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Growth inhibition of KB human oral squamous cell carcinoma by resveratrol

조선대학교 대학원

치의학과

김 성 훈

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Resveratrol에 의한 사람 구강편평세포암종 KB 세포의 성장억제

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지도교수 김 홍 중

이 논문을 치의학 박사학위신청 논문으로 제출함.

2010년 10월 일

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김 성 훈

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2010년　12월　　일

조선대학교 대학원

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ABSTRACT

Growth inhibition of KB human oral squamous cell carcinoma by resveratrol

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Resveratrol (trans-3,4′,5,-trihydroxystilbene), 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 oral cancer are not clearly established. The main goal of this study was to investigate the effect of resveratrol on cell growth and apoptosis induction in human oral cancer cells.

To determine the effect of resveratrol on cell growth and apoptosis induction in human oral cancer cells, it was examined by inhibition of cell growth (MTT assay), DNA fragmentation analysis, immunoblotting and determination of caspase activation in KB human oral squamous cell carcinoma. The results are as follows.

1. Treatment of resveratrol induced the inhibition of cell growth depended on the resveratrol treatment time and concentration in KB cells.
2. Treatment of resveratrol induced the formation of DNA ladder in a time-dependent manner in KB cells.
3. Resveratrol promoted proteolytic cleavages of procaspase-3 and procaspase-7 with the increases in the amount of cleaved caspase-3 and caspase-7.
4. The proteolytic processing of caspase-9 was increased by resveratrol treatment in KB cells.
5. Activation of caspase-3 and -7 was detected in living KB cells by fluorescence microscopy.

These results suggest that the resveratrol can induce the suppression of cell growth and cell apoptosis in KB human oral squamous cell carcinoma, and that it may have potential properties for anti-cancer drug discovery.

KEY WORDS: Resveratrol, Cell death, Apoptosis, Anti-cancer therapy, Oral cancer

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]

Resveratrol (trans-3,4′,5,-trihydroxystilbene), 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] However, resveratrol effect on oral cancer cells has been unknown at

all.

It has been known that oral cancer is the sixth most common cancer globally.[21] Despite the introduction of novel therapeutic modalities into the treatment of oral cancer, improvements in long-term survival rates have only been modest.[22] Advances in the underlying mechanisms of oral cancer are likely to be necessary to improve survival rates, which, despite the better early detection of oral cancer, have plateaued over the past two decades and remain among the worst of all cancer sites.[22] In this study, therefore, the effect of resveratrol on cell growth and the mechanism of cell death elicited by resveratrol were examined in KB human oral squamous cell carcinoma.

II. MATERIALS AND METHODS

1. Materials

The KB human oral squamous cell carcinomas were provided by American Type Culture Collection (ATCC, Rockville, MD, USA). 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, USA) and Sigma (St Louis, MO, USA), respectively. Anti-cleaved caspase-3, anti-cleaved caspase-7 and anti-cleaved caspase-9 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Cell-permeable fluorogenic substrate PhiPhiLux-G_1D_2 was purchased from OncoImmunin, Inc. (Gaithersburg, MD, USA). Other analytical reagents were purchased based on the analytical grade.

2. Cell cultures

The KB human oral squamous cell carcinomas were grown in MEM containing NEAA (nonessential amino acids) at a ratio of 100:1, and the media was supplemented with 5% fetal bovine serum (FBS).[23] The KB cells were maintained as monolayers in plastic culture plates at 37℃ in the humidified atmosphere containing 5% CO_2.

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. DNA fragmentation analysis

Following treatment with 0, 30 and 100 μM resveratrol for 48 hours, approximately 5 X 10^6 cells were collected and transferred to lysis buffer containing 100 mM NaCl, 10 mM EDTA, 300 mM Tris-HCl, pH 7.5, 200 mM sucrose, 0.5% SDS and 0.5 mg/ml proteinase K and incubated at 65℃. DNA was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1, v/v) and precipitated with ethanol. The DNA was resuspended in Tris-EDTA buffer, pH 8.0 containing 5 ㎍/ml DNase-free RNase and incubated at 37℃ for 1 hour. The DNA was visualized on 2% agarose gel in the presence of 0.5 ㎍/ml ethidium bromide.

5. Immunoblotting

The cells were treated with 0, 30 and 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, anti-cleaved caspase-7 or anti-cleaved caspase-9 antibody (1:1000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA) was used as the primary antibody.

6. 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 and 30 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).

7. Data analysis

All experiments were performed at least three times. Results are presented as mean ± SEM. Statistical significance was analyzed by using Student's t-test for two groups and one way analysis of variance for multi-group comparisons. $P<0.05$ is considered statistically significant.

Ⅲ. RESULTS

1. Cytotoxic effect of resveratrol in KB 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. From 30 to 300 μM treatment of resveratrol, 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 hours, resveratrol inhibited the proliferation of KB cells in a dose-dependent manner (Fig. 2). The IC_{50} values of resveratrol on the cell viability are shown in Table 1.

Table 1. Antiproliferative effect of resveratrol in KB cells

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

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

2. DNA fragmentation

Increased cellular apoptosis is only one among several possible mechanisms involved in reduced cell proliferation.[7-9,28] To determine if apoptosis is indeed the underlying mechanism for the reduced cell proliferation observed, the KB cells treated with resveratrol were subjected to DNA fragmentation. As shown in Fig. 3, the formation of DNA ladder in the KB cells treated with 30 and 100 μM resveratrol was observed in a time-dependent manner.

3. Activation of caspases by resveratol

The levels of cleaved caspase-3, caspase-7 and caspase-9 were examined by immunoblotting and the levels of procaspase-3 and procaspase-7 were detected by fluorescence microscopy using a selective fluorogenic substrate since caspase-3, caspase-7 and caspase-9 are effector caspases of apoptotic cell death.[29-31] Treatment with 30 and 100 μM resveratrol significantly promoted proteolytic cleavages of procaspase-3 in the KB cells, with the increases in the amount of cleaved caspase-3 (Fig. 4). Resveratrol (30 and 100 μM) also promoted proteolytic cleavages of procaspase-7 (Fig. 5) and procaspase-9 (Fig. 6), with the increases in the amount of cleaved caspase-7 and caspase-9. In addition, activation of caspase-3/-7 in resveratrol treated KB cells was confirmed by fluorescence microscopy using fluorogenic substrate. As shown in Fig. 7, resveratrol treatment led to activate the caspase-3/-7 in the living KB cells.

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] There is a need to find new anti-cancer agents that can kill cancerous cells with minimal toxicity. Resveratrol is a naturally occurring polyphenolic compound that is highly enriched in grape skin and red wine.[12-15] Resveratrol 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, resveratrol effects on oral cancer 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 KB human oral squamous cell carcinoma.

In MTT assay, resveratrol inhibited growth of KB cells in a concentration- and a time-dependent manner (Fig. 1 and 2). 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 oral cancer 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 internucleosomal DNA fragmentation in KB cells, suggesting apoptotic cell death (Fig. 3). These results indicated that resveratrol inhibits the growth of KB 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, caspase-7 and caspase-9 may serve as effector caspases of apoptotic cell death.[29-31] Caspase-3, caspase-7 and caspase-9 are synthesized as inactive proenzymes (of sizes 32 kDa, 35 kDa and 47 kDa, respectively), which require proteolytic activation to cleaved enzymes (of sizes 19 kDa, 20 kDa and 37 kDa, respectively).[29-31] Our results show that low levels of cleaved capase-3, cleaved capase-7 and cleaved capase-9 were present in resveratrol-untreated KB cells, and the amount of cleaved enzymes was increased after resveratrol treatment in the KB cells (Fig. 4, 5 and 6). In addition, the activity of caspase-3/-7 was increased by resveratrol treatment in KB cells compared with DMSO treatment as a control (Fig. 7). These results suggested that resveratrol induces apoptotic cell death through caspase-3-, caspase-7- and caspase-9-dependent processing in the KB cells. 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 results indicate that the resveratrol highly inhibits cell proliferation and induces apoptosis in KB human oral squamous cell carcinoma.

Moreover, these results suggest that the resveratrol could be a new agent of chemotherapeutic for the inhibition of oral cancer cell growth. However, to elaborate this nascent possibility, further investigation of its activity including *in vivo* and purification of bioactive compounds is now in progress.

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Ⅵ. FIGURE LEGENDS

Fig. 1. Time-dependent effect of resveratrol on the cell viability in KB human oral squamous cell carcinoma. The KB cells were treated with 10 (circle), 30 (square), 100 (triangle) and 300 μM (diamond) 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 $A_{570\ nm}$ 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).

Fig. 2. Concentration-dependent 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 hours (diamond). The cell viabilities were determined by the MTT assays. The percentage of cell viability was calculated as a ratio of $A_{570\ nm}$ 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).

Fig. 3. Fragmentation of internucleosomal DNA by resveratrol in KB cells. The KB cells were treated with 0, 30 and 100 μM resveratrol for 48 hours and nuclear DNA (5 μg) was subjected to agarose gel electrophoresis.

Fig. 4. Proteolytic cleavage of caspase-3 by resveratrol treatment in KB cells. (A) Activity of cleaved caspase-3 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". (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 KB cells. (A) Activity of cleaved caspase-7 by resveratrol was measured in KB cells. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

Fig. 6. Proteolytic cleavage of caspase-9 by resveratrol treatment in KB cells. (A) Activity of cleaved caspase-9 by resveratrol was measured in KB cells. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

Fig. 7. Activation of caspase-3/-7 by resveratrol treatment in living KB cells. The cells were treated with 0 and 30 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.

Ⅶ. FIGURES

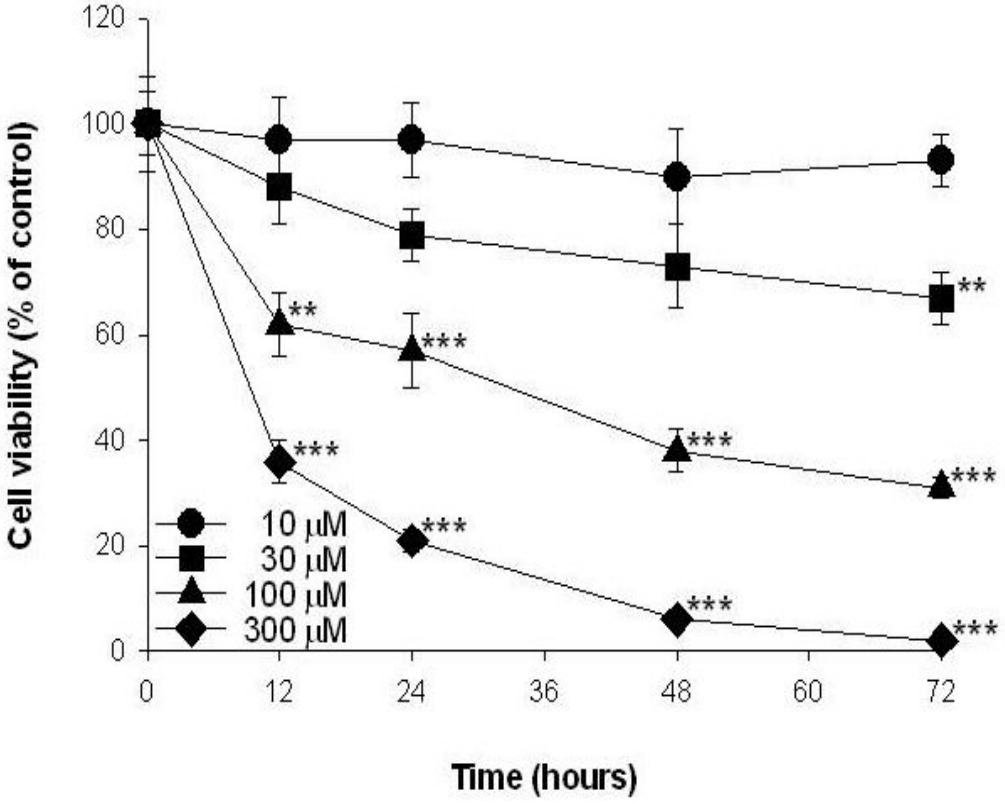

Fig. 1. Time-dependent effect of resveratrol on the cell viability in KB human oral squamous cell carcinoma.

Ⅶ. FIGURES

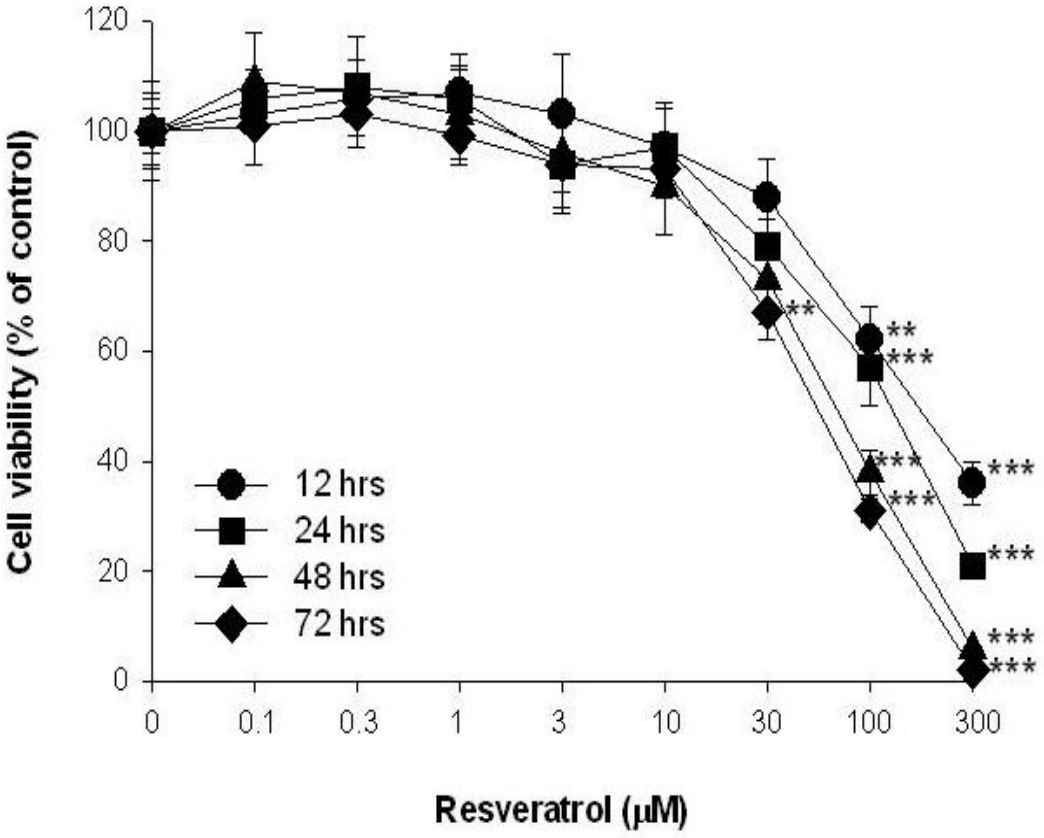

Fig. 2. Concentration-dependent effect of resveratrol on the cell viability in KB cells.

Ⅶ. FIGURES

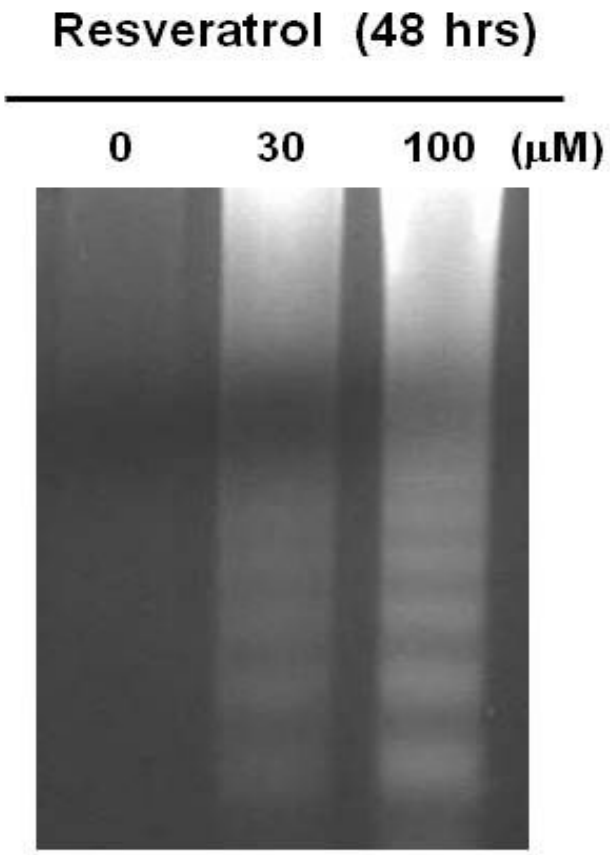

Fig. 3. Fragmentation of internucleosomal DNA by resveratrol in KB cells.

Ⅶ. FIGURES

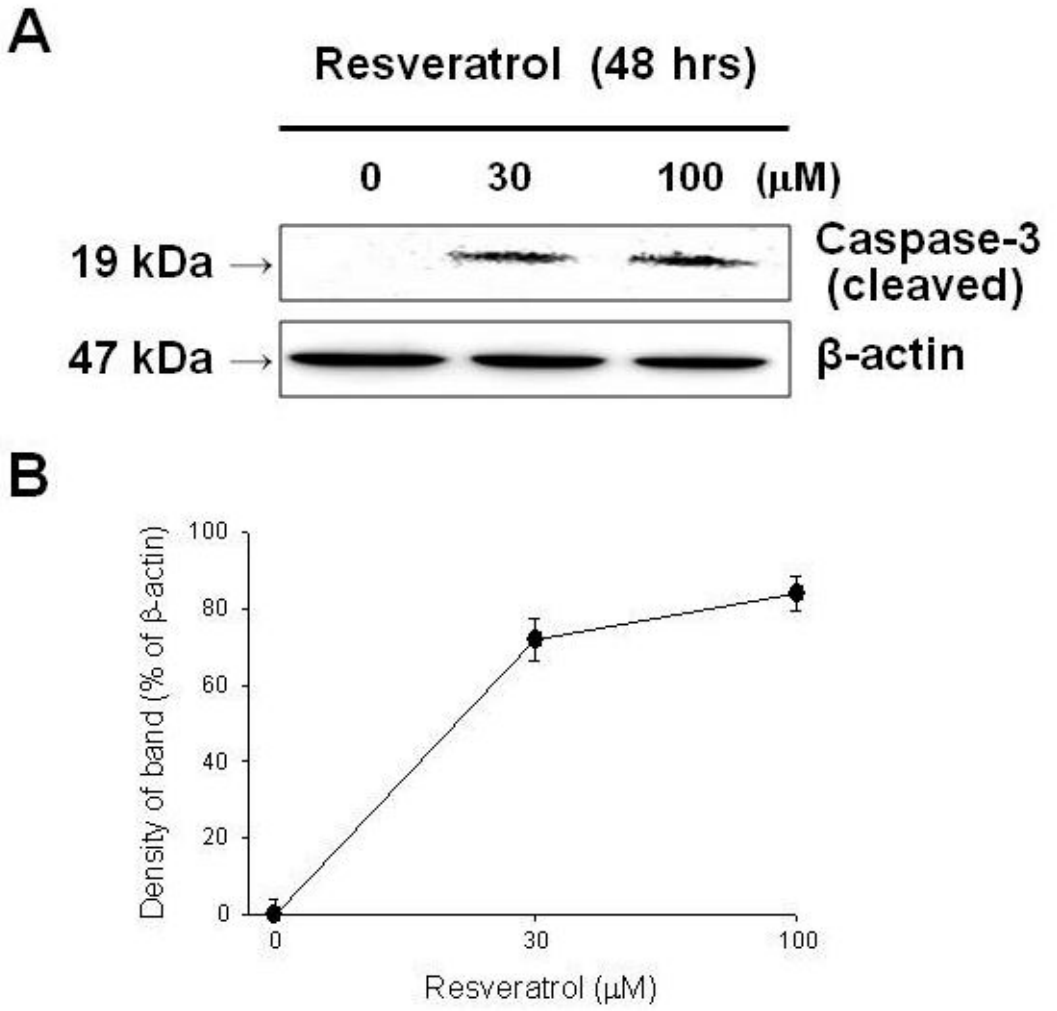

Fig. 4. Proteolytic cleavage of caspase-3 by resveratrol treatment in KB cells.

Ⅶ. FIGURES

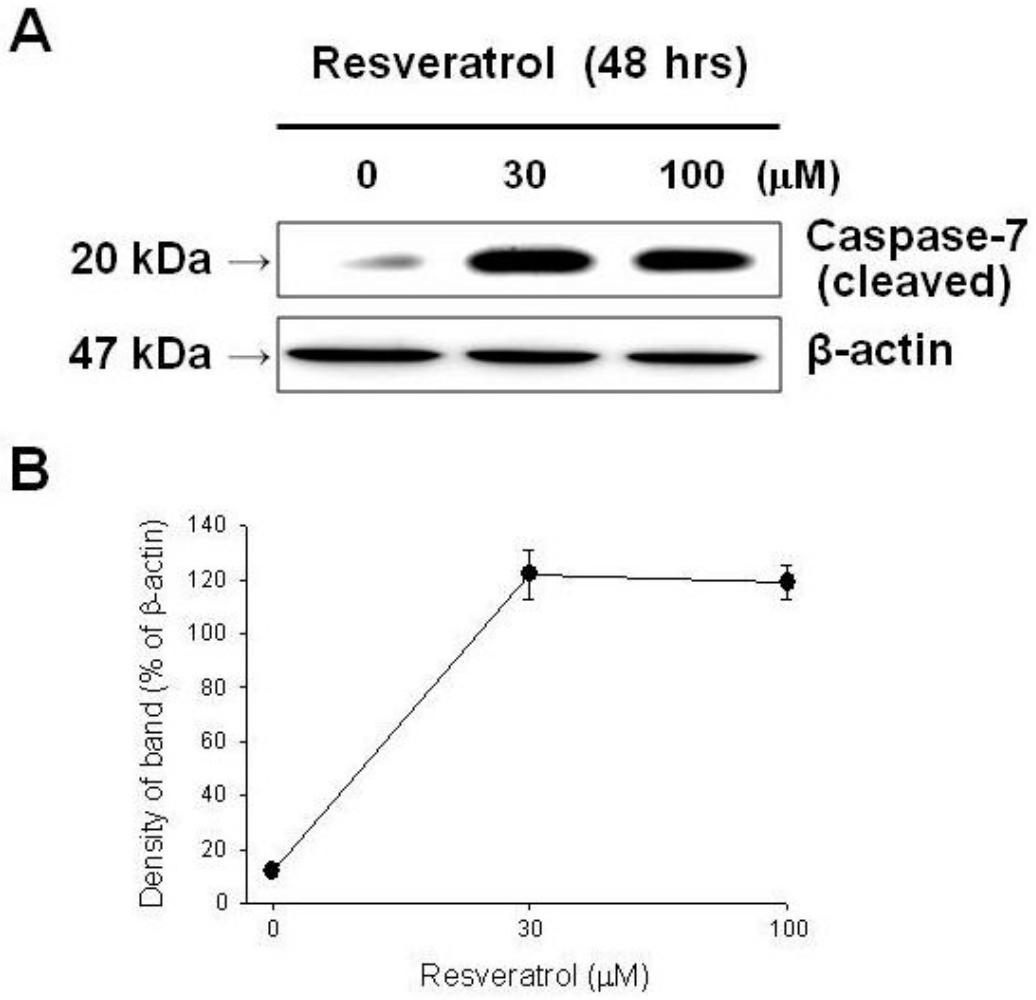

Fig. 5. Proteolytic cleavage of caspase-7 by resveratrol treatment in KB cells.

Ⅶ. FIGURES

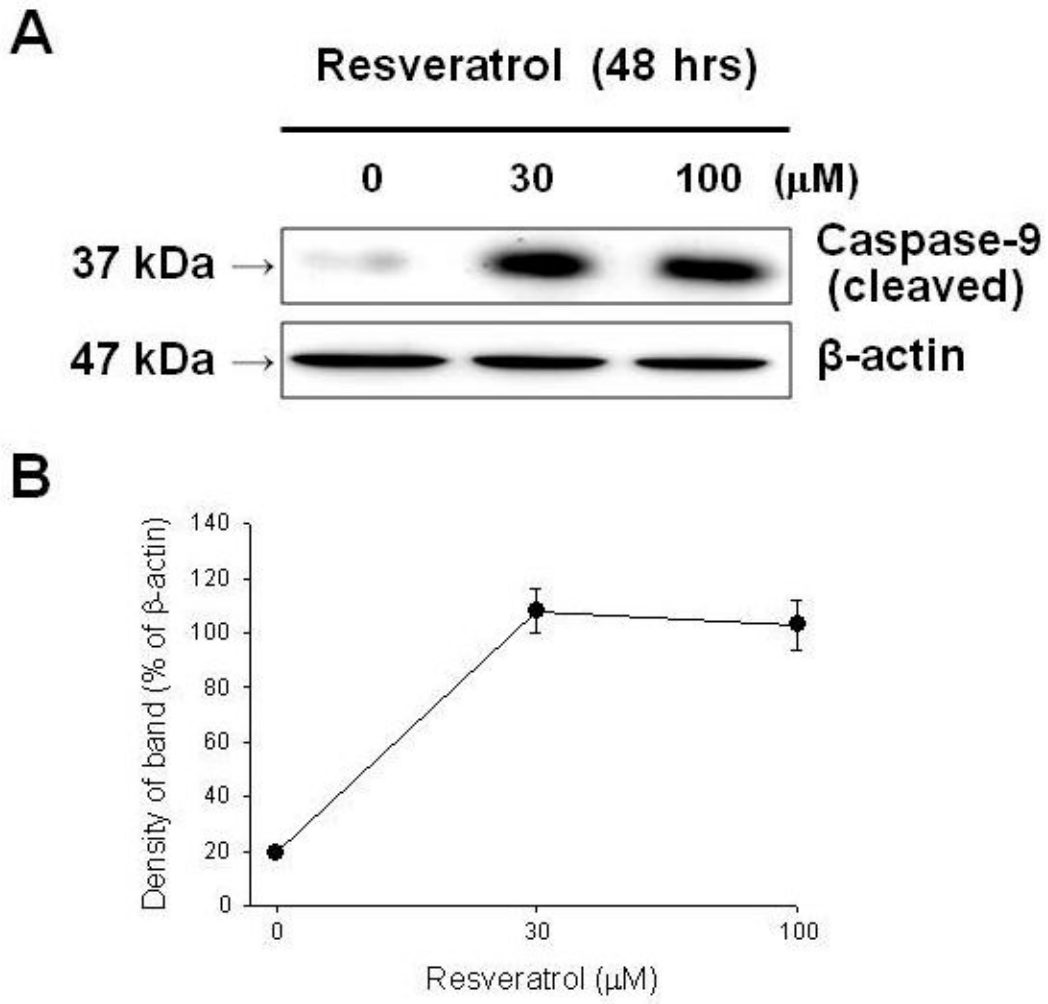

Fig. 6. Proteolytic cleavage of caspase-9 by resveratrol treatment in KB cells.

Ⅶ. FIGURES

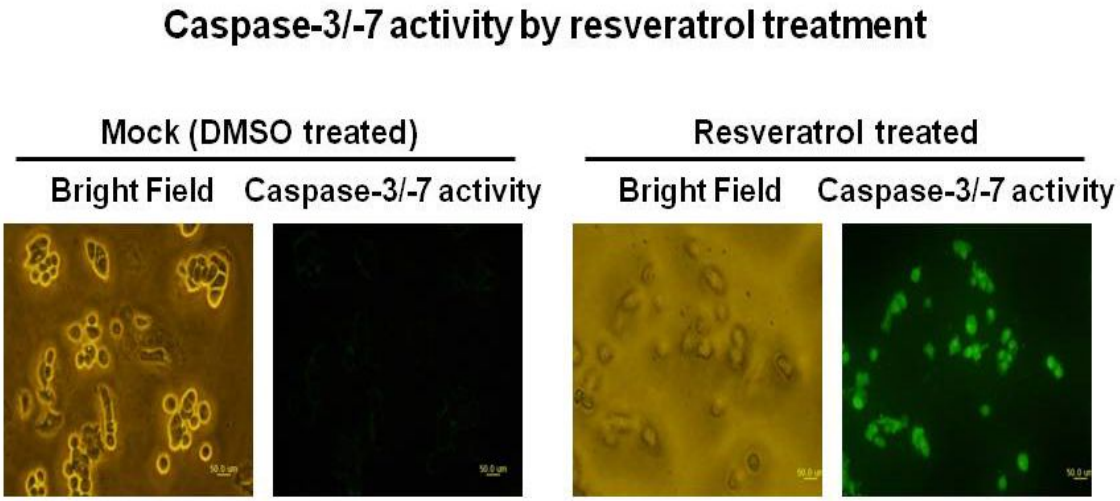

Fig. 7. Activation of caspase-3/-7 by resveratrol treatment in living KB cells.

ABSTRACT in KOREAN

Resveratrol에 의한 사람 구강편평세포암종 KB 세포의 성장억제

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

본 연구에서 resveratrol에 의한 구강암세포 성장억제와 그 기전을 조사하기 위해, KB 세포주에서 resveratrol을 이용하여 MTT 분석, DNA fragmentation 분석, immunoblotting 및 caspase 활성분석 등을 시행하였다.

사람 구강편평세포암종 KB 세포주에서 resveratrol은 구강암 세포주의 성장을 시간과 농도에 의존적으로 억제하였다. KB 세포에서 resveratrol은 DNA fragmentation을 유도하였다. KB 세포에 resveratrol을 처리한 실험군에서 procaspase-3, procasapse-7 및 procaspase-9의 proteolytic cleavage 현상을 확인할 수 있었다. KB 세포에 resveratrol을 처리한 실험군에서 caspase-3/-7의 활성화를 확인할 수 있었다.

본 연구의 결과로 resveratrol은 사람 구강편평세포암종 KB 세포주의 apoptosis를 유도하여 구강암세포 성장을 억제시키는 것으로 사료된다. 또한 본 연구의 결과로, resveratrol을 이용한 구강암세포의 성장억제에 관한 하나의 방향을 제시할 수 있을 것으로 사료된다.

중심어: Resveratrol, 세포사, Apoptosis, 항암치료제, 구강암

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본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(O) 반대( )

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