Abstract. Resveratrol is a polyphenolic compound that is extracted from Polygonum cuspidatum and is used in traditional Chinese medicine. Previous data have shown that resveratrol inhibits the growth of human gastric cancer. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] and trypan blue assays showed that resveratrol significantly decreased the survival rate of MGC803 cells in a concentration- and time-dependent manner. Our flow cytometric analysis showed that resveratrol treatment arrested the cells at the G0/G1 phase of the cell cycle. Furthermore, western blotting demonstrated that resveratrol decreased the protein expression of phospho-glycogen synthase kinase 3β (p-GSK3β), cyclin D1, phospho-phosphatase and tensin homologue (p-PTEN), phospho-phosphatidylinositol 3'-OH kinase (p-PI3K), and phospho-protein kinase B (p-PKB/Akt). We also found that resveratrol inhibited the progression of the cell cycle in MGC803 cells by repressing p-PI3K and p-Akt expression. Meanwhile, resveratrol did not decrease the phosphorylation level of Akt when the PTEN gene expression was knocked down by an siRNA in the MGC803 cells. Taken together, these results suggest that resveratrol induced cell cycle arrest in human gastric cancer MGC803 cells by regulating the PTEN/PI3K/Akt signaling pathway.

Introduction

Gastric cancer (GC) was found to be the fifth most common malignancy in 2012 (0.95 million cases, 6.8% of all cancer cases) and was responsible for the third highest mortality rate (0.72 million deaths, 8.8% of the total) worldwide. More than 70% of cases (0.67 million cases) occur in developing countries (0.45 million in men, 0.22 million in women), and 50% of the worldwide cases occur in Eastern Asia (mainly China) (1). In Southeastern Asia, 14.0 per 100,000 men and 9.8 per 100,000 women die of GC, and these mortality rates are 4.0-4.5 times higher than those in the United States and Europe (1). Currently, GC is primarily treated with surgery, chemotherapy and radiotherapy. Two recent and large randomized controlled trials (RCTs) showed that adjuvant chemotherapy reduced the recurrence rate and improved survival (2,3). Resveratrol (3,4,5-trihydroxystilbene) is a polyphenol compound that is extracted from Polygonum cuspidatum for use in traditional Chinese medicine; it has significant anti-bacterial, anti-inflammatory, anticancer, anti-hyperlipidemia, lipid peroxidation and anti-apoptotic effects (4-6). However, the mechanism of action of resveratrol in regards to the treatment and prevention of GC has not been determined in previous studies.

Cyclin D1 is an important protein related to the G0/G1 cell cycle checkpoint (7). Phospho-glycogen synthase kinase 3β (p-GSK3β) has been reported to regulate cyclin D1 expression (8). GSK3β is an important protein in the phosphatidylinositol 3'-OH kinase (PI3K)/protein kinase B (Akt) signaling pathway. Several studies have shown that PI3K/Akt signaling plays a pivotal role in the development and progression of cancer (9,10). Phosphatase and tensin homolog (PTEN), a switch gene, can inhibit tumor cell growth by downregulating the protein expression of p-PI3K and p-Akt (11,12). PTEN also regulates cell cycle arrest in the G1 phase by repressing the protein expression of p-PI3K and p-Akt (13,14).

Therefore, we explored the effects of resveratrol on the proliferation of human gastric adenocarcinoma MGC803 cells and examined the underlying mechanisms of the involvement of resveratrol in the PTEN/PI3K/Akt pathway.
Materials and methods

Cell culture. The human gastric adenocarcinoma cell line MGC803 was obtained from the Cell Bank of the Chinese Academy of Science and was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (both from Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 95% and 5% CO₂.

Reagents. Resveratrol with a purity exceeding 98% was purchased from Sigma-Aldrich (St. Louis, MO, USA). Its structure is shown in Fig. 1. It was dissolved in DMSO at the desired concentrations and diluted in media at a maximal concentration of 0.1%. The concentration of DMSO in the controls was also 0.1%.

Cell proliferation assay. The effect of resveratrol on the cell viability was evaluated using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) (Promega Corporation, Madison, WI, USA). After 24 and 48 h of exposure to different concentrations of resveratrol, 10 µl of MTT (5 mg/ml) was added to each well, and the cells were incubated for 4 h. The medium was then removed, and 150 µl of DMSO was added to each well. The absorbance was recorded at 570 nm in a microplate reader, and the inhibition ratio (I%) was calculated using the following equation: I% = [A570 (control) - A570 (treated)]/A570 (control) x 100.

Cell viability assay. After removing the cell culture medium, the MGC803 cells were gently rinsed twice with phosphate-buffered saline (PBS). To determine the effect of resveratrol on cell viability, MGC803 cells were cultured in a 24-well plate at ~1x10⁵ cells/well with serum-free medium containing the appropriate concentration of resveratrol (50, 100 and 200 µmol/l). The control was incubated in serum-free medium alone. The cells were then incubated for 24 h. At the end of the test, the cells were stained with trypan blue and counted. All assays were performed at least three times (15).

Flow cytometric analysis of cell cycle arrest. To examine cell cycle arrest, MGC803 cells (1x10⁶) were cultured in 60-mm Petri dishes and incubated for 24 h. The cells were then harvested, washed with PBS, resuspended in 250 µl of PBS, and fixed in 750 µl of 100% ethanol at 4°C. After an overnight incubation, the cell pellets were collected by centrifugation, resuspended in 1 ml of 1X Annexin V binding buffer (BD Biosciences, San Jose, CA, USA) at a concentration of 1x10⁶ cells/ml and transferred to 100 µl of solution (1x10⁵ cells) in a 5-ml culture tube. Subsequently, 5 µl of FITC Annexin V and 5 µl of propidium iodide (PI) were added to the tube, and the cells were gently vortexed and incubated for 15 min at room temperature (RT, 25°C) in the dark. Thereafter, 400 µl of 1X binding buffer was added to the tube. The fluorescence emitted by the PI-DNA complex was quantified after the excitation of the fluorescent dye by FACScan cytometry (BD Biosciences). The fraction of cells in each cell cycle stage was quantitated with ModFit LT for Mac 3.0 software (BD Biosciences).

RNA interference assay. PTEN-siRNA was purchased from GeneChem Biotech Ltd. (Shanghai, China). One day before the transfection, the MGC803 cells were allowed to adhere and were counted. The cell concentration was adjusted to 2x10⁵/ml. The cells were then grown in 6-well plates in 2.5 ml of antibiotic-free cell culture medium. For each well to be transfected, PTEN-siRNA-Lipofectamine™ RNAiMAX complexes were prepared as follows. i) PTEN-siRNA plasmid (150 pmol) was diluted in 250 µl of Opti-MEM® I Reduced Serum Medium (Gibco, Grand Island, NY, USA) and the solution was gently mixed. ii) Lipofectamine™ RNAiMAX (Invitrogen Life Technologies) was gently mixed and 12 µl of the reagent was then diluted in 250 µl of Opti-MEM® I Reduced Serum Medium. The solution was gently mixed. iii) The diluted PTEN-siRNA plasmid was added to the diluted Lipofectamine™ RNAiMAX. The solution was gently mixed and incubated for 15 min at RT in the dark. The PTEN-siRNA-Lipofectamine™ RNAiMAX complexes were then added to each well containing cells. The final volume of these solutions was 3 ml, and the final concentration of RNA was 50 nM. The plates were gently mixed by rocking the plate back and forth. Finally, the cells were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air.

Western blot analysis. The cell pellets were resuspended in lysis buffer (Beyotime, Haimen, China) and lysed on ice for 30 min. After centrifugation for 30 min, the supernatant was collected, and the protein content of the supernatant was measured using a bicinchoninic acid (BCA) protein assay kit (Beyotime). The protein lysates (20 µl) were separated by electrophoresis on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% bovine serum albumin for 2 h. The membranes were then probed with specific primary antibodies against PI3K (rabbit monoclonal, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), p-PI3K (1:1,000), Akt (1:1,000), p-Akt (Ser-473) (1:1,000), PTEN (1:1,000) and p-PTEN (1:1,000) overnight at 4°C. All primary antibodies were purchased from Cell Signaling Technology Inc. Rabbit monoclonal anti-human p-GSK3β (1:1,000) and GSK3β (1:5,000) antibodies were purchased from Abcam (Cambridge, UK). Anti-rabbit antibody was purchased from Cell Signaling Technology Inc. Anti-rabbit horseradish
peroxidase (HRP)-conjugated antibody was purchased from Merck Millipore. The membranes were incubated with the appropriate HRP-conjugated anti-rabbit antibody (1:5,000; Cell Signaling Technology Inc.).

Statistical analysis. The obtained data were statistically analyzed and are presented as the mean ± standard deviation (SD) of at least 3 independent experiments. Datasets with three or more groups were analyzed by one-way analysis of variance (ANOVA) or one-way ANOVA with repeated measures, and the last significant difference (LSD) post hoc test for multiple comparisons. Comparisons of two groups were analyzed using the Student’s t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of resveratrol on the viability and cell cycle arrest of MGC803 cells. To investigate the effects of resveratrol on the growth of MGC803 cells, MGC803 cells were exposed to seven different concentrations of resveratrol (6.25, 12.5, 25, 50, 100, 200 and 400 µM) for 24 and 48 h. Resveratrol inhibited cell growth in a dose- and time-dependent manner (Fig. 2A). The half maximal inhibitory concentration (IC50) of resveratrol for MGC803 cells was ~29.0 µg/ml (127 µM) (Fig. 2A) at 24 h of incubation, and this concentration significantly increased at 48 h of incubation (P<0.05). The cell viability assay showed that resveratrol induced cell death in a dose-dependent manner at 24 h (Fig. 2B). Because resveratrol significantly inhibited the growth of MGC803 cells, we investigated the effects of resveratrol on cell cycle progression. Resveratrol treatment arrested MGC803 cells at the G0/G1 phase of the cell cycle, whereas cell cycle progression remained unchanged in the untreated cells (Fig. 2C). Treatment with 50 µM resveratrol significantly increased the number of MGC803 cells arrested in the G0/G1 phase compared with the untreated MGC803 cells (61.21±0.51 vs. 59.70±0.36%, P<0.05). Treatment with 100 µM resveratrol treatment further increased the number of MGC803 cells arrested in the G0/G1 phase compared with the 50 µM resveratrol treatment (63.21±0.33%, P<0.05). These data suggest that resveratrol at least partly inhibits the growth of GC cells by inducing G0/G1 arrest.

Resveratrol inhibits the GSK3β-dependent cyclin D1 signaling pathway in MGC803 cells. The GSK3β-dependent cyclin D1 signaling pathway plays a critical role in the regulation of the G0/G1 transition of the cell cycle (16). We examined the effects of resveratrol on the expression of these two proteins. Cyclin D1 is a component of the core cell cycle machinery and is a key regulatory protein of the G1 phase of the cell cycle that allows the cell to progress to the S phase. The treatment of MGC803 cells with 100 and 200 µM resveratrol substantially reduced the expression of cyclin D1 (Fig. 3A). The expression of cyclin D1 is known to be mediated by GSK3β. Therefore, we

Figure 2. Inhibition of MGC803 cell growth and induction of apoptosis and G0/G1 cell cycle arrest by resveratrol. (A) Correlation of resveratrol drug doses and growth inhibition in MGC803 cells. y-axis, percentage of growth inhibition; x-axis, resveratrol concentration (µM). (B) Cell viability assay: cells were incubated for 24 h and then stained with trypan blue. (C) Cell cycle analyses were performed by flow cytometry.
examined the involvement of GSK3β in the resveratrol-induced downregulation of cyclin D1 in MGC803 cells. Although the total protein level of GSK3β did not change, resveratrol markedly suppressed GSK3β phosphorylation within 24 h in a dose-dependent manner (Fig. 3A). Moreover, the protein expression of p-GSK3β was regulated in a time-dependent manner and significantly decreased after 6 h of incubation with resveratrol (Fig. 3B). These data suggest that resveratrol inhibits GSK3β and promotes the degradation of cyclin D1, leading to cell cycle arrest in the G1 phase.

Resveratrol inhibits PI3K/Akt signaling in MGC803 cells. Previous studies have shown that the phosphorylation and subsequent inactivation of GSK3β via the PI3K/Akt signaling pathway is important for tumor cell growth (17,18). The PI3K/Akt signaling pathway is well defined and is directly associated with cell growth, proliferation and survival. After treatment for 24 h, resveratrol inhibited the protein levels of p-PI3K and p-Akt in a dose-dependent manner, as shown in Fig. 4A. Compared with the untreated cells, 100 µM resveratrol inhibited the p-PI3K and p-Akt protein levels by 50.57 and 40.77%, respectively and 200 µM resveratrol inhibited the expression of these proteins by 64.56 and 61.24%, respectively. We also found that the p-PI3K and p-Akt protein levels in MGC803 cells were decreased after 6 h of treatment with 100 µM resveratrol (Fig. 4B). Taken together, these findings suggest that resveratrol inhibits GSK3β phosphorylation and degradation and decreases the protein level of cyclin D1 at least partly by inhibiting the PI3K-Akt pathway.

Resveratrol regulates PTEN activity to suppress the PI3K/Akt pathway. PTEN negatively regulates the activity of its upstream kinase, PI3K. PTEN blocks the action of PI3K by dephosphorylating the signaling lipid phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Resveratrol was shown to decrease the protein level of p-PTEN (inactive) in a dose-dependent manner. This effect of resveratrol was apparent at concentrations ranging from 50 to 200 µM and was maximized at 200 µM (Fig. 5A). Subsequent investigations showed that 100 µM resveratrol treatment resulted in the inactivation of PTEN in a time-dependent manner (Fig. 5B).

To determine the involvement of PTEN in the inhibition of PI3K/Akt signaling by resveratrol, RNA interference technology was adopted to silence endogenous PTEN expression. PTEN protein expression was significantly downregulated by PTEN-siRNA but not by the control siRNA treatment (Fig. 6). PTEN-siRNA treatment also reduced the protein level of p-PTEN. PTEN-siRNA also increased the activity of Akt kinase in the MGC803 cells (Fig. 6). Interestingly, resveratrol treatment after PTEN-siRNA transfection for 24 h slightly upregulated PTEN, suggesting that other factors are involved in the regulation of the PTEN protein level by resveratrol; however, this upregulation of PTEN was much weaker than that in cells that were not treated with PTEN-siRNA, and the...
Figure 4. Resveratrol regulates the protein expression of phospho(p)-PI3K and p-Akt in MGC803 cells. (A) Extracts of MGC803 cells treated with 0, 50, 100 and 200 µM resveratrol were probed for p-PI3K and p-Akt protein expression. *P<0.05 and **P<0.01 compared with the control group. (B) The protein levels of p-PI3K and p-Akt were detected in MGC803 cells at the indicated time points during resveratrol treatment. The p-Akt level was obtained from one of the three individual experiments and indicates a change in expression. *P<0.05 and **P<0.01 compared with the control group.

Figure 5. Resveratrol regulates the protein expression of phospho(p)-PTEN in MGC803 cells. (A) Extracts of MGC803 cells treated with different concentrations of resveratrol were probed for p-PTEN expression, and the p-PTEN protein levels were quantified. *P<0.05 and **P<0.01 compared with the control group. (B) The p-PTEN protein levels were detected in MGC803 cells at the indicated time points during resveratrol treatment. The p-PTEN levels were obtained from one of three individual experiments and indicate a change in expression. *P<0.05 and **P<0.01 compared with control group.
were quantified. and then treated with resveratrol. PTEN, p-PTEN and p-Akt protein levels transfected with PTEN-siRNA or control siRNA, they were cultured for 24 h protein expression by resveratrol in MGC803 cells. After MGC803 cells were Figure 6. Knockdown of PTEN influences the inhibition of phospho(p)-Akt apoptotic effect of resveratrol on MGC803 cells. Previous gastric MGC803 cells induced the apoptosis of MGC803 cells (Fig. 2).

The present study was supported by Shanghai Natural Science Foundation of China (13ZR1434300) and Shanghai Science and Technology Committee Foundation of China (13495810300).
References


