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Resveratrol Induces Apoptosis of KB Human Oral Cancer Cells

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Resveratrol (trans-3,4',5,-trihydroxystilbene), a phytoalexin present in grape skin and red wine, suppresses many types of cancers by regulating cell proliferation and apoptosis through a variety of mechanisms. However, the effects of resveratrol on oral cancer are not completely understood. Thus, effects of resveratrol on cell growth and apoptosis induction were examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, DNA fragmentation, immuno-blotting, and determination of caspase activation in KB human oral cancer cells. Treatment with resveratrol induced inhibition of cell growth depending on the resveratrol treatment time and concentration in KB cells. Treatment with resveratrol induced DNA ladder formation in KB cells and promoted proteolytic cleavage of procaspase-3 and procaspase-7 with increases in the amount of cleaved caspases-3 and -7. Proteolytic processing of caspase-9 in KB cells was increased by resveratrol treatment. Activation of caspase-3/-7 was detected in living KB cells by fluorescence microscopy. These results suggest that the resveratrol can suppress cell growth and induce cell apoptosis in KB human oral cancer cells, and may have potential as an anti-cancer drug.

Key words: anti-cancer therapy, apoptosis, cell death, oral cancer, resveratrol

In recent years, there has been a global trend toward the use of natural substances present in fruits, vegetables, oilseeds and herbs as medicines and functional foods. Several of these substances, such as Taxol, Oncovin and captothecin, are potential cancer chemopreventive or therapeutic agents in the human body [Pezutto, 1997; van Poppel and van den Berg, 1997; Christou *et al*., 2001; Mukherjee *et al*., 2001]. Most of these bioactive substances exert their cancer chemotherapeutic activity by blocking cell cycle progression and triggering apoptotic cell death [Pezutto, 1997; van Poppel and van den Berg, 1997; Christou *et al*., 2001; Mukherjee *et al*., 2001]. Therefore, the induction of apoptosis in cancer cells is an important indicator in the cancer treatment response by employing a bioactive substance to reduce and control human mortality due to cancer [Smets, 1994; Paschka *et al*., 1998].

Apoptosis, which is a major form of programmed cell death, plays an important role in the regulation of tissue development and homeostasis in eukaryotes [Green and Reed, 1998; Hengartner, 2000; Kaufmann and Hengartner, 2001]. Apoptosis can occur via a death receptor-dependent extrinsic or a mitochondria-dependent intrinsic pathway, and can be induced by various chemotherapeutic agents [Kaufmann and Earnshaw, 2000; Reed, 2001].

Resveratrol (trans-3,4',5,-trihydroxystilbene), a phytoalexin that is present in grape skin and red wine, has a variety of actions to reduce superoxides, prevent diabetes mellitus, and inhibit inflammation [Elliott and Jirousek, 2008; Jiang *et al*., 2009; Vanamala *et al*., 2010; Wang *et al*., 2010]. Furthermore, resveratrol decreases plaque formation relevant to neurodegenerative diseases, such as Alzheimer disease and Huntington disease [Karuppagounder *et al*., 2009]. Of particular interest is that resveratrol acts as a chemopreventive agent and induces apoptotic cell death in various types of cancer cells [Jang *et al*., 1997;

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Banerjee *et al*., 2002; Stewart *et al*., 2003; Hope *et al*., 2008; Jiang *et al*., 2009; Vanamala *et al*., 2010]. However, the effect of resveratrol on oral cancer cells is yet unknown.

Oral cancer is the sixth most common cancer globally [Notani, 2000]. Despite the introduction of novel therapeutic modalities for the treatment of oral cancer, there has been only modest improvement in the long-term survival rates [Todd *et al*., 1997]. Advances in the underlying mechanisms of oral cancer are necessary to improve the survival rates, which, despite the earlier detection of oral cancer, have not improved over the past two decades and remain among the worst of all cancer sites [Todd *et al*., 1997]. In the present study, the effect of resveratrol on cell growth and the mechanism of cell death elicited by resveratrol were examined in KB human oral cancer cells.

Materials and Methods

Materials. The KB human oral cancer cells were provided by the American Type Culture Collection (ATCC, Rockville, MD). Resveratrol (trans-3,4',5,-trihydroxystilbene, 5-[(1E)-2-(4-hydroxyphenyl)ethenyl]-1-3-benzenediol), and 3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were supplied by Cayman Chemical (Ann Arbor, MI) and Sigma (St Louis, MO), respectively. Anti-cleaved caspases-3, -7, and -9 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Cell-permeable fluorogenic substrate PhiPhiLux-G_1D_2 was purchased from OncoImmunin, Inc. (Gaithersburg, MD). All other reagents purchased were of analytical grade.

Cell cultures. The KB human oral cancer cells were grown in Minimum essential medium (MEM) containing NEAA (nonessential amino acids) at a 100:1 ratio, and the medium was supplemented with 5% fetal bovine serum (FBS) [Kim *et al*., 2008]. The KB cells were maintained as monolayers in plastic culture plates at 37°C in a humidified atmosphere containing 5% CO_2.

Inhibition of cell growth (MTT assay). The cell viability test was performed according to the previously described method with minor modifications [Hwang *et al*., 2007; Kwon *et al*., 2008]. The cells were seeded at a concentration of 5×10^3 cells/well in 24-well plates. After 24 h growth, the cells were treated with resveratrol at various concentrations and incubation times. The cell viability was assessed using MTT assay. Four separate experiments were performed for each concentration/exposure time combination.

DNA fragmentation analysis. After treatment with 0, 30, and 100 μM resveratrol for 48 h, approximately 5×10^6 cells were collected and transferred to a lysis buffer containing 100 mM NaCl, 10 mM EDTA, 300 mM Tris-HCl, pH 7.5, 200 mM sucrose, 0.5% sodium dodecyl sulfate (SDS), and 0.5 mg/mL proteinase K, and incubated at 65°C. DNA was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1, v/v) and precipitated with ethanol. The extracted DNA was resuspended in Tris-EDTA buffer, pH 8.0, containing 5 μg/mL DNase-free RNase (Sigma, St Louis, MO) and incubated at 37°C for 1 h. The DNA was visualized on 2% agarose gel in the presence of 0.5 μg/mL ethidium bromide.

Immunoblotting. The cells were treated with 0, 30, and 100 μM resveratrol for 48 h. Immunoblotting was performed using a slightly modified method described elsewhere [Choi *et al*., 2010; Kim *et al*., 2010]. The anti-cleaved caspases-3, -7 or -9 antibody (1:1000 dilution, Cell Signaling Technology, Inc., Boston, MA) was used as the primary antibody.

Determination of caspase activation. To examine the activation of caspase-3/-7 by resveratrol in the living KB cells, the activity of caspase-3/-7 was determined using the cell-permeable fluorogenic substrate PhiPhiLux-G_1D_2 (OncoImmunin, Inc.) according to the manufacturer's instructions. The cells were treated with 0 and 30 μM resveratrol for 48 h and incubated with PhiPhiLux-G_1D_2. The activity of caspase-3/-7 was visualized by fluorescence microscopy (IX71, Olympus, Tokyo, Japan).

Data analysis. All experiments were performed at least three times. The results are presented as the mean ± SEM. Statistical significance was analyzed using a Student's t-test for the two group comparison and one way analysis of variance for the multi-group comparisons. A p-value <0.05 was considered significant.

Results

Cytotoxic effect of resveratrol in KB cells. To examine the effect of resveratrol on the cell viability, the cells were treated with resveratrol at various concentrations for 0-72 h, and MTT assay was performed. In resveratrol treatment from 30 to 300 μM, the inhibition of KB cell growth depended on the resveratrol treatment time (Fig. 1). When the KB cells were treated with resveratrol for 0–72 h, resveratrol inhibited the proliferation of KB cells in a dose-dependent manner (Fig. 1). Table 1 lists the IC_{50} values of resveratrol on the cell viability.

DNA fragmentation by resveratrol. Increased cellular apoptosis is only one of several possible mechanisms involved in reducing the level of cell proliferation [Hu and Kavanagh, 2003]. To determine if apoptosis is indeed the underlying mechanism for the reduced cell proliferation

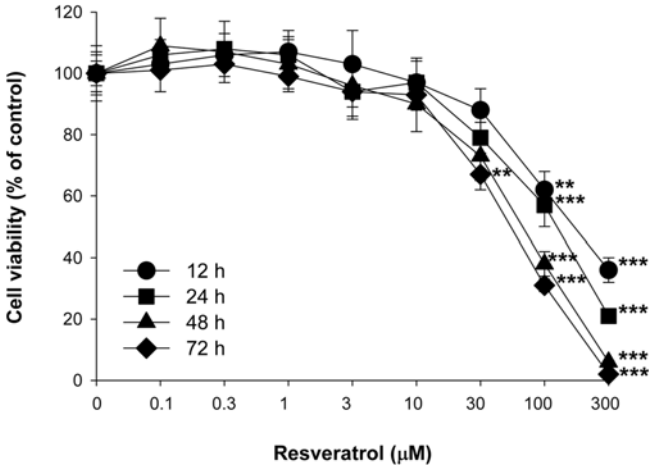

Fig. 1. The effect of resveratrol on the cell viability in KB cells. The KB cells were treated with various concentrations of resveratrol or without resveratrol for 12 (circle), 24 (square), 48 (triangle), and 72 h (diamond). The cell viabilities were determined by the MTT assays. The percentage of cell viability was calculated as a ratio of $A570_{nms}$ of resveratrol-treated cells and untreated control cells. Each data point represents the mean ± SEM of four experiments. $^{**}p < 0.01$ vs. control and $^{***}p < 0.001$ vs. control (the control cells measured in the absence of resveratrol).

Table 1. Antiproliferative effect of resveratrol in KB cells

Time (h)	IC_{50} (μM)
12	197.9±10.4
24	139.6±9.1
48	77.1±6.7
72	63.3±5.8

The IC_{50} values represent the means ± SEM of four experiments.

observed, the KB cells treated with resveratrol were subjected to DNA fragmentation, and the formation of a DNA ladder in the KB cells treated with 30 and 100 μM resveratrol was observed (Fig. 2).

Activation of caspases by resveratol. The levels of cleaved caspases-3, -7, and -9 were examined by immunoblotting, and the levels of procaspases-3 and -7 were detected by fluorescence microscopy using a selective fluorogenic substrate, because caspases-3, -7, and -9 are effector caspases of apoptotic cell death [Cohen, 1997; Datta *et al*., 1997; Liu *et al*., 1997]. Treatment with 30 and 100 μM resveratrol significantly promoted the proteolytic cleavage of procaspase-3 in KB cells, with increases in the amount of cleaved caspase-3 (Fig. 3A). Resveratrol (30 and 100 μM) also promoted the proteolytic cleavage of procaspases-7 (Fig. 3B) and -9 (Fig. 3C), with increases in the amount of cleaved caspases-7 and -9. In addition, the activation of caspase-3/-7 in the resveratrol-treated KB cells was confirmed by

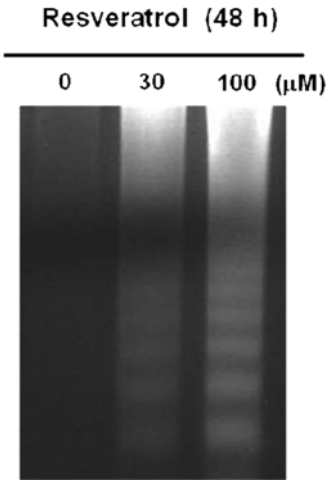

Fig. 2. Fragmentation of internucleosomal DNA by resveratrol in KB cells. The KB cells were treated with 0, 30, and 100 μM resveratrol for 48 h, and nuclear DNA (5 μg) was subjected to agarose gel electrophoresis.

fluorescence microscopy using a fluorogenic substrate; the resveratrol treatment activated the caspase-3/-7 in living KB cells (Fig. 4).

Discussion

Recent studies have shown that chemicals derived from natural materials have an inhibitory effect on cell growth in cancer cells [Cheng *et al*., 2005; Park *et al*., 2005; Tan *et al*., 2005], which alters various factors associated with the cell cycle and induces apoptotic cell death [Hoshino *et al*., 1997; Cheng *et al*., 2005; Park *et al*., 2005; Tan *et al*., 2005; Tian *et al*., 2006]. Therefore, identification of novel anti-cancer agents that can kill cancerous cells with minimal toxicity is needed. Resveratrol is a naturally occurring polyphenolic compound that is found in high concentrations in grape skin and red wine [Elliott and Jirousek, 2008; Jiang *et al*., 2009; Vanamala *et al*., 2010; Wang *et al*., 2010], and has anti-inflammatory, anti-oxidant, anti-leukemic, anti-viral, and neuroprotective properties [Elliott and Jirousek, 2008; Jiang *et al*., 2009; Vanamala *et al*., 2010; Wang *et al*., 2010]. In addition, resveratrol acts as a cancer chemopreventive and chemotherapeutic agent, inhibiting the different stages of carcinogenesis [Jang *et al*., 1997; Banerjee *et al*., 2002; Stewart *et al*., 2003; Hope *et al*., 2008]. However, the effects of resveratrol on oral cancer are not completely understood. Therefore, the present study examined the cytotoxic activity of resveratrol and the mechanism of cell death exhibited by resveratrol in KB human oral cancer cells.

MTT assay showed that resveratrol inhibited KB cell growth in a concentration- and time-dependent manner

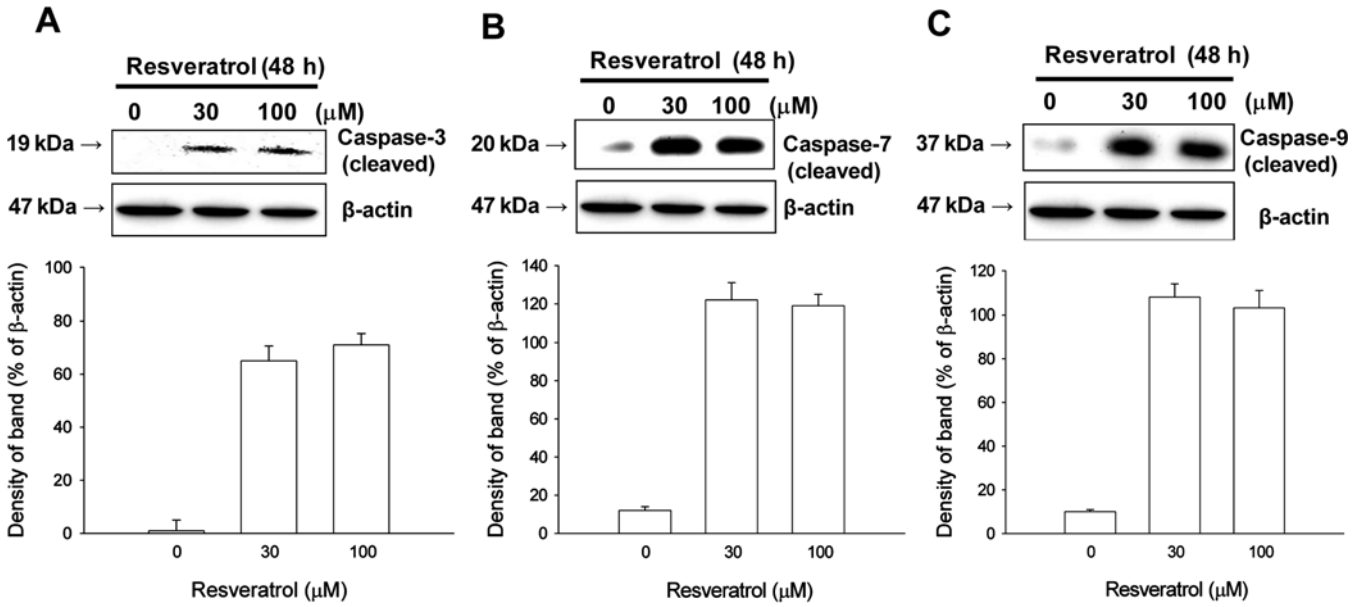

Fig. 3. Proteolytic cleavage of caspase-3, -7, and -9 by resveratrol treatment in KB cells. Activities of cleaved caspase-3 (A), -7 (B), and -9 (C) by resveratrol was measured in KB cells. The cells were treated with 0, 30, and 100 μM resveratrol for 48 hours. The cell lysate was prepared and analyzed by immunoblotting as described in "Materials and Methods". Lower panels represent the quantitative data for the activities of cleaved caspases-3, -7, and -9 analyzed by using Imagegauge 3.12 software after β-actin normalization.

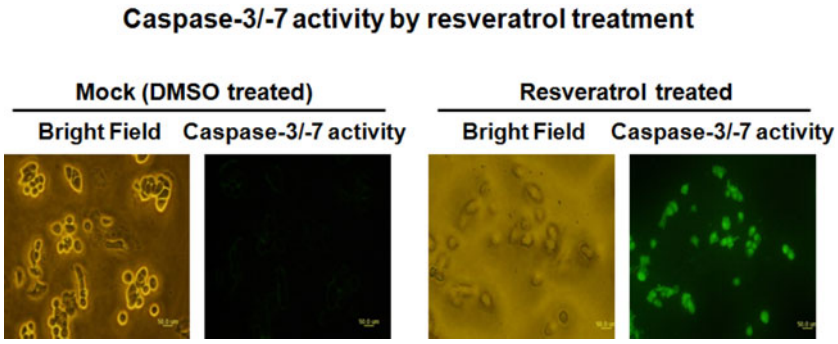

Fig. 4. Activation of caspase-3/-7 by resveratrol treatment in living KB cells. The cells were treated with 0 and 30 μM resveratrol for 48 h and added with specific cell-permeable substrate Phiphilux G_1D_2. Activity of caspase-3/-7 was visualized by fluorescence microscopy.

(Fig. 1). This corresponded to the results showing that resveratrol has anti-cancer effects via the suppression of cancer cell growth in various types of cancer cells [Jang *et al*., 1997; Banerjee *et al*., 2002; Stewart *et al*., 2003; Hope *et al*., 2008]. These results suggest that resveratrol has cytotoxicity for oral cancer cells and thus has potential value for anti-cancer drug discovery.

Apoptosis is an important function for maintaining cellular homeostasis between cell division and cell death [Green and Reed, 1998; Hengartner, 2000; Kaufmann and Hengartner, 2001]. The induction of apoptosis in cancer cells is a useful strategy for anti-cancer drug development [Hu and Kavanagh, 2003]. Therefore, many studies have been performed to screen apoptosis from natural materials. In the present study, the treatment with resveratrol induced internucleosomal DNA fragmentation in KB cells, suggesting apoptotic cell death (Fig. 2). These results suggest that resveratrol inhibits the growth of KB cells by activating cell apoptosis.

The activation of a family of intracellular cysteine proteases, known as caspases, plays an important role in the initiation and execution of apoptosis induced by various stimuli [Datta *et al*., 1997; Liu *et al*., 1997]. Among the caspases identified in mammalian cells, caspases-3, -7, and -9 may be effector caspases of apoptotic cell death [Cohen, 1997; Datta *et al*., 1997; Liu *et al*., 1997]. These caspases-3, -7, and -9 are synthesized as inactive proenzymes (of sizes 32, 35, and 47 kDa, respectively) that require proteolytic activation to cleaved enzymes (of sizes 19, 20, 37 kDa, respectively) [Cohen,

1997; Datta *et al*., 1997; Liu *et al*., 1997]. These results revealed low levels of cleaved capase-3, -7, and -9 in the untreated KB cells, whereas the amount of cleaved enzymes in the KB cells was increased after the resveratrol treatment (Fig. 3). In addition, the activity of caspase-3/-7 was increased by the resveratrol treatment in KB cells compared to the DMSO treatment as a control (Fig. 4). These results suggest that resveratrol induces apoptotic cell death of KB cells through caspases-3-, -7-, and -9-dependent processing. The mechanisms of apoptosis induced by resveratrol are not completely understood. Further studies will be needed to reveal the precise cellular and molecular mechanisms of apoptosis induced by resveratrol.

In conclusion, the results of the present studt suggest that resveratrol strongly inhibits cell proliferation and induces apoptosis in KB human oral cancer cells. Moreover, these results suggest that resveratrol can be a new chemotherapeutic agent for the inhibition of cell growth in oral cancer. Further study on the activity of resveratrol including *in vivo* and purification of bioactive compounds to elaborate this nascent possibility is currently underway.

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