Effects of Apoptosis on Induction of Resveratrol in Human Lung Cancer A549 Cells

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Resveratrol (3,5,4′-trihydroxystilbene, RSVL) is a supplement found mostly in grape skins. RSVL is known to exhibit potent antiproliferative activity against various cancer cell lines. The aim of this study was to examine the potential of RSVL to induce apoptosis in lung cancer A549 cells and to assess the mechanism of RSVL-induced apoptosis. Five different assays were employed in this study: cell viability and cytotoxicity, morphological assessments of apoptotic cells, quantitative DNA fragmentation analysis, and western blot analysis. The results indicated that RSVL has cytotoxic effects in A549 cells in a concentration- and time-dependent manner. And nuclear morphological changes of cells were observed by RSVL. RSVL regulates the cell cycle, especially Sub-G1 phase. Also, RSVL induces apoptosis of A549 cells via Caspase3, CAD and PARP-dependent fashion.

Key words : Resveratrol, A549 cells, CAD

Introduction

Resveratrol (3,4′,5-trihydroxystilbene, RSVL) is a dietary polyphenol with chemopreventive properties in grapes, peanuts, red wine, and a wide variety of food sources [15]. RSVL has been reported to elicit many cellular responses including cell cycle arrest and differentiation [17], and it has anti-inflammatory and antiviral properties [5,9,32]. RSVL dietary intake is known to be associated with a decreased risk for cardiovascular disease [13]. RSVL can inhibit several enzymes involved in carcinogenesis [12] and platelet aggregation [27]. RSVL has been shown to induce apoptotic cell death in a number of cancer cell lines, including LNCaP prostate cells [28], DU 145 prostate cells [22], mouse myeloid leukemia cells [14], human B cell chronic leukemia cells [30], as well as several other human cancer cell lines such as MCF7, SW480, HCE7, and HL60 [17]. Therefore we set out to study the mechanism of RSVL in lung cancer A549 cells.

Apoptosis does not only play an important role in the development and maintenance of tissue homeostasis but also represents an effective mechanism by which harmful cells can be eliminated. Morphological changes of apoptosis are usually accompanied by internucleosomal DNA fragmentation [4,33], and produces ladders of DNA fragments that are size of 180-200 bp [8]. Cells have a mechanism converse various extracellular signals to intracellular common signals in apoptotic process. But the mechanism of apoptosis is poorly understood yet, it is generally accepted that many different signal of apoptosis ultimately leads to activation of an endogenous endonuclease that cuts DNA between the nucleosomes in the linker regions [3]. DNA fragmentation and chromatin are two characteristic of apoptosis in most cells [25,34]. The main nuclease responsible for DNA fragmentation is CAD/DF40/CPAN3 (caspase-activated DNase/DNA fragmentation factor 40 kDa/caspase-activated nuclease) [26]. In addition, CAD is required for the completion of apoptotic chromatin condensation [6]. Apoptotic signaling and execution pathways involve the activation of caspase, which in turn cleaves key protein substrates. Also, one of the hallmarks of apoptosis is the cleavage of chromosomal DNA into nucleosomal units. The degradation of DNA in the nuclei of apoptotic cells is accomplished in a number of ways following activation of caspases. Western blots were used to measure protein expression level of several genes related to apoptosis. The enzyme poly (ADP-ribose) polymerase, or PARP, was the first protein identified as a substrate for caspases. PARP is involved in repair of DNA damage and functions by catalyzing the synthesis of poly (ADP-ribose) and by binding to DNA strand breaks and modifying nuclear proteins. The ability of PARP to repair DNA damage is prevented following cleavage of PARP. Most recently,
studies indicate that the cancer chemopreventive activity of RSVL was related to its ability to trigger apoptosis. But a few studies have focused on the possible molecular target, which could explain the apoptotic effects of RSVL. The purpose of this study is to elucidate the effects of apoptosis on induction of RSVL in human lung cancer A549 cells.

Materials and Methods

Cell culture and growth condition

The human lung cancer A549 cells were maintained in Dulbecco’s modified Eagle’s medium (D-MEM) with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO₂.

Cell viability assay

Cell viability was measured by hemocytometer using the trypan blue dye exclusion. Trypsinized cells were incubated with 0.4% trypan blue solution (Sigma) for 10 mins, and more than 2×10⁵ cells were scored on a hemocytometer. Viable and nonviable cells were counted by inverted microscopy.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

Cytotoxic effects of RSVL were determined by MTT assay [2]. The cytotoxic effect of RSVL (Sigma) in cells was estimated by MTT assay. In the MTT assay, cells were placed in a 96-well plate and incubated for 24 hr. Then cells were treated with various concentrations of RSVL. And then, the cells were treated with 1 mg/ml of MTT in growth medium. Cells were incubated at 37°C, 5% CO₂ for 4 hr. The medium was aspirated and the formazan crystals, which are formed from MTT by NADH-generating dehydrogenases in metabolically active cell, were dissolved in 200 μl DMSO (dimethyl sulfoxide). Cell viability was evaluated in comparison to the control culture (taken as 100%) by measuring the intensity of the blue color (OD at 540 nm) by a multi-well reader (Quant, Bio-Tek, Highland Park, USA). The assay was performed in triplicate.

Quantitative DNA fragmentation analysis

The quantification of DNA fragmentation was carried as described by McConkey et al. (1989) with slight modifications. Cells treated with UV in a 100 mm culture dish were lysed in 0.33 ml of buffer containing 5 mM Tris, pH 8.0, 20 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% Triton X-100. After incubation for 15 mins on ice, samples were centrifuged for 10 mins at 10,000 rpm to separate the intact chromatin (pellet) from the fragmented DNA (supernatant). Pellets were resuspended in 0.33 ml of a buffer containing 10 mM Tris, pH 8.0 and 1 mM EDTA. Pellets and supernatant fractions were separately assayed for DNA content using the diphenylamine reagent containing 1.5% diphenylamine, 1.5% sulfuric acid and 0.008% acetaldehyde in glacial acetic acid. DNA fragmentation was quantified by measuring the ratio of the DNA content in supernatant fraction to the total DNA content (supernatant plus pellet).

4′-6-diamidino-2-phenylindole (DAPI) staining assay

DAPI stain was used to observe the the apoptotic morphology of cells. For DAPI staining assay, 1×10⁶ cells were plated in 1 ml growth medium in the presence or absence of various concentrations of RSVL in 6-well plates and cultured at 37°C in 5% CO₂. Cells were fixed with 4% paraformaldehyde in phosphate buffered saline for 1 hr, stained by DAPI (500 μl) for 5 min, and then subjected to fluorescence microscopy.

Cell cycle analysis

Cells were harvested in PBS-EDTA, fixed in cold 70% ethanol, and stored at -20°C. Fixed cells were subsequently washed, treated with 100 μg/ml RNase A, and stained with 50 μg/ml propidium iodide. Cells were sorted based on expression of green fluorescent protein and DNA content was analyzed in these cells.

Western blot analysis

Cells were washed three times in cold PBS. Protein content was determined using the bradford assay. Samples were then diluted with an equal volume of 2× SDS sample buffer and heated for 5 min at 100°C. Samples were loaded to equivalent amount (30 μg/lane) on one-dimensional SDS-polyacrylamide gel and subjected to electrophoresis. Membrane was soaked in methanol for 10 secs and washed in distilled water for 5 min. Blots probed for PARP, CAD and Caspase3 were detected with HRP-linked secondary antibodies and enhanced chemiluminescence western blotting reagents (Amersham Pharmacia Biotech), according to manufacturer’s protocols.
Results

Concentration and time-dependent cell death induced by RSVL in A549 cells

To assess the cytotoxic effect of RSVL on lung cancer A549 cells, we analyzed cell viability by trypan blue exclusion or MTT assay. To confirm cell viability of cells, above mentioned trypan blue assay is performed in A549 cells. Cell viability was decreased with increasing treatment dose or time of RSVL. Therefore, RSVL treatment of A549 cells decreased cell viability in a dose-dependent and time-dependent decreased manner (Fig. 1). To examine RSVL-induced growth arrest and apoptosis in A549 cells, we assessed the effect of RSVL on survival and proliferation of these cells by treating them with various concentrations or incubation time of RSVL followed by MTT assays. The results presented in Fig. 2 revealed that RSVL at 0.01, 0.1, 1, 10, and 100 μM reduced proliferation and survival of A549 cells in a dose-dependent fashion. Significant decreases of RSVL-induced cell growth were observed at the concentrations of 10 and 100 μM (Fig. 2A). To determine the time-dependent increase of cell death, cells were treated with 10 μM of RSVL for 12, 24, 48 and 72 hr. Significant decreases of RSVL-induced cell growth were observed at the 48 and 72 hr time points (Fig. 2B).

RSVL-induced apoptotic cell death in lung cancer A549 cells

To confirm morphological changes (fragmentation) of A549 cells by RSVL, 4’6-Diamidino-2-phenylindole (DAPI) staining assay was performed. To determine the time-dependent increase of cell death, cells were treated with 10 μM of RSVL for 6, 12, 24, 48 and 72 hr. Significant decreases of RSVL-induced cell growth were observed at the 48 and 72 hr time points (Fig. 3A). Fig. 3B represents the quantitative analysis of fragmented DNA in A549 cells treated with RSVL and then incubated for various times. The amount of nucleosomal fragmented DNA in cells treated with RSVL was most increased after 48 hr incubation.

RSVL-induced subG1 growth arrest

RSVL, through its stabilizing effects on microtubules, induces Sub-G1 arrest. To determine whether RSVL controlled Sub-G1 arrest in A549 cells, flow cytometric cell cycle analyses were performed following the PI staining of nuclei. Fig. 4 shows the results from a representative experiment in which the cells were incubated for 48 hr with various concentration of RSVL. In results confirmed that the Sub-G1 peak representing apoptotic cells is enhanced following treatment with RSVL in a dose-dependent manner.

Fig. 1. A549 cells (1×10⁴ cells/well in 6-well) were incubated with various concentrations of RSVL for 24 or 48 hr. Trypan blue-positive cells were considered as non-viable and their percentage was estimated by bright-field microscopy.

Fig. 2. Cytotoxic effect of RSVL in A549 cells. Cells (1×10⁴ cells/well in 48-well plates) were treated with increasing concentrations or treatment time of RSVL, and cell survival induced by RSVL was determined by MTT assay.
Fig. 3. Percentage of cell viability or apoptotic cells (A), and quantitative assay of fragmented DNA (B) during apoptosis induced by RSVL in A549 cells. After treatment with RSVL, the cells were incubated for various times. At least 400 cells were scored in each treatment.

Fig. 4. Effect of RSVL on DNA content in A549 cells. The cells were stained with PI and analyzed by flow cytometry. The sub-G1 population was calculated to estimate the apoptotic cell population.

Apoptosis related proteins were detected by western blot analysis

Fig. 5 represents the western blot analysis of expression of PARP, CAD, and Caspase3 proteins in lung cancer A549 cells treated with RSVL for 8, 16 or 24 hr. RSVL induced Caspase3 cleavage and activation from 8 hr. Intact 32 kDa Caspase3 and its 20 kDa cleaved products are indicated. As shown in Fig. 5, treatment of the cells with 10 μM RSVL for 8 hr increased the cleavage of PARP to its 89 kDa active forms in these cells. After treatment of RSVL the A549 cells, the increase of the cleavage of the PARP protein by activated Caspase3. Cleavage of ICAD (inhibitor caspase-activated DNase) by Caspase3 activates the nuclease activity of CAD. After treatment of RSVL the A549 cells, the increase of the CAD protein. These results suggested that Caspase3 or PARP cleavage in the cells affected to apoptosis. And apoptosis was increased when the CAD overexpressed.

Discussion

Numerous studies have focused on the targeted induction of apoptotic cell death in order to control the cancer cells. RSVL has been reported to induce apoptosis in numerous cancer cell lines. RSVL has also been shown to inhibit cell proliferation and induce apoptosis in human breast carcinoma MCF-7 cells [18]. However, the effect of RSVL in human lung cancer A549 cells is still not well understood. Our study intends to examine the effect of RSVL in A549 cells. Our results show that RSVL can induce both dose- and time-dependent apoptosis in A549 cells. Thus, RSVL caused preferentially apoptotic cell death on A549 cells. In this study, RSVL produces a significant
dose-dependent decrease in cell proliferation and induced an apoptotic-type cell death. Apoptosis, as described by Wyllie et al. (1980), involves the condensation of chromatin, restructuring of the cytoplasm, blebbing of cytoplasmic membranes, and finally fragmentation of the cells into apoptotic bodies that are phagocytosed by neighbouring cells, and features that distinguish the process from necrotic cell death. Apoptosis is initially characterized by morphological changes of dying cells. Morphological change (DNA fragmentation) by various concentrations of RSVL was observed. RSVL has been reported to induce the cell cycle arrest at the S phase in human promyelocytic leukemia HL-60 cells [29], T cell-derived lymphocytic leukemia cell line CEM-C7H2 [7] and prostate LNCAp cells [19]. RSVL can partially disrupt G1-S transition in androgen-nonresponsive DU 145, PC-3, and JCA-1 human prostate cancer cells, but has no effects in androgen-responsive LNCAp cells [16]. RSVL induced arrest of the cell cycle at the G1-phase followed by apoptosis has been reported in human epidermoid carcinoma A431 cells [1]. The role of cell cycle regulation on the extent of RSVL-induced apoptosis requires further demonstration of the correlation of cell cycle regulation and apoptosis. Also, our results demonstrated that RSVL induced sub-G1 arrest in a dose-dependent manner.

Apoptosis can be triggered in various ways (Lowe et al., 1993). The caspases have cleverly targeted the proteins within signalling pathways that will assist in their purpose to destroy the cell [10]. Thus death receptor mediated caspase cleavage of substrates can engage the mitochondria to amplify the pro-apoptotic signal (Bosch-Weitzen et al, 1999). Caspase3 had been perceived as the principle enzyme responsible for cleaving PARP [20, 21]. PARP plays the active role of "nick sensor" during DNA repair and apoptosis, when it synthesizes ADP-ribose from NAD⁺ in the presence of DNA strand breaks. Moreover, PARP becomes a target of apoptotic caspases, which originate two proteolytic fragments of 89 and 24 kDa [31]. RSVL-induced apoptosis requires the activation of Caspase3. CAD proteins is involved in apoptosis and prevents the accumulation of chromosomal damage. It has been reported that CAD proteins expression was increased in a benign disease and apoptosis. In this study, RSVL treatment induced the increase of CAD proteins. And, RSVL treatment demonstrated that the expression of Caspase3 and PARP cleaved in time-dependent manner. RSVL induced apoptosis in A549 cells is associated with Caspase3 and PARP activation and CAD. The results presented here indicate that RSVL is capable of killing A549 cells through apoptosis mechanism. In conclusion, all observations indicate that RSVL may provide a superior therapeutic index and advantage in the clinic for the treatment of A549 cells.

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References

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초록: 사람 A549 폐암세포에 resveratrol 유도가 세포자멸사에 미치는 영향

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Resveratrol (RSVL)은 포도껍질에 많이 함유된 성분으로 다양한 암세포에 대해 antiproliferative activity가 존재한다고 보고된 바 있다. 이에 본 연구에서는 폐암 A549세포에서 RSVL이 세포자멸사를 유도하는지와 관련된 세포자멸사 기작에 대해 알아보고자 한다. 그리하여 본 연구에서는 5가지 방법으로 즉, 세포생존율검사, 세포특성 검사, apoptotic cell의 형태학적 변화, 정량적 DNA 분석 분석, western blot 분석법을 사용하여 수행하였다. 본 연구의 결과로 RSVL을 처리한 A549세포에서 형태학적(분절화) 변화가 관찰되었고, flow cytometric 분석에서는 RSVL이 세포에서 세포주기 특히 subG1기로 조절함을 알 수 있었다. 그리고 RSVL을 처리한 A549세포에서는 apoptosis와 관련된 caspase3와 PARP cleavage가 유발되었고 CAD 단백질 증가도 보였다.