Ginsenosides are the major pharmacologically active components of ginseng and they exhibit various biological effects such as anti-inflammatory and anticancer effects\[12\]. Ginsenoside Rh2 is one of the main bioactive components in ginseng extracts and has been reported in both in vitro and in vivo studies to possess potent antitumor activity, including inhibition of cell growth and induction of apoptosis in various tumor cells. However, there are no relevant reports about the effects of ginsenoside Rh2 on pancreatic cancer. In this study, we investigated the effects of Rh2 on apoptosis, proliferation, invasion and migration of the human pancreatic cancer cell line Bxpc-3.

The typical characteristics of the abnormal proliferation of tumors are out-of-control cell reproduction and strong growth. Therefore, inhibiting tumor cell proliferation is the key to controlling tumor development. Studies have shown that Rh2 can inhibit cell viability by inducing cell cycle arrest in human breast cancer cells\[13\]. In the cell cycle, cyclin D1 regulates cell proliferation by encoding a key regulator of the cell cycle transition from the G1 to S phase. We demonstrated that the expression of cyclin D1 was depressed in Bxpc-3 cells treated with Rh2. This result is consistent with MTT, clonogenic assay and flow cytometry results. Flow cytometry showed that Rh2 arrested Bxpc-3 cells in G1 phase. The data suggested that Rh2 may downregulate cyclin D1 and regulate cell cycle transition, thereby suppressing cancer cell proliferation.
Except for cell proliferation, we investigated apoptosis in Bxpc-3 cells treated with Rh2. We examined cell apoptosis rates through flow cytometry and Hoechst 33342 fluorescence staining. Our experiments showed that Rh2 could induce Bxpc-3 cell apoptosis in a concentration-dependent manner. What is the mechanism of apoptosis induced by Rh2? Recent reports have shown that Rh2 induces two types of cell death (caspase-dependent apoptosis and caspase-independent paraptosis-like cell death) in colorectal cancer cells through the activation of p53\(^{[16]}\), and Rh2 inhibits cell viability by inducing Bcl-2 family protein-mediated apoptosis in human breast cancer cells\(^{[17]}\). Therefore, we investigated several apoptosis-related proteins, including Bcl-2, Bax, survivin, cleaved caspase-3, caspase-8 and caspase-9. The Bcl-2 and caspase families are considered to be the most important proteins regulating apoptosis, which can be divided into two types: antiapoptotic and proapoptotic proteins\(^{[18]}\). Dominant in the two families, Bcl-2 is an antiapoptotic protein, however, Bax, cleaved caspase-3, caspase-8 and caspase-9 are proapoptotic proteins. The activation pathway of apoptotic protein is divided into the endogenous and exogenous pathways. In the endogenous pathway, caspase-3 is activated, which initiates the apoptotic program\(^{[19]}\). The other above-mentioned apoptotic proteins, except for caspase-8, belong to the endogenous pathway. Activation of endogenous and exogenous apoptosis-related proteins regulates the expression of cleaved caspase-3, which plays a different apoptotic role. Another important apoptosis inhibiting factor, survivin, is an important member of the inhibitors of apoptosis family and promotes many tumor cells including pancreatic cancer cell survival by regulating the G1 checkpoint and G2/M phase of the cell cycle and directly inhibiting caspase-3 and caspase-7 activation\(^{[20-22]}\). Survivin has attracted growing attention as a potential target for cancer treatment because its expression has been found in primary and cultured tumor cells and its overexpression is associated with poor prognosis\(^{[23]}\). Our results showed that Rh2 downregulated Bcl-2 and survivin, and upregulated Bax, cleaved caspase-3 and caspase-9. However, it had no effect on cleaved caspase-8. This indicated that Rh2 could induce apoptosis of Bxpc-3 cells mainly...
through initiating the endogenous apoptotic pathway. However, it remains unclear how Rh2 induced apoptosis of Bxpc-3 cells by initiating the endogenous apoptotic pathway instead of the exogenous pathway. This mechanism merits further investigation in the future.

Another typical characteristic of pancreatic cancer is aggressive metastasis[24], including local invasion of adjacent structures and metastasis to lymph nodes and liver in the very early stages. Therefore, efforts must be focused on not only targeting the primary tumor but also controlling metastases of pancreatic cancer. Therefore, we examined the effect of Rh2 on invasion and migration of Bxpc-3 cells by using a Matrigel invasion assay and a scratch wound healing assay. Our results showed that Rh2 significantly inhibited cell invasion and migration in a concentration-dependent manner. What is the mechanism of Rh2 in inhibiting Bxpc-3 cell invasion and migration?

In invasion and metastasis of tumors, cataplasia of the extracellular matrix is an important component. In such processes, MMPs, as a family of endopeptidases, play a major role and can induce extracellular matrix degradation related to cancer cell invasion and metastasis. Among all members of the MMP gene family, MMP-2 and MMP-9 are considered to be especially important in the degradation of the extracellular matrix that is associated with malignant behavior in a variety of tumor cells[25-30], including pancreatic cancer[31]. We therefore examined the expression of MMP-2 and MMP-9, and found that Rh2 significantly downregulated MMP-2 and MMP-9. This suggested that Rh2 prevented Bxpc-3 cells from invasion and migrating through downregulating MMP-2 and MMP-9.

In summary, we demonstrated the effects of Rh2 on Bxpc-3 pancreatic cancer cells for the first time: (1) Rh2 inhibits cell proliferation by downregulating cyclin D1 and arresting cells in G1 phases; (2) Rh2 induces cell apoptosis mainly through initiating the endogenous apoptotic pathway; and (3) Rh2 prevents cells from invading and migrating through downregulating MMP-2 and MMP-9. Our study shows that Rh2 can inhibit the proliferation and invasion and induce apoptosis of Bxpc-3 pancreatic cancer cells. As a natural and safe medicine, Rh2 may have future utility in clinical applications for treating pancreatic cancer. Our study will provide a new experimental basis for the clinical application of Rh2 in pancreatic cancer treatments.

COMMENTS

Background
Several studies have revealed that ginsenoside Rh2 could inhibit the growth of many kinds of cancer cells, including breast cancer, prostate cancer, hepatoma, gastric cancer and colon carcinoma. However, little is known about the role of ginsenoside Rh2 in pancreatic cancer. In this study, the authors investigated the effects of ginsenoside Rh2 on the proliferation, apoptosis and migration of cells in the human pancreatic cancer cell line Bxpc-3, and the potential mechanisms were explored.

Research frontiers
This is the first report on ginsenoside Rh2 relevant to pancreatic cancer cells.

Figure 6 Effects of Rh2 on protein and mRNA expression of matrix metalloproteinase-2 and matrix metalloproteinase-9 in Bxpc-3 cells. A, B: Reverse transcriptase-polymerase chain reaction was used to examine the expression of matrix metalloproteinase (MMP)-2 and MMP-9 mRNA. Rh2 (45 μmol/L) downregulated expression of MMP-2 and MMP-9 mRNA; C, D: Enzyme-linked immunosorbent assay was used to examine protein expression of MMP-2 and MMP-9. Rh2 downregulated protein expression of MMP-2 and MMP-9 in supernatants of Bxpc-3 cells in a dose-dependent manner, *P < 0.05 vs 0 μmol/L group.
Innovations and breakthroughs
By studying the growth-inhibiting, apoptosis-inducing and migration-inhibiting effects of ginsenoside Rh2 in the human pancreatic cancer cell line Bcap-3, the authors concluded that Rh2 could induce apoptosis and inhibit the growth and migration of cells in the human pancreatic cancer cell line Bcap-3.

Applications
As a natural and safe medicine, Rh2 may have future utility in clinical applications for treating pancreatic cancer. This study will provide a new experimental basis for the clinical application of Rh2 in pancreatic cancer treatments.

Terminology
Ginsenoside is the main effective component of ginseng, which has been widely used in oriental countries for thousands of years. Ginsenoside Rh2 is a pure compound extracted from ginsenosides.

Peer review
The authors present new findings that constitute the first evidence that this compound may have potential in pancreatic cancer treatment. The data are consistent and provide a solid foundation for future work. The data presented by the authors are interesting. The experiments were well controlled and executed.

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