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Resveratrol induces apoptosis of human malignant B cells by activation of caspase-3 and p38 MAP kinase pathways

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ABSTRACT

Red wine polyphenol, trans-resveratrol (trans-3,4′,5-trihydroxy stilbene), has potent chemopreventive effects against various tumors. In this study, we found for the first time that resveratrol rapidly induces S phase cell cycle arrest of human malignant B cells including myeloma cells in dose- and time-dependent manners, followed by S phase cell cycle arrest through ATM/Chk pathway. Resveratrol-induced apoptosis occurs in association with the activation of caspase-3 and the loss of mitochondrial transmembrane potentials. In addition, resveratrol induces the phosphorylation of p38 MAP kinase, and specific inhibition of p38 MAP kinase abolishes the resveratrol-induced apoptosis, indicating that activation of the p38 MAP kinase pathway is required for inducing apoptosis in malignant B cells. These results suggest that resveratrol may have potential as a novel therapeutic agent for the patients with B cell malignancies including multiple myeloma.

1. Introduction

Resveratrol (trans-3,4′,5-trihydroxystilbene), a naturally occurring polyphenolic phytoalexin, is found in a wide variety of plants. Resveratrol has been produced by the skin of red grapes, Japanese knotweed (*Polygonum cuspidatum*), peanuts, and mulberries [1]. It has structural similarities to estradiol and diethylstilbestrol [2]. In plants, resveratrol functions microbiologically as a phytoalexin that protects against fungal infection [1]. The epidemiological finding of an inverse relationship between consumption of red wine and the incidence of cardiovascular disease has been called the "French paradox" [3]. For a variety of reasons, the cardioprotective effects of red wine have been attributed to resveratrol [4]. These effects have been reported to include suppression of platelet aggregation, anti-oxidant, anti-inflammatory, and vasorelaxant activities [5]. In addition, numerous reports have indicated that resveratrol has anti-viral and anti-bacterial effects [6,7]. Recently, in addition to cardioprotective effects, resveratrol has also been found to exhibit anti-cancer properties, as suggested by its ability to suppress proliferation of a wide variety of tumor cells, including leukemia and cancers of the breast, lung, stomach, colon, liver, pancreas, and prostate [8]. However, there have been no reports regarding resveratrol's biological effect on the proliferation of cells from B cell malignancies including multiple myeloma. In addition, the precise mechanisms of resveratrol-induced apoptosis in tumor cells remain unclear.

Multiple myeloma is a typical B cell malignancy characterized by latent accumulation in bone marrow of secretory

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plasma cells with a low proliferative index and extended life span [9]. Various anti-cancer agents are used for the treatment of myeloma, including combinations of vincristine, bis-2-chloroethylnitrosourea (BCNU), melphalan, cyclophosphamide, adriamycin, and prednisolone or dexamethasone [10]. Recently, patients younger than 65 years have been treated with high-dose melphalan with autologous stem-cell support, and older patients who cannot tolerate such intensive treatment have received standard-dose oral melphalan and prednisolone (MP) therapy. Despite these treatments, multiple myeloma still remains an incurable hematological malignancy, with a complete remission rate of approximately 5% and a median survival of 30–36 months [11,12]. Severe side effects and complications such as serious infection due to anti-cancer drugs are also major problems in clinical settings. In particular, side effects of anti-cancer drugs can be fatal in older patients or immunocompromised patients. In addition, repeated episodes of relapse of disease may lead to refractory or chemotherapy-resistant multiple myeloma. Novel, effective, and less toxic therapeutic strategies are therefore desired in order to improve the outcomes of patients with multiple myeloma.

In the present study, we investigated the effects of resveratrol on cellular growth, cell cycle distribution, and apoptosis in human malignant B cell lines, IM9 and HS-sultan cells. Resveratrol was found to induce inhibition of cellular growth of both malignant B cell lines in dose- and time-dependent manners as mediated through induction of apoptosis. Interestingly, treatment of myeloma cells with resveratrol was found to markedly induce S phase cell cycle arrest followed by apoptosis. We further investigated the molecular mechanisms of resveratrol-induced apoptosis in human malignant B cells.

2. Materials and methods

2.1. Cells and cell culture

Human malignant B cell lines including myeloma cell line (IM9), and Burkitt's lymphoma cell line (HS-sultan) were cultured in RPMI1640 medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL) in a humidified atmosphere with 5% CO_2. These malignant B cell lines were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan). The morphology was evalu-

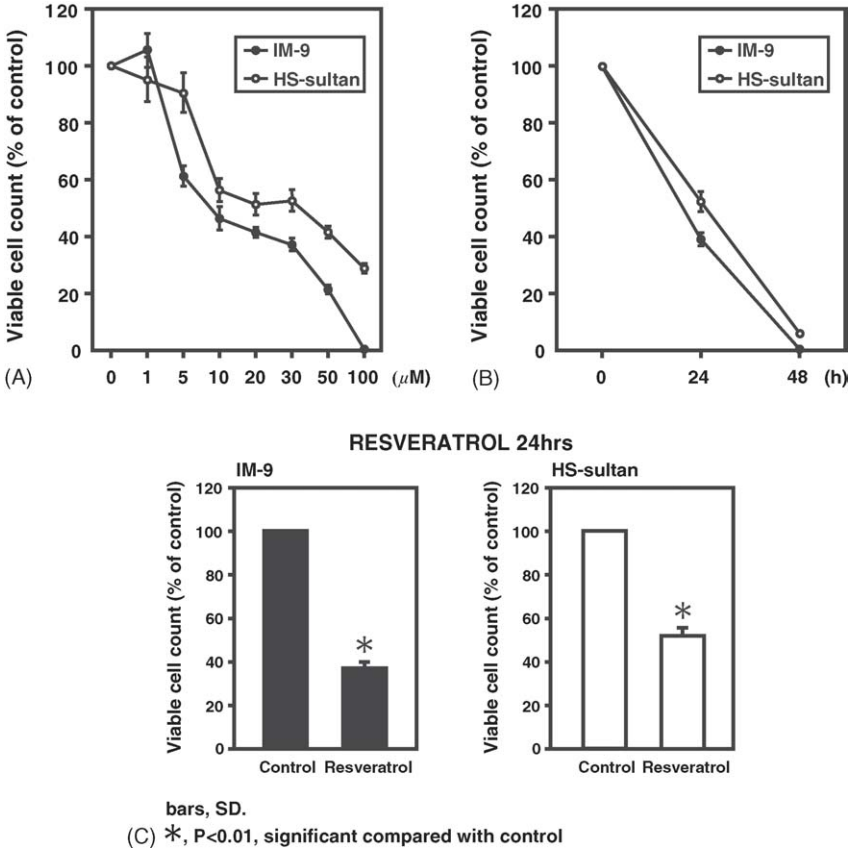

Fig. 1 – Resveratrol inhibits the growth of malignant B cells via the induction of apoptosis. (A) Human malignant B cell lines, IM9 and HS-sultan cells, were treated with resveratrol at various concentrations (0–100 μM) for 24 h. (B) IM9 and HS-sultan were treated with 30 μM resveratrol for 0, 6, 12, 24, and 48 h. Cell viability was assessed by trypan blue dye exclusion. Results are expressed as the mean of three different experiments, and the S.D. was within 10% of the mean. (C) Both IM9 and HS-sultan cells were treated with 30 μM resveratrol for 24 h, and then cell viability was assessed by trypan blue dye exclusion. Data are mean ± S.D. of two independent experiments in triplicate. Effect of resveratrol is statistically significant in comparison with control ($P < 0.01$).

ated by cytospin slide preparations with Giemsa staining and the viability was assessed by trypan blue dye exclusion.

2.2. Reagents

Trans-resveratrol (trans-3,4′,5-trihydroxy stilbene) and p38 MAP kinase-specific inhibitor (SB203580) was purchased from Sigma Chemical Co. (St. Louis, MO). Resveratrol was dissolved in DMSO.

2.3. Cell cycle analysis

Cells (1×10^5) were suspended in hypotonic solution (0.1% Triton X-100, 1 mM Tris–HCl (pH 8.0), 3.4 mM sodium citrate, 0.1 mM EDTA) and stained with 50 μg/ml of PI. The DNA content was analyzed by flow cytometry. The population of cells in each cell cycle phase was determined using ModiFIT software (Becton Dickinson).

2.4. Assays for apoptosis

Apoptosis was determined by morphological change as well as by staining with annexin V-FITC and -PI labeling. Apoptotic cells were quantified by annexin V-FITC and -PI-double staining by using a staining kit purchased from PharMingen (San Diego, CA). The mitochondrial transmembrane potential ($\Delta\Phi_m$) was determined by flow cytometry (FACS Calibur; Becton Dickson, San Jose, CA). Briefly, cells were washed twice with PBS and incubated with 1 μg/ml Rhodamine 123 (Sigma) at 37 °C for 30 min. The Rhodamine 123 intensity was determined by flow cytometry.

2.5. Caspase assays

Activation of caspase-3 was analyzed using a commercially available caspase-3 assay kit from PharMingen. Briefly, FITC-conjugated antibody against the active form of caspase-3 provided in the kit was used for FACS analysis according to the manufacturer's instructions (PharMingen). In the caspase inhibitors assay, cells were pretreated with a synthetic pan-caspase inhibitor (50 μM, Z-VAD-FMK) or caspase-3 specific inhibitor (5 μM, DEVD-CHO) for 1 h prior to addition of resveratrol (30 μM). Pan-caspase inhibitor and caspase-3 specific inhibitor were purchased from Calbiochem (La Jolla, CA).

2.6. Cell lysate preparation and Western blotting

Cells were collected by centrifugation at 700 × *g* for 10 min, and the pellets were then resuspended in lysis buffer (1% NP-40, 1 mM phenylmethysulfonyl fluoride (PMSF), 40 mM Tris–HCl (pH 8.0), 150 mM NaCl) at 4 °C for 15 min. Protein concentrations were determined using a protein assay DC system (Bio-Rad, Richmond, CA). Cell lysates (20 μg protein/lane) were fractionated in 12.5% SDS polyacrylamide gels prior to transfer to the membranes (Immobilon-P membranes, Millipore, Bedfold, MA) using a standard protocol. Antibody binding was detected by using an enhanced chemiluminescence kit for Western blotting detection with Coomassie brilliant blue to confirm equal amounts of protein extract on each lane. The following antibodies were used in this study (1:1000 dilution): Bcl-2, Mcl-1, Chk1, Chk2, Cyclin A, Cyclin E, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), Bax (MBL, Nagoya, Japan), phospho-ATM (Rockland,

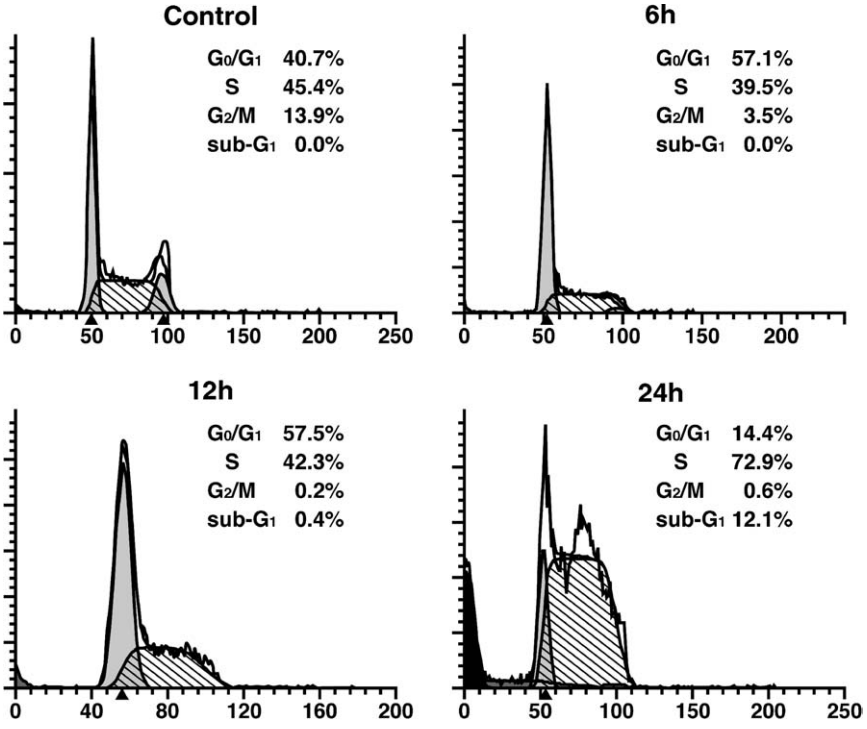

Fig. 2 – Cell cycle analysis of IM9 cells cultured with resveratrol. Cells were cultured with 30 μM resveratorol for 24 h and then stained with PI. The DNA content was analyzed by means of flow cytometry. G_0/G_1, G_2/M, and S indicate cell phase, and sub-G_1 DNA content refers to apoptotic cells. Each phase was calculated using the ModiFIT program. Data shown are from representative experiments repeated three times with similar results.

Gilbertsville, PA), phospho-Chk1 (Ser345), phospho-Chk2 (Thr160), phospho-CDK2 (Thr160), p38 MAP kinase, phospho-p38 MAP kinase (Cell Signaling, Beverly, MA). Secondary antibodies (1:3000 dilution) conjugated with horseradish peroxidase were obtained from Kirkegaard Perry Laboratories Inc. (Gaithersburg, MA).

2.7. Statistical analysis

Experiments were repeated at least three times, and probability (P) was calculated using a Student *t*-test. *P* values of $>.05$ were considered statistically significant.

3. Results

3.1. Resveratrol inhibited cellular proliferation of human malignant B cells

We first examined whether resveratrol-induced inhibition of the growth of human malignant B cells including myeloma cells (IM9), and Burkitt's lymphoma cell line (HS-sultan). We examined the inhibitory effects of IM9 and HS-sultan cells cultured with 0–50 μM resveratrol for 0–48 h (Fig. 1A and B). Resveratrol inhibited proliferation of IM9 and HS-sultan cells in dose- and time-dependent manners. In addition, resveratrol significantly inhibited proliferation of both IM9 and HS-sultan cells ($P < 0.01$) (Fig. 1C). IM9 cells were more sensitive to resveratrol with an IC_{50} (7.5 μM) compared to HS-sultan cells; we therefore used myeloma cell line IM9 cells for the additional experiments.

3.2. Resveratrol-induced S phase cell cycle arrest and subsequent apoptosis

The effects of resveratrol on cell cycle progression were investigated using IM9 cells. The cells were treated with 30 μM resveratrol for 0–24 h and analyzed for cell cycle distribution by means of flow cytometry. Cultivation with resveratrol of IM9 cells increased the population of cells in the S phase, with a reduction of cells in the G_1 and G_2/M phase (Fig. 2). In addition, induction of apoptosis was shown by the appearance of a haplodiploid DNA peak with sub-G_1 DNA contents at 24 h after treatment (Fig. 2). These results

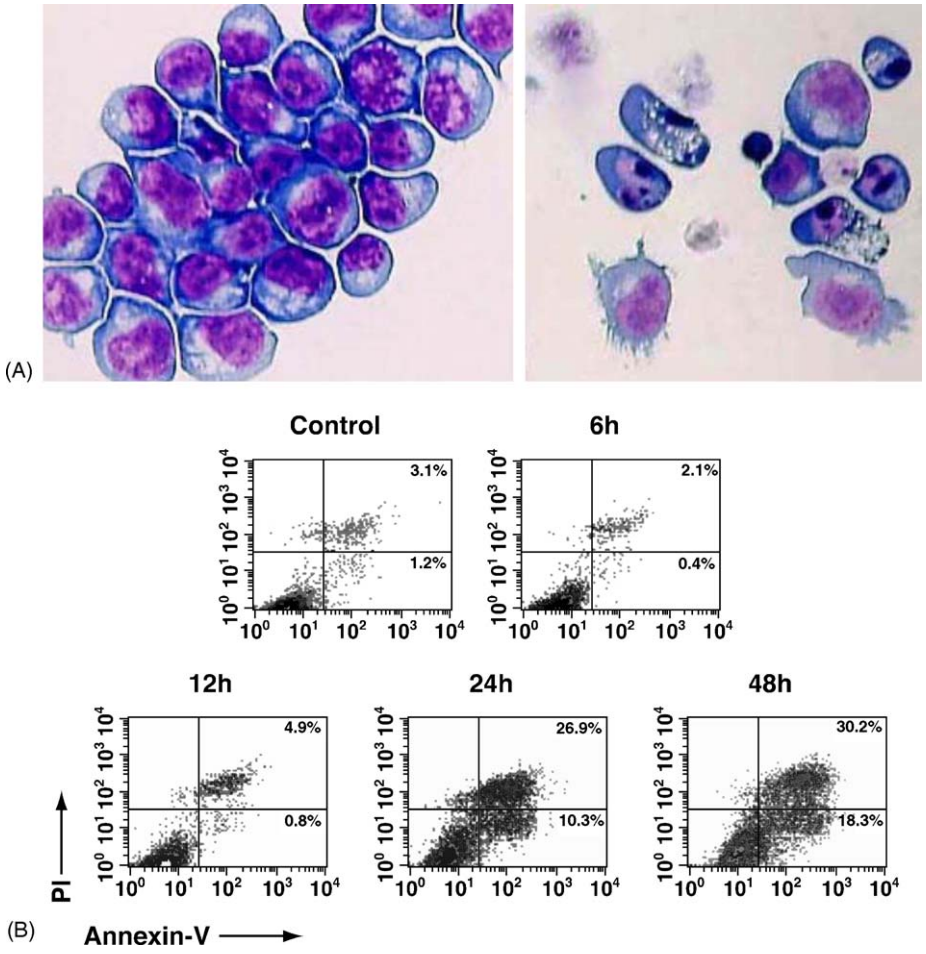

Fig. 3 – (A) Morphological changes characteristic of apoptosis in IM9 cells. IM9 cells were treated with 30 μM resveratorol for 24 h, and cytospin slides were then prepared and stained with Giemsa (original magnification, 400×). (B) Detection of apoptotic cells by annexin V and PI-double-staining. IM9 cells were cultured with 10 μM resveratrol for 0, 6, 12, 24, and 48 h, stained with annexin V-FITC and -PI labeling, and were analyzed by flow cytometry. The percent digits refer to the annexin V positive cells. Three independent experiments were performed, and all gave similar results.

indicate that resveratrol led to cell cycle arrest at the S phase followed by apoptosis.

3.3. *Resveratrol-induced apoptosis in IM9 cells*

We confirmed resveratrol-induced apoptosis by means of morphology and annexin V/PI-double staining. IM9 cells was cytospun onto glass slides for morphological study after treatment with 30 μM resveratrol for 24 h. Apoptotic bodies were easily found by Giemsa staining in resveratrol-treated cells but not in control cells (Fig. 3A). To quantify the percentage of apoptosis caused by resveratrol, IM9 cells were subjected to annexin V/PI-double staining. Annexin V positive IM9 cells dramatically increased in a time-dependent manner, indicating that resveratrol-induced apoptosis of myeloma cells had occurred (Fig. 3B).

3.4. *Effects of resveratrol on caspase-3 activity*

Caspases are believed to play a central role in mediating various apoptotic responses. To address the apoptotic pathway in resveratrol-treated IM9 cells, we next examined the activation of caspase-3 by FACS analysis after incubation with 30 μM resveratrol for 24 h. The percentage of IM9 cells expressing the active form of caspase-3 was increased (Fig. 4A). Furthermore, to elucidate the functional role of

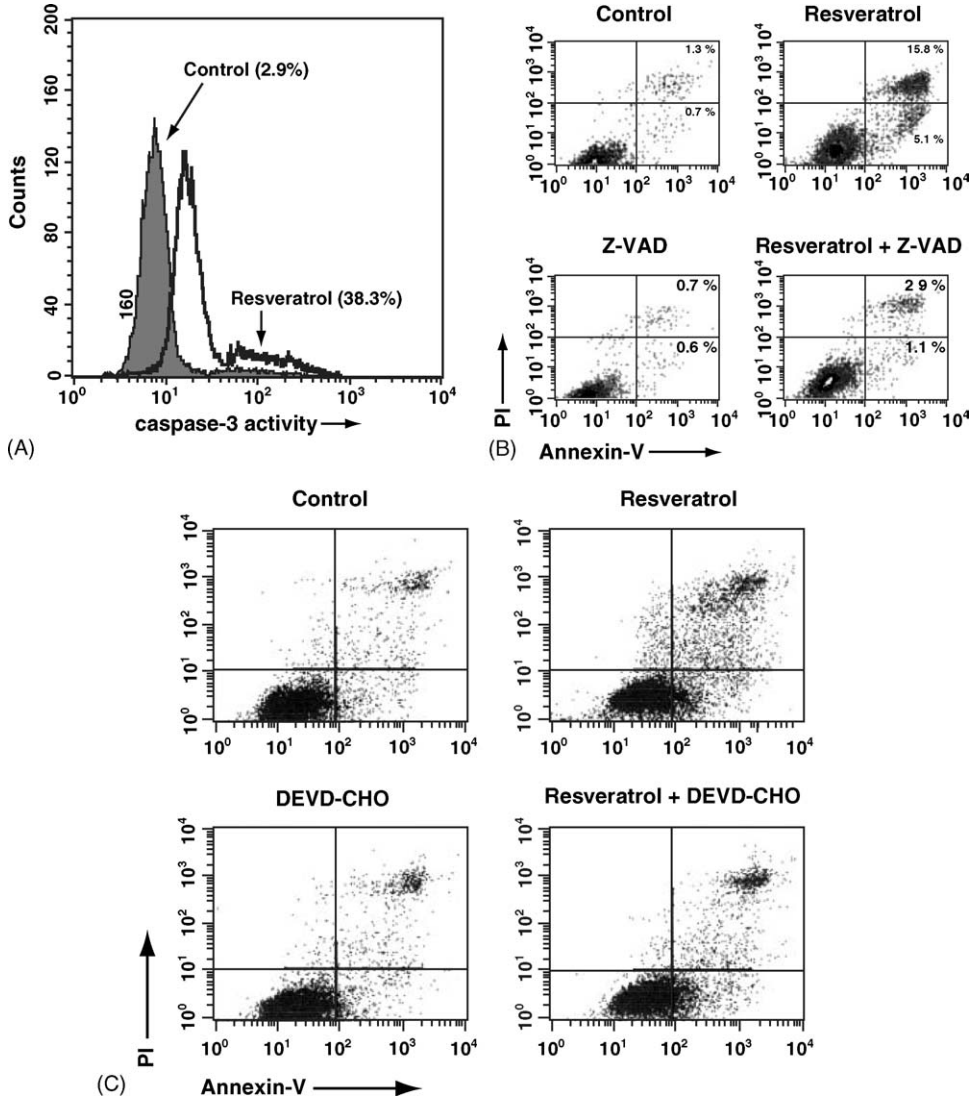

Fig. 4 – Effects of resveratrol on caspase activation. (A) Detection of the active form of caspase-3 by flow cytometry. IM9 cells were treated with 30 μM resveratrol for 24 h. The percentage of cells expressing the active form of caspase-3 was determined using the specific antibody by flow cytometry. (B) Effects of caspase inhibitor on resveratrol-treated IM9 cells. Inhibition of resveratrol-induced apoptosis of IM9 cells was estimated in a co-culture with the pan-caspase inhibitor, Z-VAD-FMK. Cells were preincubated with Z-VAD-FMK for 1 h prior to the addition of 30 μM resveratrol. The percent digits mean the annexin V positive cells. Three independent experiments were performed, and all gave similar results. (C) Effect of caspase-3 specific inhibitor (DEVD-CHO) on resveratrol-treated IM9 cells. IM9 cells were pre-treated 5 μM DEVD-CHO for 1 h before the addition of 30 μM resveratrol. Cells were then stained with annexin V-FITC and PI labeling and were analyzed by flow cytometry. Three independent experiments were carried out, and all gave similar results.

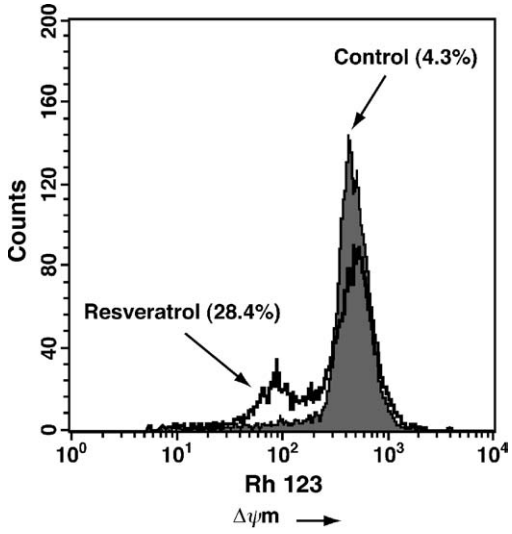

Fig. 5 – Flow cytometric analysis of $\Delta\psi_m$ as estimated by Rh123 intensity. IM9 cells were cultured with 30 μM resveratorol for 24 h, and Rh123 fluorescence was analyzed by flow cytometry.

caspase-3 in resveratrol-induced apoptosis, experiments were performed with a caspase inhibitor. IM9 cells were treated with 30 μM resveratrol for 24 h, either alone or in combination with Z-VAD-FMK (pan-caspase inhibitor). Resveratrol-induced apoptosis was completely blocked by the treatment with Z-VAD-FMK (Fig. 4B). In addition, pre-treatment of caspase-3 specific inhibitor (DEVD-CHO) inhibited resveratrol-induced apoptosis in IM9 cells (Fig. 4C). These results suggest that resveratrol-induced apoptosis is associated with the activation of caspase-3.

3.5. *Resveratrol-induced death signaling is mediated through the mitochondrial pathway*

Recent studies have suggested that mitochondria play an essential role in death signal transduction. Mitochondrial changes, including permeability transition pore opening and the collapse of the mitochondrial Δψm, subsequently cause apoptosis by the activation of caspases. After treatment with 30 μM resveratrol for 24 h, Rh123 staining in IM9 cells indicated an increase in the loss of mitochondrial $\Delta\psi_m$ (Fig. 5). The loss of $\Delta\psi_m$ appeared in paralell with the activation of caspase-3, as well as with apoptosis (data not shown).

3.6. *Expression of cell cycle- and apoptosis-associated proteins in resveratrol-treated IM9 cells*

To characterize the mechanism of resveratrol-induced S phase cell cycle arrest and subsequent apoptosis in IM9 cells, the expression of cell cycle- and apoptosis-associated proteins was examined during treatment with resveratrol. Resveratrol was found to induce the phosphorylation of ATM, the Ser345 residue of Chk1 and the Thr68 residue of Chk2 kinases (Fig. 6A). In addition, resveratrol treatment induced activation of CDK2 and expression of Cyclin A and E (Fig. 6A). We next examined the expression of apoptosis-associated proteins during treatment with resveratrol. Resveratrol-induced expression of pro-apoptotic Bax protein and reduced expression of anti-apoptotic Mcl-1 protein (Fig. 6B). However, anti-apoptotic Bcl-2 protein was not modulated in the treatment of resveratrol (Fig. 6B).

3.7. *Resveratrol-induced apoptosis via phosphorylation of p38 MAP kinase*

We observed that resveratrol-induced caspase-dependent apoptosis in IM9 cells. To investigate the resveratrol-induced intracellular signaling pathways for apoptosis, we analyzed the effects of p38 mitogen-activated protein (MAP) kinase inhibitor SB203580 on resveratrol-induced apoptosis in myeloma cells. IM9 cells were treated with 30 μM resveratrol for 24 h, either alone or in combination with SB203580. Resveratrol-induced apoptosis was completely blocked by the treatment with SB203580 (Fig. 7A). In addition, we analyzed the levels of p38 MAP kinase phosphorylation after treatment with resveratrol in IM9 cells by Western blotting. Resveratrol (30 μM) was found to have induced higher levels of p38 MAP kinase phosphorylation on 30 min after treatment with IM9 cells (Fig. 7B). Taken together, these results indicate that resveratrol induces apoptosis of myeloma cells via phosphorylation of p38 MAP kinase.

4. Discussion

The mechanisms of resveratrol-induced suppression of cellular proliferation have been reported to involve the induction of apoptosis through Fas/CD95, mitochondrial, and p53 mediated pathways [13–15]. In addition, resveratrol has been reported to suppress NF-κB and protein kinase C activity, and to modulate the angiogenesis and expression of various growth factors as well as NO/NOS [16–20]. In most experiments, resveratrol was used at concentrations ranging from 5 to 100 μM [21].

In the present study, we have demonstrated that resveratrol induces apoptosis in human malignant B cells including myeloma cells. We found that resveratrol induces activation of caspase-3 and reduces the mitochondrial electric potential in IM9 myeloma cells. The pan-caspase inhibitor (Z-VAD-FMK), and caspase-3 specific inhibitor (DEVD-CHO) prevented the occurrence of resveratrol-induced apoptosis. Apoptotic bodies were observed in IM9 cells 24 h after treatment with resveratrol. We demonstrated that resveratrol-induced expression of pro-apoptotic Bax protein, but down-regulated the expression of anti-apoptotic Mcl-1 protein. However, the level of Bcl-2 was not changed by exposure of the cells to resveratrol. These results indicate that resveratrol-induced apoptosis is mediated through caspase-3 and mitochondrial pathways.

Several reports have indicated that resveratrol inhibits proliferation of cells by inhibiting cell cycle progression at different stages of the cell cycle. The arrest of cells in G_1 phase, S phase, M/G_2 phase and G_2 phase of the cell-cycle have been reported [16,19,22,23]. We found that resveratrol was able to induce S phase cell cycle arrest followed by apoptosis in IM9

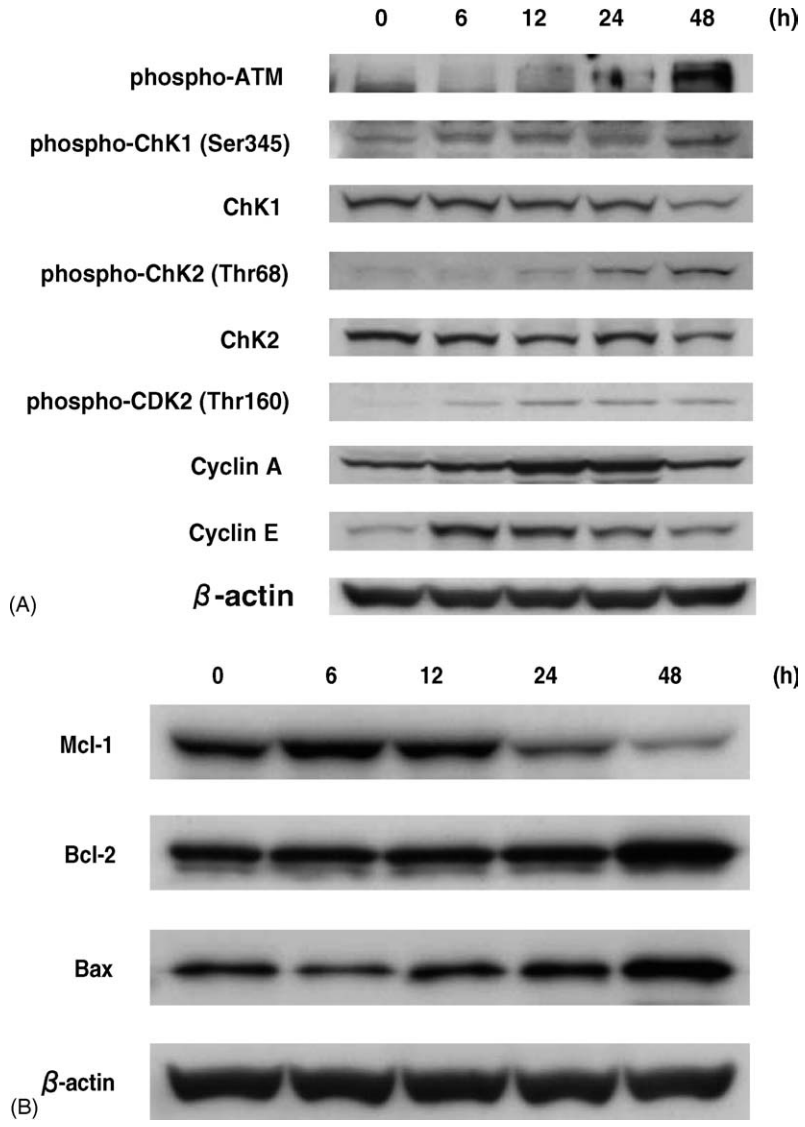

Fig. 6 – Expression of cell cycle (A)- and apoptosis (B)-associated proteins. IM9 cells were treated with 30 μM resveratrol for the indicated times. Cell lysates (20 μg/lane) were fractionated on 12.5% SDS-polyacrylamide gels and analyzed by Western blotting. Re-blotting with β-actin staining demonstrated that equal amounts of proteins were present in each lane.

myeloma cells. Our present study identified resveratrol activity in IM9 cells via ATM check point signaling-dependent S phase arrest. We also found that ATM activation by resveratrol induces Chk1 (Ser345) and Chk2 (Thr68) kinases followed by the activation of CDK2, and the accumulation of Cyclin A and E. These results are compatible with the previous study that resveratrol causes S phase arrest in human ovarian cancer cells through ATM/Chk pathway [24].

The p38 MAP kinase cascade is primarily activated by environmental stresses such as osmotic shock, ultraviolet irradiation, heat shock, and pro-inflammatory cytokines [25,26]. Abundant evidence of p38 MAP kinase involvement in apoptosis currently exists and is based on concomitant activation of p38 MAP kinase and apoptosis-induced by a variety of agents [27]. p38 MAP kinase may function both upstream and downstream from the signaling cascade of caspases in apoptosis [28]. While p38 MAP kinase signaling has been shown to promote cell death in some cell lines, p38 MAP kinase has been shown to enhance survival, cell growth, and differentiation in different cells. It must be mentioned that the role of p38 MAP kinase in apoptosis is dependent on cell types and stimuli [29], but its role in pathogenesis in multiple myeloma is unclear. In the mouse epidermal cell line, resveratrol activated p38 MAP kinase and then formed a complex with p53. It has reported that p38 MAP kinase mediates resveratrol-induced activation of p53 and p53-dependent apoptosis [30]. It has also been reported that the specific p38 MAP kinase inhibitor VX-745 inhibits paracrine myeloma cell growth associated with the down-regulation of IL-6 and VEGF secretion in bone marrow stromal cells [31]. Furthermore, p38 MAP kinase inhibition blocks TNFα-induced IL-6 secretion in bone marrow stromal cells, thereby inhibiting myeloma cell growth and survival [32]. In the present study, we first found that resveratrol induces the phosphorylation of

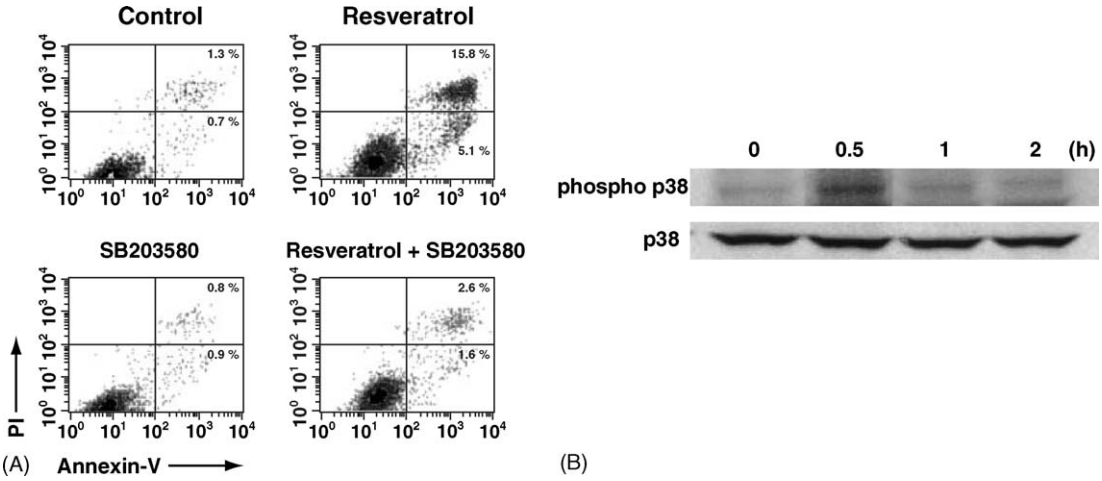

Fig. 7 – Resveratrol induces apoptosis via phosphorylation of p38 mitogen-activated protein (MAP) kinase in IM9 cells. (A) Effects of p38 MAP kinase inhibitor on resveratrol-treated IM9 cells. Inhibition of resveratrol-induced apoptosis of IM9 cells was estimated in a co-culture with p38 MAP kinase inhibitor SB203580. Cells were preincubated with SB203580 (10 μM) for 1 h prior to the addition of 30 μM resveratrol. The percent digits mean the annexin V positive cells. Three independent experiments were performed, and all gave similar results. (B) Western blot analyses of phosphorylation of p38 MAP kinase in resveratrol-treated cells. IM9 cells were treated with 30 μM resveratrol for the indicated times. Phosphorylation of p38 MAP kinase was analyzed using phosphor-specific antibody (upper lane). Membranes were re-probed with anti-p38 MAP kinase antibody to confirm equal protein loading (lower lane).

p38 MAP kinase in IM9 myeloma cells. The specific inhibitor of p38 MAP kinase, SB203580, prevented the resveratrol-induced apoptosis of IM9 cells, indicating that resveratrol induces apoptosis in myeloma cells through the activation of p38 MAP kinase. These results are consistent with recent reports that have demonstrated a requirement of the p38 MAP kinase pathway in UV- and γ-irradiation-induced G_2/M cell cycle arrest followed by apoptosis [33–35]. While these observations suggest that activation of p38 MAP kinase may account for the anti-proliferative activity of resveratrol in IM9 myeloma cells, the precise mechanisms may be complex and remain to be clarified.

One of the potential mechanisms by which myeloma cells could develop resistance to apoptosis is through the activation of NF-κB, a nuclear transcription factor that regulates the expression of various genes [36]. Resveratrol exhibits anti-inflammatory, cell growth-modulatory, and anti-carcinogenic effects, that it mediates these effects by suppressing NF-κB. In addition, it has reported that resveratrol suppresses the production of IL-1β and its effects on activation of NF-κB in myeloid leukemic cells [16,37]. We therefore investigated the effect of resveratrol on NF-κB activation in myeloma cells, but found that it did not suppress NF-κB activity (data not shown).

Resveratrol, a component of red wine, is a natural compound, and it appears to be safer than current chemotherapeutic drugs. Therefore, resveratrol might be developed as a new potent anti-cancer agent for the management of hematologic malignancies, because whereas the inhibitory effects of resveratrol on hematopoietic progenitors is partially reversible [38], the effects of myeloma cells are largely irreversible, as shown in this study. In particular, resveratrol might be useful in older patients or in immunocompromised patients because of its safety and lack of known toxicity. In conclusion, this component of red wine may have potential as a novel therapeutic agent to replace or augment the more cytotoxic agents currently used to treat patients with human B cell malignancies including multiple myeloma.

Acknowledgments

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