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Induction of apoptosis associated with activation of caspases by resveratrol in HTB-41 human salivary gland epidermoid carcinoma cells

조선대학교 대학원

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사람 타액선 편평상피암세포주 HTB-41에서 resveratrol에 의한
caspase 관련 apoptosis의 유도

2015년 2월 25일

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이 논문을 치의학 박사학위신청 논문으로 제출함.

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ABSTRACT

Induction of apoptosis associated with activation of caspases by resveratrol in HTB-41 human salivary gland epidermoid carcinoma cells

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Trans-3,4′,5,-trihydroxystilbene (resveratrol), a phytoalexin that is present in grape skin and red wine, suppresses many types of cancers by regulating cell proliferation and apoptosis through a variety of mechanisms. However, resveratrol effects on salivary gland tumors including squamous carcinomas are not clearly established. The main goal of this study was to investigate the effect of resveratrol on cell growth and apoptosis induction in salivary gland epidermoid carcinoma cells.

To determine the effect of resveratrol on cell growth and apoptosis induction in salivary gland epidermoid carcinoma cells, it was examined by inhibition of cell

growth (MTT assay), nuclear staining with DAPI, determination of caspase activation and immunoblotting in HTB-41 human submaxillary salivary gland epidermoid carcinoma cells. The results are as follows.

1. Treatment of resveratrol induced the inhibition of cell growth depended on the resveratrol treatment time and concentration in HTB-41 cells.
2. Treatment of resveratrol induced the nuclear condensation and fragmentation in HTB-41 cells.
3. Activation of caspase-3 and -7 was detected in living HTB-41 cells by fluorescence microscopy.
4. Resveratrol promoted proteolytic cleavages of procaspase-3, -7, -8 and -9 with the increases in the amount of cleaved caspase-3, -7, -8 and -9.
5. Cleaved PARP was increased by resveratrol in HTB-41 cells.

These results suggest that the resveratrol can induce the suppression of cell growth and cell apoptosis in HTB-41 human submaxillary salivary gland epidermoid carcinoma cells, and that it may have potential properties for anti-cancer drug discovery.

KEY WORDS: Resveratrol, Cell death, Apoptosis, Anti-cancer therapy, Salivary gland epidermoid carcinoma cells

I. INTRODUCTION

In recent years, there has been a global trend toward the use of natural substances present in fruits, vegetable, oilseeds and herbs as medicine and functional food. Several of these substances, such as Taxol, Oncovin and captothecin, are shown to have potential values as cancer chemopreventive or therapeutic agents within the human body.[1-4] Most of these bioactive substances exert their cancer chemotherapeutic activity by blocking cell cycle progression and triggering apoptotic cell death.[1-4] Therefore, induction of apoptosis in cancer cells has become an important indicator of the cancer treatment response in employing a bioactive substance to reduce and control human mortality due to cancer.[5,6]

Apoptosis, which is a major way of programmed cell death, plays an important role in the regulation of tissue development and homeostasis in eukaryotes.[7-9] Apoptosis may occur via a death receptor-dependent extrinsic or a mitochondria-dependent intrinsic pathway and apoptosis is induced by treatment of chemotherapeutic agents.[10,11]

Trans-3,4′,5,-trihydroxystilbene (resveratrol), a phytoalexin that is present in grape skin and red wine, exerts a variety of actions to reduce superoxides, prevent diabetes mellitus and inhibit inflammation.[12-15] Furthermore, resveratrol decreases plaque formation relevant to neurodegenerative diseases such as Alzheimer disease and Huntington disease.[16] Of particular interest is that resveratrol acts as a chemopreventive agent and induces apoptotic cell death in various types of cancer cells.[12-14,17-20]

Salivary gland tumors are a morphologically and clinically diverse group of neoplasms, which may present considerable diagnostic and management challenges to the pathologist or surgeon.[21] Salivary gland tumors are rare with an overall incidence in the Western world of about 2.5-3.0 per 100,000 per year and among salivary gland tumors, squamous cell carcinomas are rare.[22] However, although this tumor has a poor prognosis, unlike most other salivary gland malignancies, survival at 5 years is prognostically significant.[22] Furthermore, resveratrol effect on salivary gland tumors including squamous carcinomas has been unknown at all.

In this study, therefore, the effect of resveratrol on cell growth and the mechanism of cell death elicited by resveratrol were examined in HTB-41 human submaxillary salivary gland epidermoid carcinoma cells.

II. MATERIALS AND METHODS

1. Materials

Trans-3,4′,5,-trihydroxystilbene,5-[(1E)-2-(4-hydroxyphenyl)ethenyl]-1-3-benzenediol (resveratrol) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were supplied by Cayman Chemical (Ann Arbor, MI, USA) and Sigma (St Louis, MO, USA), respectively. Anti-cleaved caspase-3, -7, -8, -9 and anti-cleaved PARP antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Cell-permeable fluorogenic substrate PhiPhiLux-G1D2 was purchased from OncoImmunin, Inc. (Gaithersburg, MD, USA). Other analytical reagents were purchased based on the analytical grade.

2. Cell line and cell cultures

The HTB-41 human submaxillary salivary gland epidermoid carcinoma cells were provided by American Type Culture Collection (ATCC, Rockville, MD, USA). The HTB-41 cells were grown in modified McCoy′s media with 1.5 mM L-glutamine adjusted to contain 2.2 g/L sodium bicarbonate with 10% FBS in accordance with ATCC′s instruction. The cells were maintained as monolayers in plastic culture plate at 37°C in a humidified atmosphere containing 5% CO_2.[23)]

3. Inhibition of cell growth (MTT assay)

The cell viability test was performed according to the previously described method with minor modifications.[24,25] The cells were seeded at a concentration of 5 X 10^3 cells/well in 24-well plates. After 24 hours 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.

4. Nuclear staining with DAPI

Nuclear staining with DAPI (40,60-diamidino-2-phenylindole) was performed to examine the level of apoptosis. The HTB-41 cells were cultured in 24-well plates at a seeding density of 5 X 10^3 cells/well. After 24 hours growth, the cells were treated with 0, 100 or 300 μM resveratrol for 48 hours. The treated HTB-41 cells were fixed with 1% paraformaldehyde for 30 min at room temperature and washed twice with PBS. The cells were permeated with ice-cold ethanol for 5 min at room temperature and washed twice with PBS. The fixed cells were stained with DAPI (300 nM) for 5 min at room temperature in the dark, washed twice with PBS and examined by fluorescent inverted microscopy (IX71, Olympus, Japan).

5. Determination of caspase activation

The activity of caspase-3/-7 was determined using the cell-permeable

fluorogenic substrate PhiPhiLux-G_1D_2 (OncoImmunin, Inc. Gaithersburg, MD, USA), which was used according to the manufacturer' s instructions. The cells were treated with 0, 100 or 300 μM resveratrol for 48 hours and incubated with PhiPhiLux-G_1D_2. The activity of caspase-3/-7 was visualized by fluorescence microscopy (IX71, Olympus, Japan).

6. Immunoblotting

The HTB-41 cells were treated with 0 or 100 μM resveratrol for 48 hours. Immunoblotting was performed according to the previously described method with minor modifications.[26,27] The anti-cleaved caspase-3, -7, -8, -9 or anti-cleaved PARP antibody (1:1000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA) was used as the primary antibody.

7. Data analysis

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

Ⅲ. RESULTS

1. Cytotoxic effect of resveratrol in HTB-41 cells

To analyze the effect of resveratrol on the viability of cells, the cells were treated with resveratrol at various concentrations for 0 - 72 hours, and then the MTT assay was performed. When the HTB-41 cells were treated with resveratrol for 0 - 72 hours, resveratrol inhibited the proliferation of HTB-41 cells in a dose-dependent manner (Fig. 1A), suggesting that resveratrol induces HTB-41 cell death. From 10 to 1000 μM treatment of resveratrol, the inhibition of HTB-41 cell growth depended on the resveratrol treatment time (Fig. 1B). The IC_{50} values of resveratrol on the cell viability are shown in Table 1.

Table 1. Anti-proliferative effect of resveratrol in HTB-41 cells

Time	IC_{50} (μM)
24 hours	293.6 ± 28.7
48 hours	120.3 ± 9.6
72 hours	22.1 ± 3.6

The IC_{50} values represent the mean ± SEM for four experiments.

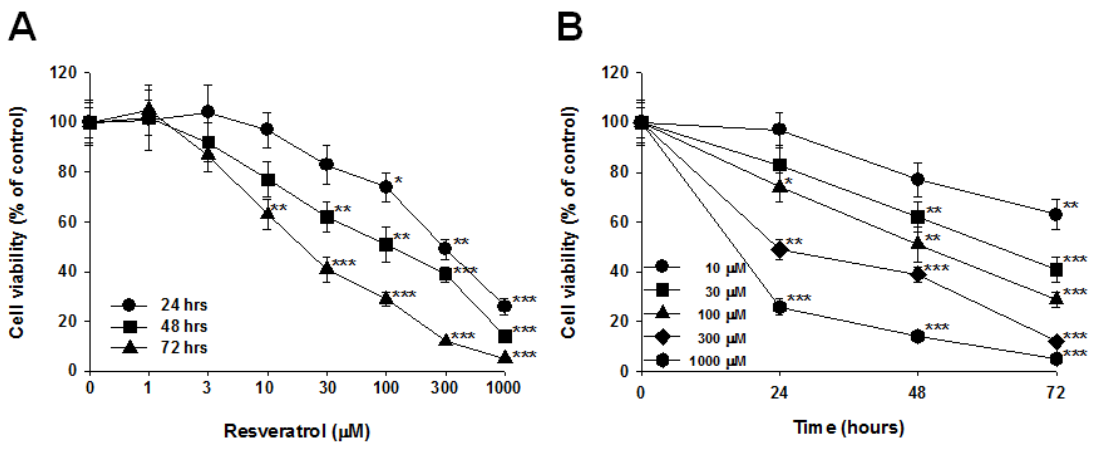

Fig. 1. Concentration- and time-dependent effects of resveratrol on the cell viability in HTB-41 human submaxillary salivary gland epidermoid carcinoma cells. (A) Concentration-dependent effect of resveratrol on the cell viability in HTB-41 cells. The HTB-41 cells were treated with various concentrations of resveratrol or without resveratrol for 24 (circle), 48 (square) and 72 hours (triangle). (B) Time-dependent effect of resveratrol on the cell viability in HTB-41 cells. The HTB-41 cells were treated with 10 (circle), 30 (square), 100 (triangle), 300 (diamond) and 1000 μM (hexagon) resveratrol for 0 - 72 hours. 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. $^{*}P<0.05$ vs. control, $^{**}P<0.01$ vs. control and $^{***}P<0.001$ vs. control (the control cells measured in the absence of resveratrol).

2. Changes in the nuclear morphology by resveratrol

The nuclear morphological changes were assessed by DAPI staining. As shown in Fig. 2A, the nuclei of the control HTB-41 cells (Control) had a normal regular and oval shape. Treatment with 100 or 300 μM resveratrol for 48 hours resulted in nuclear condensation and fragmentation, which are the characteristics of apoptosis (Fig. 2A). As quantified in Fig. 2B, 100 and 300 μM resveratrol increased the apoptotic rate of HTB-41 cells significantly to 26.9 ± 4.1% and 48.7 ± 6.8%, respectively.

3. Activation of caspase-3/-7 by resveratrol

The activation of caspase-3/-7 in the resveratrol-treated HTB-41 cells was confirmed by fluorescence microscopy using a fluorogenic substrate. As shown in Fig. 3, the resveratrol treatment led to the activation of caspase-3/-7 in the living HTB-41 cells.

4. Activation of caspases by resveratol

The levels of cleaved caspase-3, -7, -8 and -9 were examined by immunoblotting since caspase-3, -7, -8 and -9 are effector caspases of apoptotic cell death.[28-31] Treatment with 100 μM resveratrol significantly promoted proteolytic cleavages of procaspase-3 (Fig. 4) and -7 (Fig. 5) in the HTB-41 cells, with the increases in the amount of cleaved caspase-3 and -7. Resveratrol (100 μM) also promoted proteolytic cleavages of procaspase-8 (Fig. 6) and -9 (Fig. 7), with the increases in the amount of cleaved caspase-7 and -9.

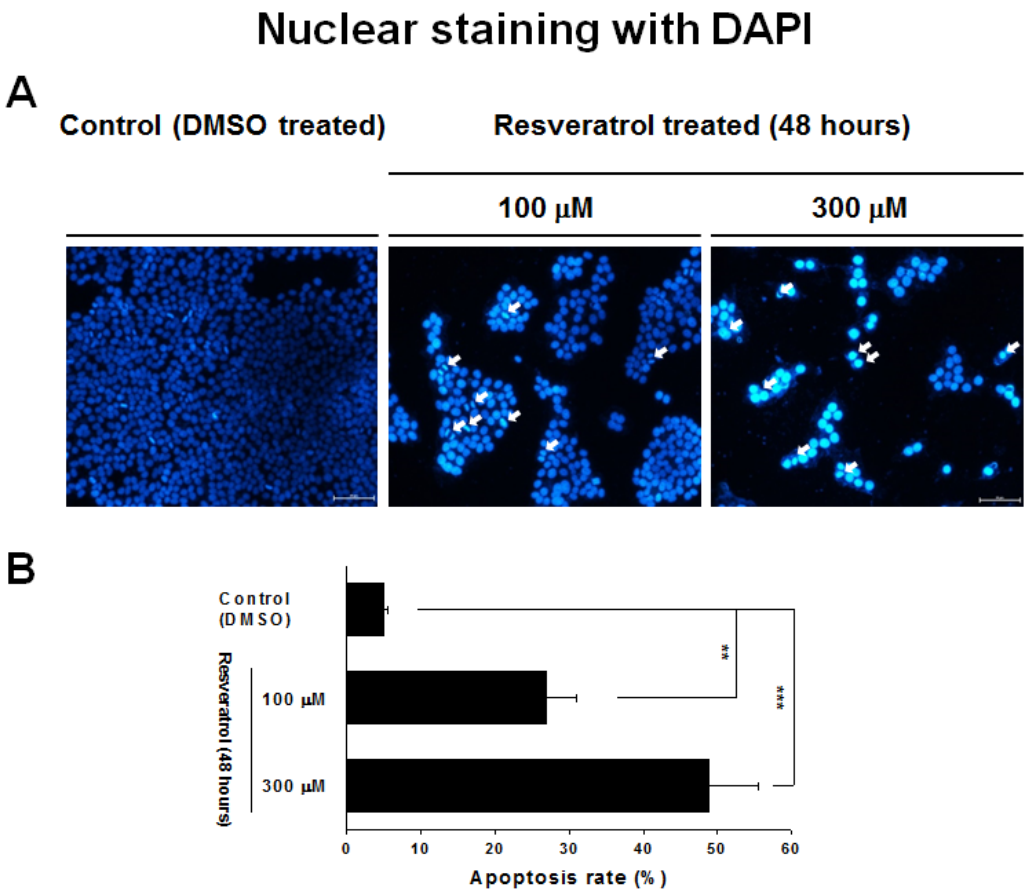

Fig. 2. Induction of apoptosis by resveratrol in HTB-41 cells. (A) Changes in nuclear morphology by resveratrol. The cells were treated with 0, 100 or 300 μM resveratrol for 48 hours. Representative fluorescence photomicrographs show the nuclei morphology of HTB-41 cells. The arrows indicate chromatin condensation, reduced nuclear size and nuclear fragmentation typically observed in apoptotic cells. (B) The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells.

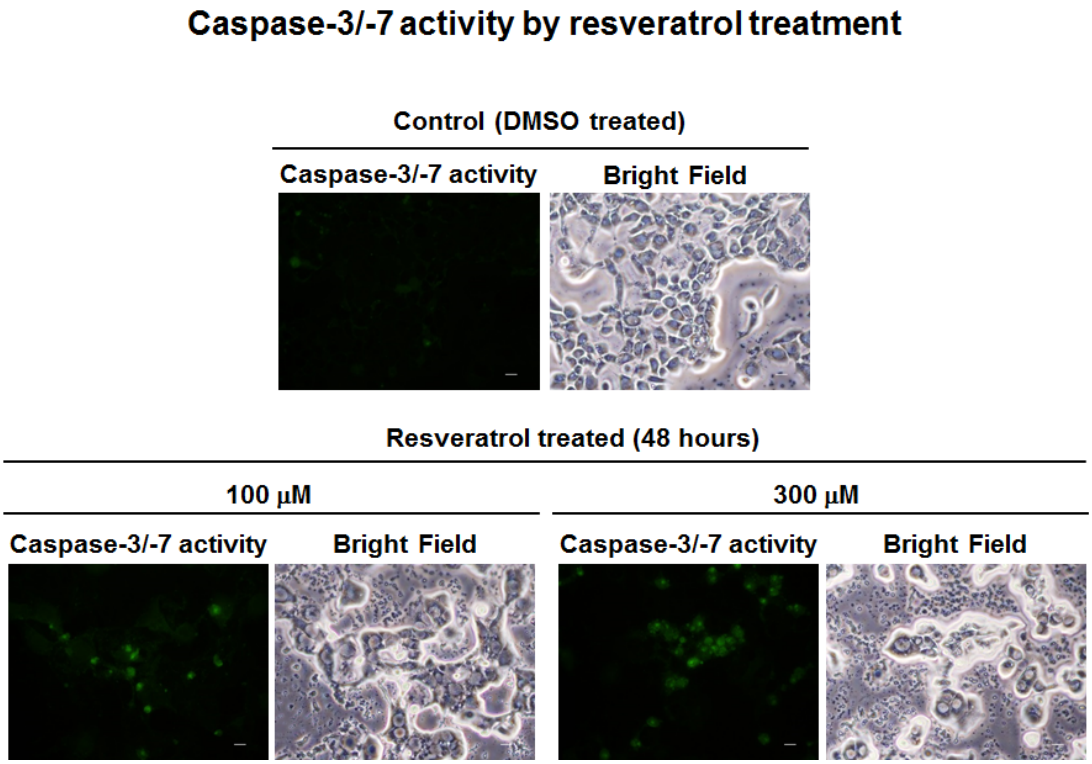

Fig. 3. Activation of caspase-3/-7 by resveratrol treatment in living HTB-41 cells. The cells were treated with 0, 100 or 300 μM resveratrol for 48 hours and added specific cell-permeable substrate Phiphilux G_1D_2. Active of caspase-3/-7 was visualized by fluorescence microscopy.

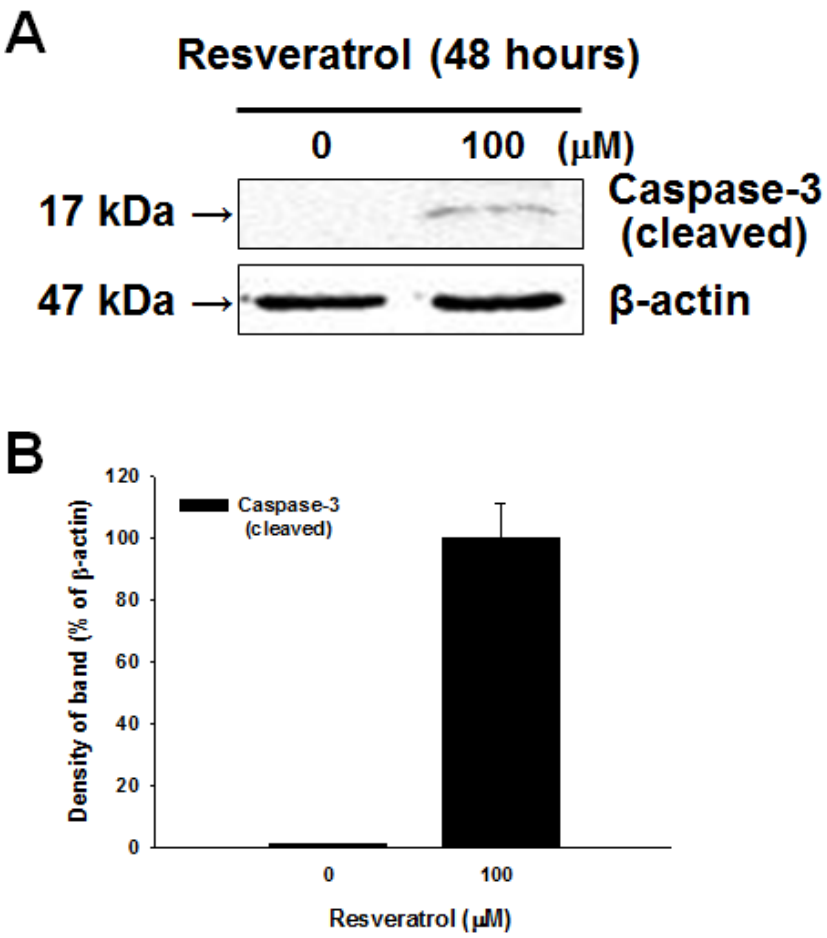

Fig. 4. Proteolytic cleavage of caspase-3 by resveratrol treatment in HTB-41 cells. (A) Activity of cleaved caspase-3 by resveratrol was measured in HTB-41 cells. The cells were treated with 0 or 100 μM resveratrol for 48 hours. The cell lysate was prepared and analyzed by immunoblotting as described in "MATERIALS AND METHODS". (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

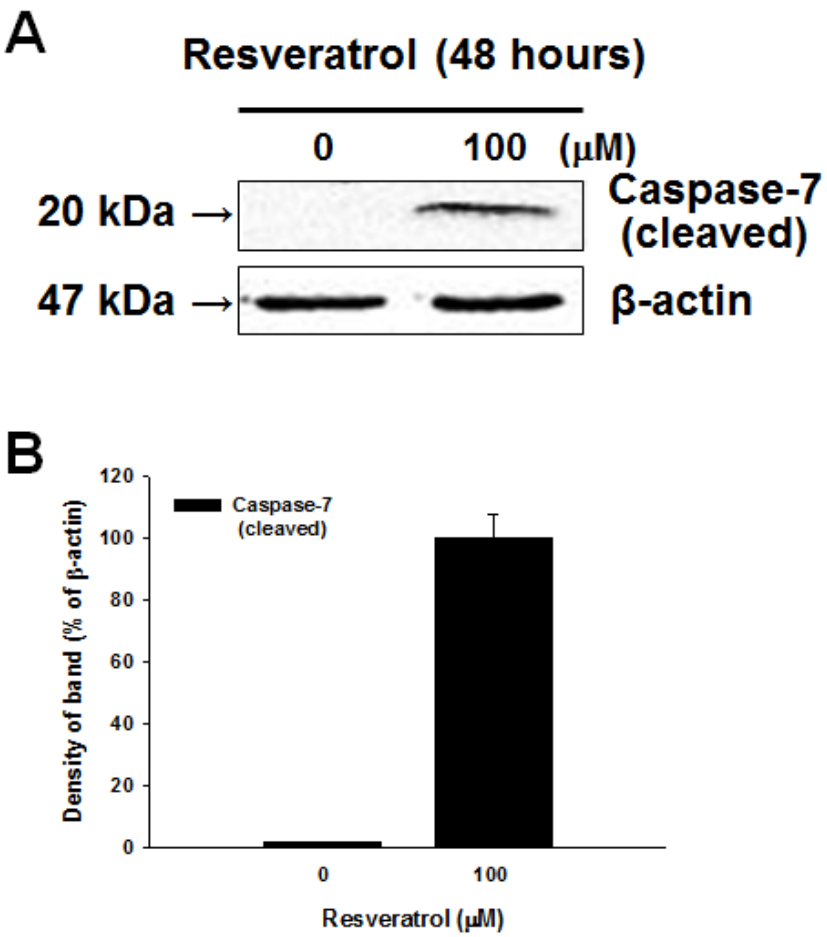

Fig. 5. Proteolytic cleavage of caspase-7 by resveratrol treatment in HTB-41 cells. (A) Activity of cleaved caspase-7 by resveratrol was measured in HTB-41 cells. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

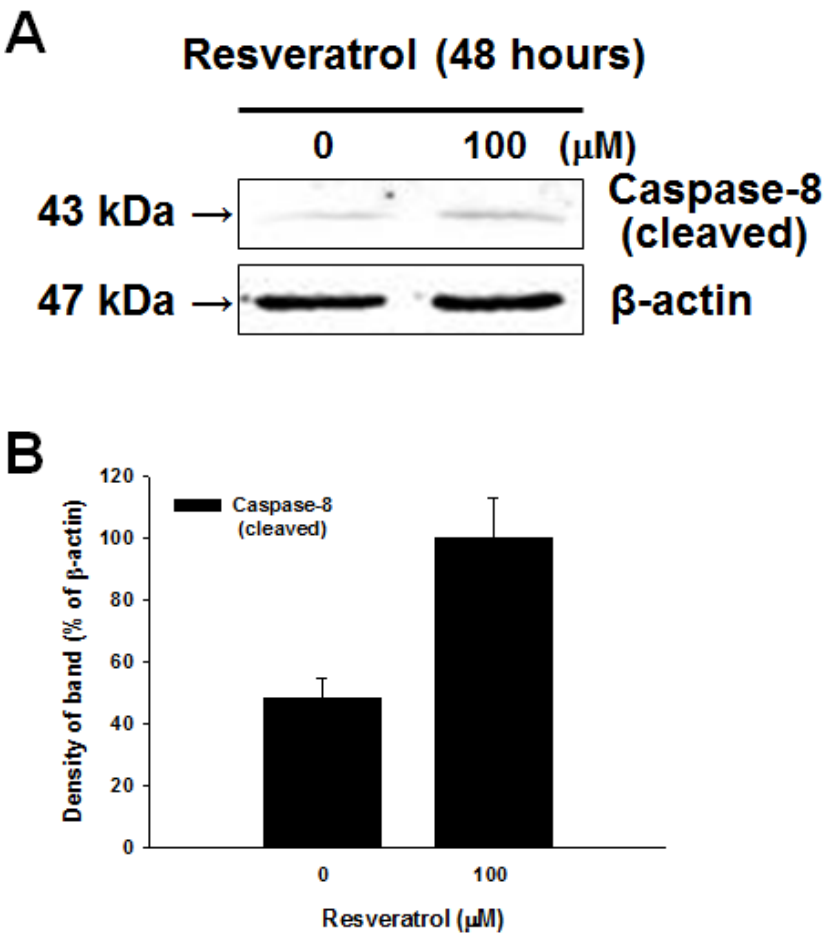

Fig. 6. Proteolytic cleavage of caspase-8 by resveratrol treatment in HTB-41 cells. (A) Activity of cleaved caspase-8 by resveratrol was measured in HTB-41 cells. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

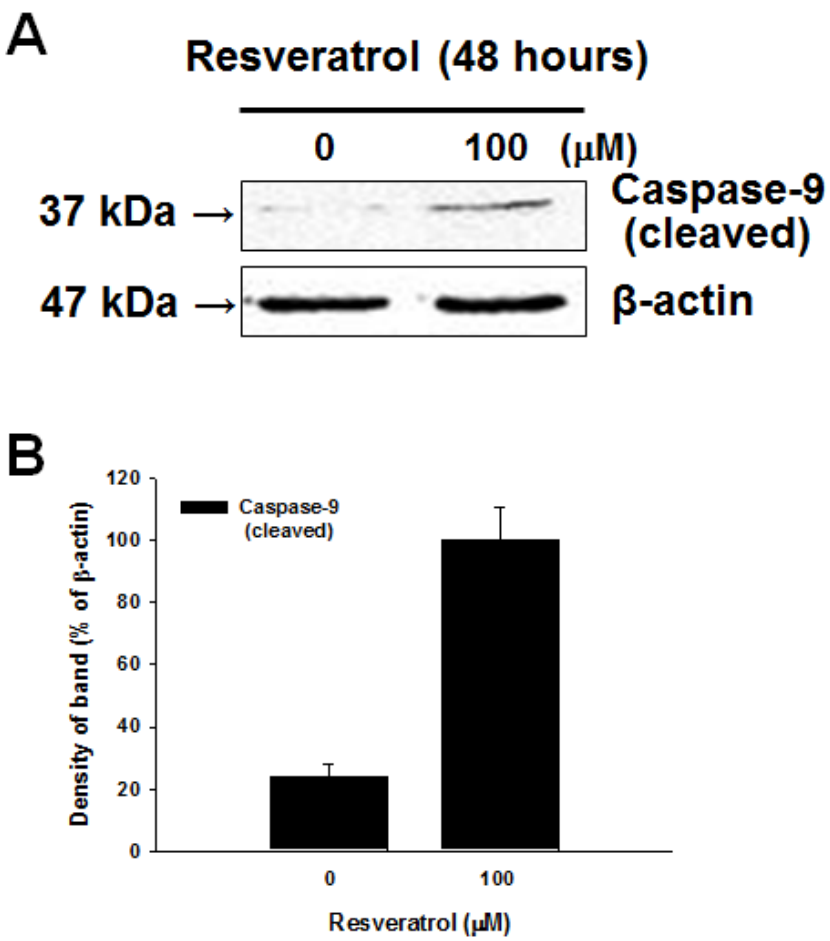

Fig. 7. Proteolytic cleavage of caspase-9 by resveratrol treatment in HTB-41 cells. (A) Activity of cleaved caspase-9 by resveratrol was measured in HTB-41 cells. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

5. Apoptosis mediated via PARP by resveratrol

To determine how resveratrol induce the apoptosis of HTB-41 cells, immunoblotting was performed to measure the expression of the PARP at the protein level. As shown in Fig. 8, cleaved PARP was increased by resveratrol compared to the control.

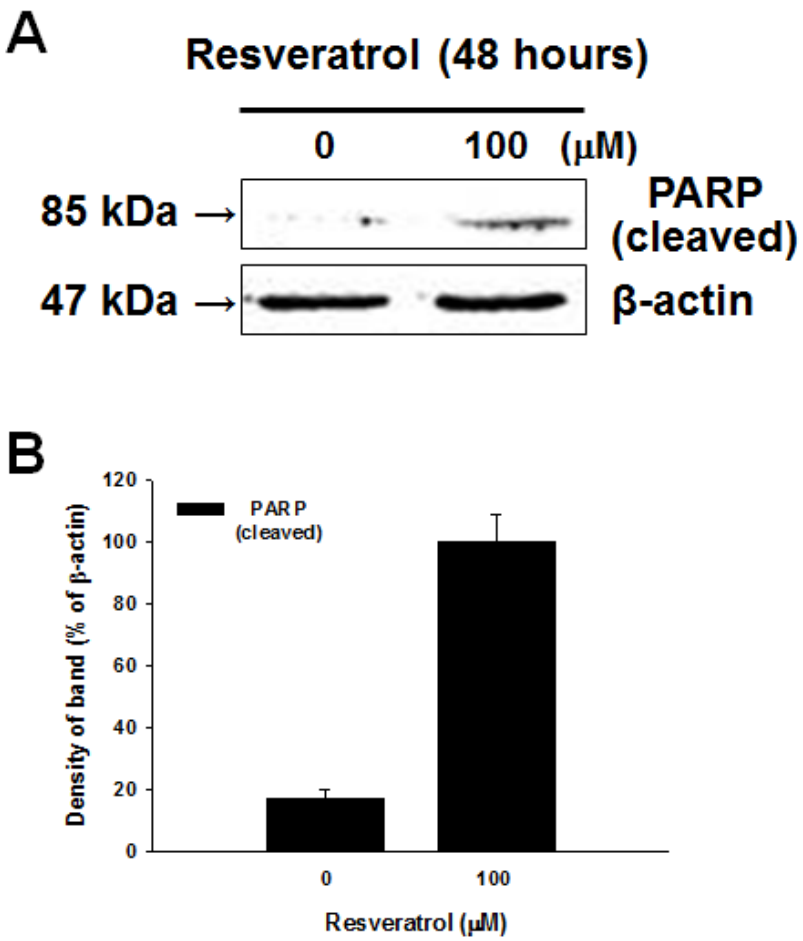

Fig. 8. Activation of cleaved PARP by resveratrol treatment in HTB-41 cells. (A) The activity of cleaved PARP by resveratrol was measured in HTB-41 cells. The HTB-41 cells were stimulated with 0 or 100 μM resveratrol for 48 hours, harvested and lyzed using a cell lysate buffer. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

Ⅳ. DISCUSSION

Recent studies have shown that such chemicals derived from natural materials have an inhibitory effect on the cell growth in cancer cells.[32-34] It has been reported that this effect alters various factors associated with the cell cycle and thereby induce the apoptotic cell death.[32-36] Finding new anti-cancer agents that can kill cancerous cells with minimal toxicity is very important. Resveratrol is a naturally occurring polyphenolic compound that is highly enriched in grape skin and red wine.[12-15] It has anti-inflammatory, anti-oxidant, anti-leukemic, anti-viral and neuroprotective properties.[12-15] In addition, resveratrol acts as a cancer chemopreventive and chemotherapeutic agent, inhibiting different stages of carcinogenesis.[12-14,17-20] However, the resveratrol effects on salivary gland tumors including squamous carcinomas are not clearly established. In this study, therefore, the cytotoxic activity of resveratrol and the mechanism of cell death exhibited by resveratrol were examined in HTB-41 human submaxillary salivary gland epidermoid carcinoma cells.

In MTT assay, resveratrol inhibited growth of HTB-41 cells in a concentration- and a time-dependent manner (Fig. 1). This corresponded with the results of resveratol that has anti-cancer effects via the suppression of cancer cell growth in various types of cancer cells.[12-14,17-20] These results speculated that resveratrol has cytotocity for salivary gland epidermoid carcinoma cells also and potential value for anti-cancer drug discovery.

Apoptosis is an important way to maintain cellular homeostasis between cell

division and cell death.[7-9] The induction of apoptosis in cancer cells is one of useful strategies for anti-cancer drug development.[28] So, many studies were performed for screening of apoptosis from natural materials. In this study, treatment with resveratrol induced the nuclear condensation and fragmentation (Fig. 2) and the activation of caspase-3/-7 (Fig. 3) in living HTB-41 cells, suggesting apoptotic cell death. These results indicated that resveratrol inhibits the growth of HTB-41 cells by activating cell apoptosis.

The activation of a family of intracellular cysteine proteases, called caspases, is known to play an important role in the initiation and execution of apoptosis induced by various stimuli.[29,30] Among the caspases identified in mammalian cells, caspase-3, -7, -8 and -9 can serve as effector caspases of apoptotic cell death.[29-31] Caspase-3, -7, -8 and -9 are synthesized as inactive proenzymes, which require proteolytic activation to cleaved enzymes (of sizes 17 kDa, 20 kDa, 43 kDa and 37 kDa, respectively).[29-31] Our results show that low levels of cleaved capase-3, -7, -8 and -9 were present in resveratrol-untreated HTB-41 cells, and the amount of cleaved enzymes was increased after resveratrol treatment in the HTB-41 cells (Fig. 4, 5, 6 and 7). Also, the cleaved PARP, which is an important regulatory factor of death receptor-mediated extrinsic apoptotic pathway, was increased by resveratrol in the HTB-41 cells (Fig. 8). These results suggest that resveratrol induces apoptotic cell death through both the mitochondria-mediated intrinsic pathway and the death receptor-mediated extrinsic pathway by the activation of caspases-3, -7, -8, -9 and PARP in HTB-41 cells. However, the mechanisms of apoptosis induced by resveratrol are not yet completely understood. Further studies will reveal the precise cellular and molecular mechanisms of apoptosis induced by

resveratrol.

In conclusion, these *in vitro* results suggest that the resveratrol inhibits cell proliferation and induces apoptotic cell death in HTB-41 human submaxillary salivary gland epidermoid carcinoma cells through both the mitochondria-mediated intrinsic pathway and the death receptor-mediated extrinsic pathway. Moreover, these results suggest that the resveratrol could be a new agent of chemotherapeutic for the inhibition of cancer cell growth. On the other hand, to elaborate this nascent possibility, a further study of its activity including *in vivo* and the purification of bioactive compounds is currently underway.

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ABSTRACT in KOREAN

사람 타액선 편평상피암세포주 HTB-41에서 resveratrol에 의한 caspase 관련 apoptosis의 유도

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항독성 물질(파이토알렉신) 중 하나인 trans-3, 4′, 5,-trihydroxystilbene(resveratrol)은 특정 암세포의 성장을 조절할 수 있다고 보고되어져 왔다. 그러나 타액선암과 관련된 resveratrol에 관한 자료는 거의 없다. 따라서 본 연구에서는 사람 타액선 편평상피암세포주 HTB-41 세포주를 이용하여 resveratrol의 타액선 편평상피암세포 성장억제에 미치는 효과와 세포성장 억제기전을 분석하였다.

본 연구에서 resveratrol에 의한 타액선 편평상피암세포 성장억제와 그 기전을 조사하기 위해, HTB-41 세포주에서 resveratrol을 이용하여 MTT 분석, DAPI를 이용한 핵 염색 분석, caspase 활성 분석 및 immunoblotting 등을 시행하였다.

사람 타액선 편평상피암세포주 HTB-41에서 resveratrol은 타액선 편평상피암세포주의 성장을 시간과 농도에 의존적으로 억제하였다. HTB-41 세포에서 resveratrol은 핵의 응집과 파쇄를 유도하였다. HTB-41 세포에 resveratrol을 처리한 실험군에서 caspase-3/-7의 활성화를 확인할 수 있었다. HTB-41 세포에 resveratrol을 처리한 실험군에서 procaspase-3, -7, -8 및 -9의 proteolytic cleavage 현상을 확인할 수 있었다. HTB-41 세포에 resveratrol을 처리한 실험군에서 PARP의 proteolytic cleavage 현상을 확인할 수 있었다.

본 연구의 결과로 resveratrol은 사람 타액선 편평상피암세포주 HTB-41의 apoptosis를 유도하여 타액선 편평상피암세포 성장을 억제시키는 것으로 사료된다. 또한 본 연구의 결과로, resveratrol을 이용한 타액선 편평상피암세포의 성장억제에 관한 하나의 방향을 제시할 수 있을 것으로 사료된다.

중심어: Resveratrol, 세포사, Apoptosis, 항암치료제, 타액선 편평상피암세포