Resveratrol induces cell cycle arrest and apoptosis in epithelioid malignant pleural mesothelioma cells

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Resveratrol epitelioid malign pleval mezotelyoma hücrelerinde hücre siklüsü arrestini ve apoptozisi uyarmaktadır

Abstract

Background: Resveratrol is a natural anti-carcinogenic polyphenol. Malignant pleural mesothelioma (MPM) is an aggressive tumor with poor prognosis. In this study, we investigated the effects of resveratrol on epithelioid MPM.

Material and methods: Human epithelioid MPM cell line (NCI-H2452) was exposed to resveratrol (5–200 μM) for 24 or 48 h. Cell viability was assessed by WST-1 assay. Flow cytometry analyses were performed to evaluate the effects of resveratrol on cell cycle distribution and apoptosis. Western blot analysis was used to determine protein expression levels of antioxidant enzymes, cyclin D1 and p53. Reactive oxygen species (ROS) were measured using H2DCFDA.

Results: Resveratrol reduced cell viability of the cells in a concentration and time dependent manner. After treatment, the cells accumulated in G0/G1 phase and the percentage of cells in G2/M phase was reduced. Resveratrol decreased cyclin D1 and increased p53 expression in cell lysates. Treated cells exhibited increased apoptotic activity. ROS were elevated with resveratrol treatment, but there was no change in the expression of superoxide dismutase (SOD)-1, SOD-2 and glutathione peroxidase.

Conclusion: Our results revealed that resveratrol exhibits anti-cell viability effect on epithelioid MPM cells by inducing cell cycle arrest and apoptosis. Resveratrol may become a potential therapeutic agent for epithelioid MPM.

Keywords: Resveratrol; Malignant pleural mesothelioma; Oxidative stress; Apoptosis; Cell cycle.

Özet


Bulgular: Resveratrol, hücrelerin canlılığı, doza ve zamana bağlı olarak düştü. Uygulamadan sonra, hücrelerin G0/G1 fazında biriktiği ve G2/M fazındaki
hücrelerin yüzdesinin azaldığı görüldü. Resveratrol, hücre lizatlarındaki, siklin D1 ekspresyonunu düşürdü ve p53 ekspresyonunu artırdı. Resveratrol uygulanan hücreler artmış apoptotik aktivite gösterdi. Resveratrol uygulaması ile ROT artarken, süperoksid dismutaz (SOD)-1, SOD-2 ve glutatyon peroksidaz enzimlerinin ekspresyonunda herhangi bir değişiklik gözlenmedi.

Sonuç: Bulgularımız, resveratrolün, epitelioid MPM hücrelerinin hücre canlılığını azaltıcı etkisini, hücre siklüsünü durdurarak ve apoptozisi uyararak gerçekleştirdiğini göstermiştir. Resveratrol, epitelioid MPM için potansiyel bir tedavi edici ajan olabilir.

Anahtar kelimeler: Resveratrol; Malign pleval mezote-lyoma; Oksidatif stress; Apoptosis; Hücre döngüsü.

Introduction

Resveratrol (3,5,4′-trihydroxystilbene) is a natural poly-phenol composed of two aromatic rings and found mainly in the skin of grapes and also in various fruits, vegetables and other plants such as pines, legumes, peanut, soybean, and pomegranate. It is an estrogen agonist, exhibits anti-oxidant, anti-inflammatory, cardioprotective, anti-diabetic properties and has chemopreventive and chemotherapeutic potential [1]. The first report on chemopreventive effects of resveratrol was published in 1997 [2]. Thereafter, its inhibitory effect on tumor initiation and promotion has been shown in multiple cancer types including hepatoma, neuroblastoma, fibrosarcoma, leukemia, skin, gastric, colorectal, lung, breast, prostate and pancreatic cancer [3].

Malignant pleural mesothelioma (MPM) is an aggressive tumor arising from the mesothelial surface of the pleural cavity. It is a rare tumor but its incidence is increasing throughout the world because of increased asbestos exposure. According to WHO histologic classification, MPM includes epithelioid, sarcomatoid and biphasic histologic subtypes, epithelioid being the most common [4]. The prognosis of MPM patients is poor, with a median survival time of 12 months [5]. Although multimodality treatments, including surgery, radiotherapy and chemotherapy, are performed, unfortunately there is no effective cure for MPM [6]. High resistance to apoptosis results in chemoresistance of MPM cells [7, 8]. Therefore, effective agents are needed to enhance apoptotic signals in order to improve survival of MPM cells.

There are few reports explaining the antiproliferative effect of resveratrol in MPM cells. Resveratrol induces apoptosis and affects cell cycle distribution of MPM cells [9–12]. However the exact mechanisms are not well understood. In our previous study, we observed that resveratrol induced apoptosis and showed antimetastatic effect on biphasic MPM cells [13]. Effect of resveratrol on cell viability depends on the histologic type of mesothelioma cell lines [12]. In this study our aim was to examine the in vitro effects of resveratrol on cell viability, apoptosis and cell cycle distribution of NCI-H2452 epithelioid MPM cells, and evaluate if reactive oxygen species (ROS) play a role on these effects.

Materials and methods

Cell culture and resveratrol treatment

Human epithelioid MPM cell line (NCI-H2452) was obtained from American Tissue Culture Collection (VA, USA). All cell culture materials were purchased from PAN Biotech GmbH (Aidenbach, Germany). The cells were grown in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin and incubated in 5% CO2 in a humidified atmosphere at 37°C.

Preparation of resveratrol

Resveratrol (Sigma Aldrich, MO, USA) was dissolved in DMSO to attain a stock concentration of 500 mM. This stock solution was kept at –20°C until used and diluted in culture medium to obtain the desired concentrations. The final concentration of DMSO was below 0.1% which is not cytotoxic for the cells.

Cell viability assay

Cell viability was evaluated by WST-1 assay kit (Roche, Mannheim, Germany). Briefly, cells were seeded into 96-well plates and left to adhere overnight. After the cells were treated with different resveratrol concentrations (5–200 μM) for 24 or 48 h, the medium was replaced with 100 μL fresh growth medium and then 10 μL reagent was added to each well. Subsequently the plate was incubated for an additional 2 h at 37°C and the amount of formazan compound produced by viable cells was measured in a spectrophotometric microtiter plate reader (Molecular Devices, CA, USA) at 440 nm wavelength. The absorbance values of treated cells were presented as percentage of the absorbance versus non-treated cells.
Cell cycle analysis

The effect of resveratrol on cell cycle distribution was evaluated using flow cytometry. Cells were seeded and then incubated with resveratrol. After incubation, the cells were trypsinized and collected in PBS. Centrifuged cells were suspended in PBS and fixed with 70% cold ethanol. Fixed cells were washed with PBS and then treated with 50 mg/mL PI and 2 mg/mL DNase-free RNase (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature in the dark [14]. The cellular DNA content was analyzed with FACS Calibur (BD, Franklin Lakes, NJ, USA). Sub G1, G0/G1, S and G2/M phases were calculated using Cell Quest software (Becton Dickinson, Tokyo, Japan).

Apoptosis analysis

Apoptosis was evaluated with Annexin V-fluorescein isothiocyanate (FITC) and PI staining (Chemicon International, Temecula, CA, USA) using flow cytometry. Briefly, the cells were seeded in a 6-well plate and treated with resveratrol. After harvesting, the cells were washed with PBS, resuspended in binding buffer and stained with PI and Annexin V-FITC according to the manufacturer’s instructions. At least 1 × 10⁴ stained cells were detected with a flow cytometer (FACS Calibur BD Biosciences, San Jose, CA, USA) and results were calculated with Cell Quest software (Becton Dickinson, San Diego, CA, USA). The percentage of the cells without treatment was set as 100%. Annexin V−/PI− cells were considered as the healthy cells, Annexin V−/PI− cells were considered as the early apoptotic cells and Annexin V+/PI+ cells were considered as the late apoptotic cells.

Intracellular ROS generation

Intracellular ROS production was evaluated by flow cytometry using the total ROS marker 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein-diacetate (H₂DCFDA) (Molecular Probes Co. OR, USA). Following the treatment with resveratrol, cells were collected and incubated with H₂DCFDA at 37°C for 30 min in the dark. The cells were washed with PBS and counted immediately using a FACS Calibur flow cytometer (BD Biosciences BD, USA). Data were acquired and analyzed with CellQuest software (Becton-Dickinson, Japan). The fluorescent signals were displayed as histogram and ROS generation in resveratrol-treated cells were compared to that in untreated cells.

Western blot analysis

The protein expressions of superoxide dismutase (EC1.15.1.1) (SOD-1, SOD-2), glutathione peroxidase (EC1.11.1.9) (GPX-4), p53 and cyclin D1 in the cells were analyzed by Western blot analysis. The cells were treated and then lysed. The whole-cell lysates were collected and centrifuged. Supernatants were transferred to fresh tubes and stored at −80°C for further use. The protein concentrations were measured with BCA Protein Assay Kit (Thermo Fisher Scientific, IL, USA). The proteins were separated with SDS-PAGE and then transferred on to nitrocellulose membranes. The membrane was blocked with 5% non-fat dry milk (Applichem, Darmstadt, Germany) and incubated with the primary antibodies against p53, SOD-1, SOD-2, GPX-4, actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin D1 and GAPDH (Thermo Fisher Scientific, IL, USA). Immunoblots were then probed with secondary antibodies conjugated with HRP: mouse anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-mouse IgG (Thermo Fisher Scientific, IL, USA). The protein of interest was detected by the Enhanced Chemi Luminescence detection kit (Santa Cruz Biotechnology, CA, USA) and then photographed by ChemiDoc XRS system (Bio-Rad). Digital blots were analyzed for relative band intensity using ImageJ gel analyzing software. The amount of each protein was calculated relative to the controls after normalization against the loading controls (GAPDH and actin).

Statistical analysis

All assays were performed at least three independent times, and each experiment was conducted in triplicate. The results were expressed as mean ± SD. SPSS software (version 13.0; SPSS Inc., IL, USA) was used for the assessment. Comparison of two groups were performed using Student’s t-test while comparison of more than two groups were performed using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. p < 0.05 was considered as statistically significant.

Results

Resveratrol reduces the cell viability of the NCI-H2452 cells in a time and dose dependent manner

We have first evaluated the effect of resveratrol on the viability of the NCI-H2452 cells. As shown in Figure 1A,
resveratrol reduced cell viability in a dose and time dependent manner. There was insignificant and minimal reduction on cell viability at the concentrations <100 μM of resveratrol after 24 h treatment but a significant reduction occurred at 5 μM resveratrol concentration after 48 h treatment (p < 0.05). After 24 h, at 200 μM concentration, the cell viability of treated cells was decreased by ~50% when compared to control cells (p < 0.05). Therefore 200 μM concentration and 24 h time point were chosen for subsequent experiments.

In the meanwhile the percentage of cells in G2/M phase significantly decreased when compared with the control group (treated vs. control; 17 ± 3% and 31 ± 5%, p < 0.05) (Figure 1B). Additionally, it was observed that resveratrol decreased the expression of cyclin D1 in total cell lysates (Figure 2A).

**Resveratrol stimulates apoptosis and increases the expression of p53 in NCI-H2452 cells**

The apoptotic index was determined by flow cytometry, in order to figure out if anti-cell viability effect of resveratrol was caused by induction of apoptosis, besides cell cycle arrest. Resveratrol induced apoptotic cell death. The percentage of apoptotic cells increased from 11 ± 7% in control cells to 23 ± 7% in treated cells (p < 0.05), while the percentage of the healthy cells decreased from 79 ± 3% to 56 ± 11% (p < 0.05) (Figure 2B). Additionally, to assess the role of p53 in resveratrol-induced cell cycle accumulation...
at G0/G1 phase and induction of apoptosis, we investigated the expression of p53 after resveratrol treatment. Treatment increased p53 protein expression by \( \sim 139\% \) (\( p < 0.05 \)) (Figure 2C).

### Discussion

Resveratrol is a natural polyphenol present at high levels in grapevines, pines, legumes and nuts. It has beneficial effects to improve health and prevent and/or treat many diseases. Its anti-tumor effects have also been reported previously [1]. Initially Jang et al. concluded that resveratrol might be a possible chemopreventive agent for skin cancer [2]. Following this report, anti-cancer effects
of resveratrol have been observed in many other cancer types such as lymphoma, leukemia, melanoma, breast, colon, pancreatic, gastric, prostate, liver, lung, thyroid, ovarian and endometrial cancers through multiple regulatory mechanisms [15].

The effects of resveratrol on MPM cell lines were also investigated in a few studies. It was proved that resveratrol decreases the cell viability of MPM cells [9, 12]. We observed that resveratrol decreased cell viability of epithelioid MPM cells in a time and dose dependent manner consistent with previous findings. A biphasic effect of resveratrol on tumor cell viability is well known. In a previous study, at lower concentrations (<30 μM) resveratrol increased the cell viability in different cancer cells such as liver and breast cancer cells. However, at higher concentrations (≥50 μM) the cytotoxicity of resveratrol was increased in a dose-dependent manner [16]. IC50 values reported in different studies for resveratrol in MPM cells vary [12, 17]. The change in these values depends on the exposure time and the subtype of MPM cells. It was shown that resveratrol (60 μM) decreased cell viability of MSTO-211H (biphasic MPM) cells to ~65% after 24 h treatment whereas ~55% after 48 h treatment [17]. It was also proven that biphasic MPM cells are more sensitive to resveratrol than epithelioid MPM cells. Resveratrol (120 μM) treatment for 48 h decreased the cell viability of NCI-H2452 cells to ~45% but the cell viability of MSTO 211H cells to ~35% [12].

The mechanisms under anti-tumor effects of resveratrol are not clear but the focus is on (1) anti-oxidant and pro-oxidant activity, (2) modulation of transcription factors, (3) regulation of inflammation, (4) regulation of cell cycle progression, (5) stimulation of apoptosis, and (6) inhibition of tumor invasion and angiogenesis [3].

The effect of resveratrol on cell cycle distribution depends on the cell type. For example resveratrol induced cell cycle arrest in S phase in HepG2 (liver cancer) cells and MCF-7 (breast cancer) cells whereas in MDA-MB-231 (breast cancer) cells in G1 phase at the same concentration [16]. There are also reports that did not observe any difference in cell cycle distribution with resveratrol [18]. The different phase accumulation was also observed depending on the concentration of resveratrol. It induced S phase arrest at lower concentration (150 μM), but caused an increase in G1 phase at higher concentration (250 μM) in cervical cancer cells [19]. We have shown that resveratrol induced cell cycle arrest in G0/G1 phase in NCI-H2452 cells at 200 μM concentration with 24 h treatment alone. Lee et al. [12] observed a decreased percentage of cells in G1 and S phases but an increased percentage of cells in G2/M phase with resveratrol alone or combination with cisplatin in same cell line.

Because resveratrol induced cell cycle arrest in G0/G1 phase in the current study, we were interested in cyclin D1 protein expression level, which has a critical cell cycle
Resveratrol induces ROS production at a low dose (30 μM) and exposure time [28]. It was shown that oxidant and anti-oxidant properties depending on the variety of cancer cells [26, 27]. Resveratrol has both pro-apoptosis through mitochondrial ROS production in NCI-H2452 cells. This effect might be caused by the decreased expression of cyclin D1 at the translation level in nucleus or increased degradation of cyclin D1 in cytosol. Cyclin D1 can act as an oncogene and contributes to chemoresistance of cancer cells [21]. Cytoplasmic sequestration of cyclin D1 and defective degradation of cyclin D1 in cytoplasm of some cancer cell lines has been shown [22]. It was also hypothesized that cytoplasmic cyclin D1 could be a marker of metastatic potential [23]. Consistent with our data it was observed that resveratrol decreases cyclin D1 levels in other types of cancer cells. It is believed that the effect of resveratrol on cyclin D1 degradation is cell line specific [15].

Apoptosis, which is another important mechanism to eliminate cancer cells, might be triggered by resveratrol [24]. MPM cells are resistant to apoptosis, thus current treatments are ineffective in MPM [25]. Lee et al. [9] showed that resveratrol caused increased apoptotic cell death in MSTO-211H, biphasic MPM cells. We observed that resveratrol induced apoptosis of NCI-H2452, epithelioid MPM cells. This data is consistent with a previous study which showed resveratrol down-regulated Mcl-1, an anti-apoptotic protein [10].

p53, a tumor suppressor protein, plays a central role in cell cycle and apoptosis and is regulated by resveratrol [15, 19]. In a previous study treatment of MPM cells with 15 μM resveratrol for 24 h did not change the protein expression of p53 in whole cell lysates [11], however in another study, treatment with 30 μM of resveratrol for 48 h caused increased protein expression of p53 in the same type of MPM cells [12]. In our study we observed increased expression of p53 in treated cells compared to controls at 200 μM resveratrol concentration after 24 h. These results suggest that resveratrol induces apoptosis and causes cell cycle arrest through activation of p53 in NCI-H2452 cells.

Previous studies indicated that resveratrol induces apoptosis through mitochondrial ROS production in a variety of cancer cells [26, 27]. Resveratrol has both pro-oxidant and anti-oxidant properties depending on the concentration and exposure time [28]. It was shown that resveratrol induces ROS production at a low dose (30 μM) after 48 h treatment in MPM cells [12]. In this study, we detected increased ROS production in the cells treated with higher resveratrol concentration (200 μM) for a shorter time (24 h). This suggests that resveratrol-induced apoptosis in NCI-H2452 cells involves the production of ROS at this concentration and time point.

GPX, SOD-1 and SOD-2 are antioxidant enzymes, which protect tissues against the harmful effects of ROS. They might counteract increased ROS production caused by resveratrol. It was found that the effect of resveratrol on the activity of antioxidant enzymes is disproportional and cancer-specific. It also depends on the concentration of resveratrol and cell type [29]. In our previous study, we observed that SOD-2 was induced by resveratrol in biphasic MPM cells, which is another histologic subtype of MPM [13]. On the other hand, here we found that resveratrol did not have any effect on the expression of GPX, SOD-1 and SOD-2 enzymes in NCI-H2452 cells at this concentration and time point. We therefore speculate that ROS generation was increased by resveratrol and increased antioxidant enzymes were used to eliminate this excess ROS generation and decreased to normal levels in the cells.

Optimal antioxidant enzyme levels are crucial for protecting cells from cancer development. Decreased activity or expressions of antioxidant enzymes were shown in some cancer cells [30]. It is known that decreased antioxidant enzyme activity and expression in a cell can cause carcinogenesis. On the other hand, it was also found that the activity of SOD was significantly up-regulated but the activity of GPX was unchanged by resveratrol in prostate, liver and breast cancer cells [29]. In the meanwhile, increased antioxidant activity by resveratrol may cause unwanted effects. Since the mode of action of some chemotherapeutics involves increasing free radical levels in cancer cells and increased activity of antioxidants can cause increased survival of cancer cells and development of drug resistance. So, no effect of resveratrol on antioxidant enzymes observed in the current study might be favorable.

In conclusion, our study showed that resveratrol causes a decrease in cell viability in a concentration and time dependent manner in epithelioid MPM cells. Some of the reasons for this inhibition may result from G0/G1 phase arrest in cell cycle and apoptosis. Our results indicate that these effects might be related to down-regulation of cyclin D1 and up-regulation of p53. Another reason of resveratrol-induced cell viability inhibition might be its ability to induce ROS production. Accordingly, resveratrol may be a potential complementary agent in the treatment of epithelioid MPM.
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