

Resveratrol suppresses proliferation and induces apoptosis of uterine sarcoma cells by inhibiting the Wnt signaling pathway

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Abstract. Resveratrol, a natural product and peroxisome proliferator-activated receptor (PPAR) agonist, has been reported to exert anti-cancer effects in several tumor models. A previous study by our group reported that prostaglandin J2, a PPAR γ ligand, inhibited cell proliferation in a uterine sarcoma cell line. The aim of the present study was to investigate the role of the Wnt signaling pathway in resveratrol-induced apoptosis and inhibition of cell proliferation in the MES-SA human uterine sarcoma cell line. A WST-1 assay demonstrated that resveratrol inhibited cell proliferation in the MES-SA cell line, and flow cytometry revealed that the number of apoptotic cells increased in a resveratrol dose-dependent manner. The mechanisms underlying these effects of resveratrol were speculated to involve the expression of β -catenin and its target gene, c-myc, which were examined using western blot analysis. The results revealed a dose-dependent downregulation of this β -catenin and c-myc. This effect was blunted by a pharmacological inhibitor of glycogen synthase kinase 3 β . Therefore, it is likely that resveratrol inhibited the cell proliferation and increased the number of apoptotic cells, at least partially, via the Wnt signaling pathway. The present results suggest that resveratrol is a potential candidate for the treatment of uterine sarcoma.

Introduction

Among gynecological tumors, uterine sarcoma has one of the poorest prognoses, and the most effective treatment is complete resection. At present, no effective therapeutic drugs are available for cases of recurrent and advanced-stage uterine sarcoma. In recent years, pazopanib and trabectedin have been used to

treat soft tissue sarcoma containing uterine sarcoma; however, their effects are limited. Thus, novel drugs are urgently required for the treatment of uterine sarcoma.

Trans-3,4',5-trihydroxystilbene (resveratrol) is a natural polyphenolic phytoalexin present in grape skin. It has been reported to function as an agonist of PPAR γ (1). In 1997, Jang *et al* (2) reported on the anti-cancer effects of resveratrol. Previous studies on resveratrol demonstrated that it inhibits the proliferation and induces apoptosis in different cancer cell types, including breast, prostate, stomach, colon, pancreatic and thyroid cancers (3). Resveratrol has potential as a novel drug with minimal side effects. A previous study by our group reported that PGJ2, a PPAR γ ligand, inhibited cell proliferation in a uterine sarcoma cell line (4). Resveratrol, which also acts as a PPAR γ agonist, has potential as an agent in the chemoprevention of uterine sarcoma.

The canonical Wnt signaling pathway is important in embryonic development. The activation of the Wnt signaling pathway is involved in the onset of certain carcinomas, including colon cancer (5). The Wnt protein induces the accumulation of β -catenin in the cytoplasm, which then translocates to the nucleus and causes the transcriptional activation of the target genes c-myc and cyclin D (6). Previous studies reported that resveratrol inhibited Wnt signaling via the β -catenin pathway in osteosarcoma, gastric cancer and colon cancer (7-10). However, the efficacy of resveratrol in human uterine sarcoma and the underlying mechanisms of action have remained elusive. At present, no drugs are available that target the Wnt signaling pathway; however, since this pathway is associated with various cancer types, it may be regarded as an attractive target for novel treatments (11). Resveratrol has potential as a novel therapeutic agent that targets the Wnt signaling pathway.

In the present study, the effect of resveratrol to inhibit the proliferation of uterine sarcoma cells and on the Wnt signaling pathway were examined.

Materials and methods

Cell line and culture. The MES-SA human uterine sarcoma cell line (European Collection of Cell Cultures, Salisbury, UK) was cultured in McCoy's 5A medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) with 10% fetal bovine serum,

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penicillin (100 U/ml) and streptomycin (100 $\mu\text{g/ml}$) in an incubator at 37°C with air containing 5% CO_2 .

Drugs and reagents. Resveratrol was purchased from Tokyo Chemical Industry (Tokyo, Japan). The glycogen synthase kinase (GSK)-3 β inhibitor CHIR99021 was purchased from Cayman Chemical (Ann Arbor, MI, USA). The primary antibodies for western blot analysis were β -catenin (cat. no. 8480), c-myc (cat. no. 13987) and β -actin (cat. no. 4967), and the secondary antibody was a horseradish peroxidase-conjugated anti-rabbit antibody (cat. no. 7074). All were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

WST-1 cell proliferation assay. Cells were seeded on a 96-well microplate at a density of 2×10^3 cells/well in 100 μl culture medium and incubated for 24 h. The cells were then treated with different concentrations of resveratrol (0, 10, 20 and 40 $\mu\text{g/ml}$) for 24, 48 or 72 h. WST-1 reagent (10 μl) was added to each well, and the cells were incubated at 37°C for 1 h. The absorbance was measured at 450 nm using a microplate reader (Thermo Scientific Varioskan Flash Multimode reader; Thermo Fisher Scientific, Inc., Waltham, MA, USA). At least 6 wells were used for each concentration of the tested reagent.

Analysis of cell apoptosis by flow cytometry. The Annexin V/fluorescein isothiocyanate (FITC) apoptosis detection kit (cat. no. 2375; Beckman Coulter, Inc., Brea, CA, USA) was used to detect cell apoptosis. Samples were washed with cold PBS twice and centrifuged at 500 \times g at 4°C for 3 min. The cell density was adjusted to 5×10^5 cells/ml in binding buffer. A 100- μl aliquot of the cell suspension was mixed with 5 μl Annexin V-FITC prior to incubation in the dark on ice for 15 min. The suspension was then mixed with 2.5 μl propidium iodide on ice. Samples were examined using a flow cytometer (BD FACSVers; BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. Cells were cultured to 80% confluence in 60-mm dish. The cells were then treated with different concentrations of resveratrol (0, 10, 20 and 40 $\mu\text{g/ml}$) for 72 h at 37°C. A group was prepared with resveratrol alone and fixed with 10 ng/ml GSK3 β inhibitor (CHIR99021) at each resveratrol concentration (10, 20, 40 $\mu\text{g/ml}$) for 72 h at 37°C. The cells were lysed in M-PER Mammalian Protein Extraction reagent (Thermo Fisher Scientific, Inc.). Protein concentrations were determined using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Protein samples (20 μg) were separated by 7.5% SDS-PAGE gels (TGX precast gel; Bio-Rad Laboratories, Hercules, CA, USA) and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 3% bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 at room temperature for 1 h and was treated with primary antibodies (1:1,000) overnight at 4°C, followed by secondary antibodies (1:2,000) for 1 h at room temperature. The signal was detected using an ECL Plus system (GE Healthcare, Little Chalfont, UK). Band intensities were quantified using ImageJ software version 1.51 (National Institutes of Health, Bethesda, MA, USA).

Statistical analysis. Statistical analysis were performed using Ekuseru-Toukei 2012, version 1.16 (Social Survey Research

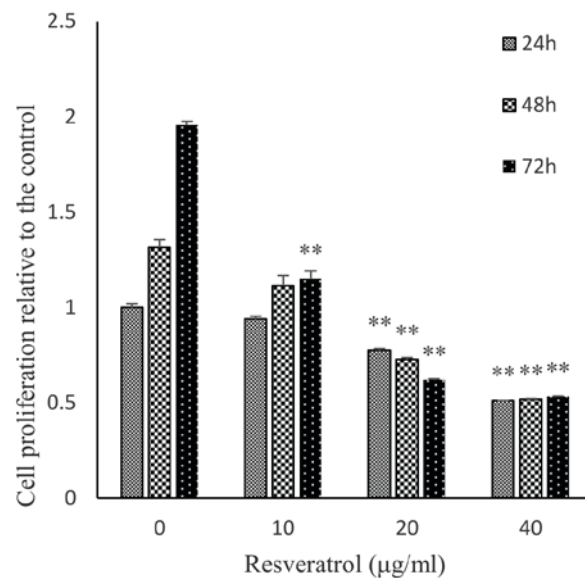


Figure 1. Effects of resveratrol on the proliferation of the human MES-SA uterine sarcoma cell line. MES-SA cells were treated with resveratrol at 0, 10, 20 or 40 $\mu\text{g/ml}$ for 24, 48 or 72 h, and the resulting number of viable cells was determined using the WST-1 assay. The absorbance is expressed as the mean \pm standard error of the mean ($n=6$). ** $P<0.01$ vs. the control.

Information Co., Ltd., Tokyo, Japan). Values are expressed as the mean \pm SEM. The significance of differences in the experimental and control groups was assessed using Dunnett's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Resveratrol inhibits the proliferation of MES-SA cells. The WST-1 assay was performed to determine the proliferation rate of human MES-SA cells treated with resveratrol. MES-SA cells were treated with 0, 10, 20 and 40 $\mu\text{g/ml}$ resveratrol for 24, 48 or 72 h. The inhibitory rates of uterine sarcoma cell proliferation were 61% at 40 $\mu\text{g/ml}$ resveratrol, 45% at 20 $\mu\text{g/ml}$ resveratrol at 48 h. The cell proliferation was suppressed in a concentration- and time-dependent manner with ≥ 20 $\mu\text{g/ml}$ resveratrol for 24 h or longer (Fig. 1).

Resveratrol induces apoptosis of MES-SA cells. Apoptotic changes in human MES-SA cells treated with resveratrol were assessed by flow cytometry. The results revealed that after 72 h of incubation with resveratrol at various concentrations, the apoptotic rate was increased in a dose-dependent manner. The apoptotic rate was 36.2% in the cells treated with 40 $\mu\text{g/ml}$ resveratrol and 3.8 and 10.6% in those treated with 10 and 20 $\mu\text{g/ml}$ resveratrol, respectively (Fig. 2).

Resveratrol inhibits Wnt signaling in MES-SA cells. Cells were treated with resveratrol or a combination of resveratrol and CHIR99021, a GSK3 β inhibitor, at several concentrations for 48 h. Western blot analysis indicated that the expression of β -catenin and its downstream target c-myc in the Wnt signaling pathway was downregulated in a resveratrol dose-dependent manner (Fig. 3). Furthermore, the expression of β -catenin and c-myc were increased with the combination of CHIR 99021

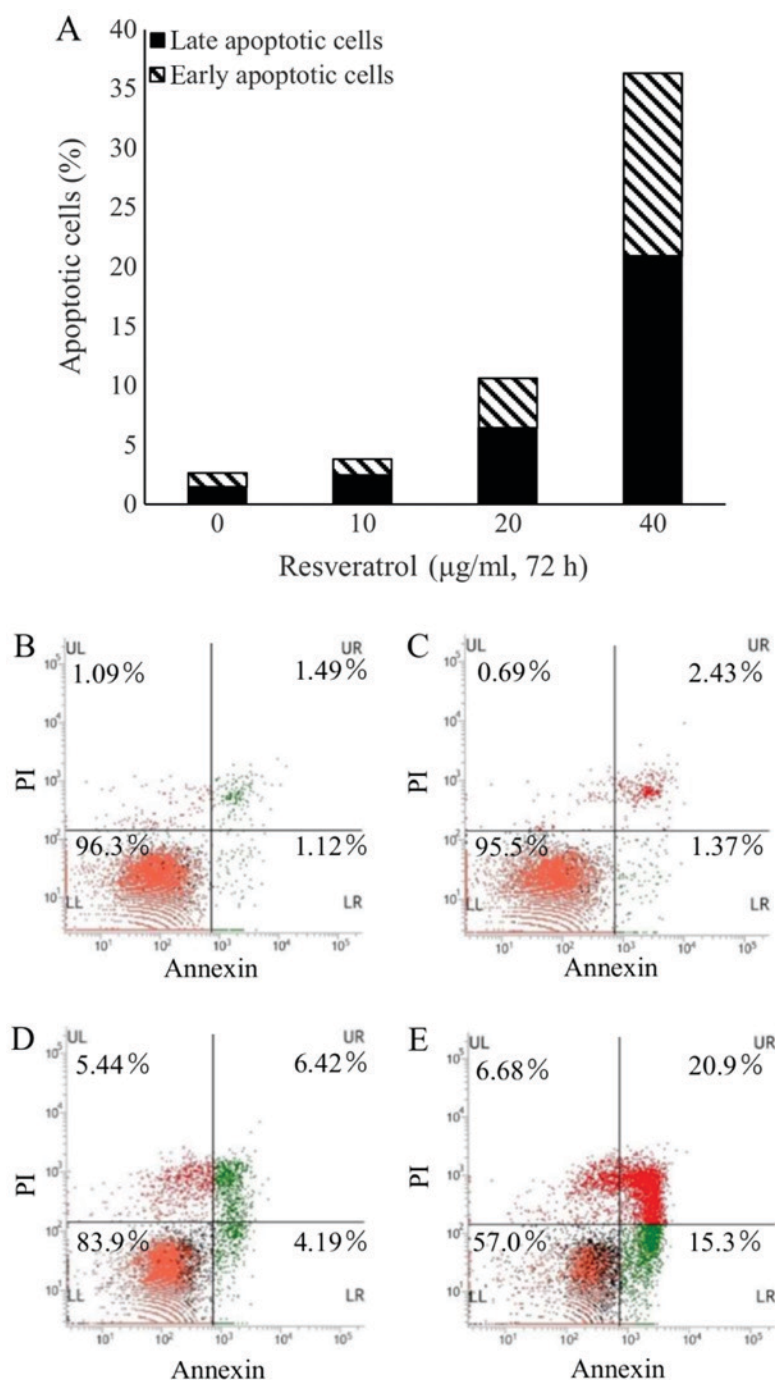


Figure 2. Effect of resveratrol on apoptosis in the human MES-SA uterus sarcoma cell line. Cells were treated with resveratrol for 72 h, and apoptosis was determined by flow cytometry using an Annexin V kit. (A) Quantified amounts of cells in early and late apoptosis in groups treated with different concentrations of resveratrol. (B-E) Representative flow cytometry dot plots of MES-SA cells treated with resveratrol at (B) 0 µg/ml, (C) 10 µg/ml, (D) 20 µg/ml and (E) 40 µg/ml. Quadrants: UL, necrotic cells; UR, late apoptotic cells; LL, live cells; LR, early apoptotic cells. PI, propidium iodide; UL, upper left; UR, upper right; LL, lower left; LR, lower right.

and resveratrol compared with the resveratrol alone group. CHIR99021 reduced the level of active β -catenin and c-myc by blunting the effect of resveratrol. This indicated that resveratrol inhibited upstream of GSK3 β in the Wnt signaling pathway.

Discussion

The present results revealed that resveratrol inhibited the proliferation of cells and induced apoptosis in a dose-dependent manner. Although the effects of resveratrol were dependent on

its dose, cytostatic effects were observed in uterine sarcoma cells following a prolonged exposure to a low dose. The expression of β -catenin in uterine sarcoma cells was detected by western blot analysis, revealing that its expression was inhibited by resveratrol in a dose-dependent manner. The expression of c-myc, the target gene of β -catenin, was similarly inhibited. GSK3 β disintegrates as it phosphorylates β -catenin, thereby stabilizing β -catenin (12). When the phosphorylation of β -catenin is inhibited, accumulation of β -catenin activates the Wnt signaling pathway (13). The expression of β -catenin and suppression of

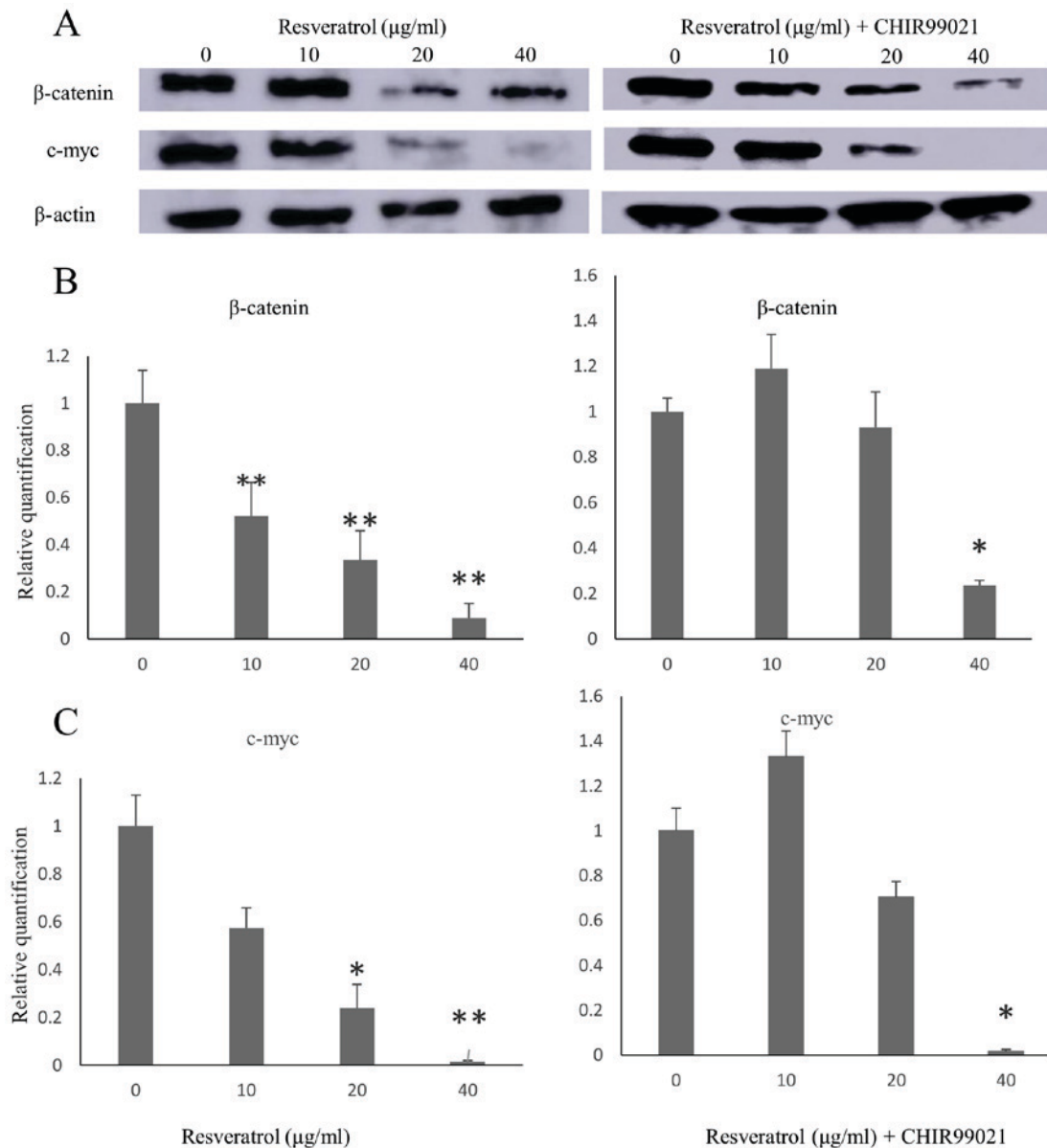


Figure 3. β -Catenin and c-myc expression levels in the human MES-SA uterine sarcoma cell line following treatment with resveratrol for 48 h. (A) Representative western blots for determining the expression of β -catenin and its target gene c-myc. (B and C) Quantified expression levels of β -catenin and c-myc (B) after resveratrol treatment at different concentrations and (C) after treatment with resveratrol vs. resveratrol and CHIR99021 (10 ng/ml). β -actin was used as the loading control. Values are expressed as the mean \pm standard error of the mean (n=3). *P<0.05 and **P<0.01 vs. the control.

c-myc were increased in cells treated with the combination of the GSK3 β inhibitor and resveratrol. Resveratrol inhibits the proliferation of uterine sarcoma cells via the β -catenin/Wnt signaling pathway.

A previous study reported the activation of Wnt signaling in bone sarcoma cells (14). Resveratrol has been demonstrated to inhibit Wnt signaling in human osteosarcoma cells (15). After exposure to 40 μ g/ml resveratrol for 72 h, the apoptotic rates of osteosarcoma and uterine sarcoma were 27.5% (15). In colorectal cancer, resveratrol led to an apoptosis rate of 30% at 150 μ M (7). In the present study, an apoptotic rate of 43% was determined at the same concentration. The cytostatic effects of resveratrol were marked at 40 μ g/ml, and the expression of β -catenin and c-myc was not upregulated by the GSK3 β inhibitor. The cytostatic effects of resveratrol at a lower concentration may be greater in

uterine sarcoma cells than in osteosarcoma cell and colon cancer cell.

The Wnt/ β -catenin signaling pathway has an important role in normal developmental processes, and is activated in neoplastic cells (16). Thus, the inhibition of this signal may cause side effects due to stem cell dysfunction (11).

Antioxidant (17) and cranial nerve protective effects (18) have been reported at low doses of resveratrol, and preventive effects regarding colon cancer have been demonstrated (19). A previous study reported the absence of severe side effects in healthy human subjects administered 5 g resveratrol (20). Resveratrol may exert anti-tumor effects without any serious adverse effects by inhibiting the Wnt signaling pathway in a concentration-dependent manner.

One of the limitations of resveratrol is its low bioavailability (21). Previous studies reported on the effects of resveratrol

on breast, liver, pancreas, prostate, and bladder cancer *in vivo*. In uterine sarcoma, the detailed mechanisms of action of resveratrol have remained elusive, and further *in vivo* research similar to that performed for other cancer types (22,23) is required in order to elucidate the underlying mechanisms.

In conclusion, the present study demonstrated that resveratrol inhibited the proliferation of human MES-SA uterine sarcoma cells, possibly by downregulating the expression of β -catenin and c-myc in the Wnt signaling pathway. The present results indicate the potential of resveratrol as a novel treatment for uterine sarcoma.

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Availability of data and materials

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AM, ET and TaK analyzed and interpreted the data regarding the effect of resveratrol in uterine sarcoma. AM performed all experiments, and was a major contributor in writing the manuscript. MN, ToK, AA and MI were involved in writing and revising the manuscript. All authors read and approved the final manuscript.

Ethical approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests to disclose.

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