Induction of Apoptosis by Vitamin E Succinate in Human Erythroleukemia K562 Cells

Chang Deug Jang, Jong Myoung Kim[1], Won G. An[2] and Hye Ryoun Park*

School of Dentistry, Pusan National University, 602-739
[1]Department of Aquaculture, PuKyong National University, 608-737
[2]Department of Nanomedical Engineering, Pusan National University, 609-735

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Regulation mechanism of apoptosis has been known to be important for understanding the pathogenesis of a number of human diseases including cancers. The effects of RRR-α-tocopheryl succinate (vitamin E succinate, VES) on the cell viability, generation of ROS, expression of proteins involved in apoptosis, and growth of human chronic myelogenous leukemia K562 cells were analyzed in this study. VES treatment not only induced the generation of the ROS but also increased the levels of NF-κB, COX-2, and $p21^{WAF1/CIP1}$ in K562 cells. It modulates the levels of pro-apoptotic proteins such as Bax provoking the apoptosis in K562 cells. The cleavage of PARP into 89 kDa was also increased upon VES treatment in a dosage-dependent manner. Induction of an apoptosis was evident by the increase of sub-G1 peak and cell shrinkage condensed chromatin in K562 cells treated with VES. It also resulted in an inhibition of tumor growth by 50% and prolonged survival of the lymphoma-induced mice. This potentiation of VES obtained in *vitro* and *in vivo* may indicate the feasibility of more effective chemotherapy in chronic myelogenous leukemia.

Key words – VES (Vitamin E succinate), apoptosis, cancer

Introduction

Apoptosis is a major component of normal development, preservation of tissue homeostasis, and elimination of damaged cells. Deregulation of apoptosis has been shown to contribute to the pathogenesis of a number of human diseases including cancers [31]. Induction of an apoptosis was known to be mediated by genes such as p53, Bcl-2 and Bax [6]. The mitochondria-dependent apoptotic signaling pathway was responsible for extracellular signals and internal injuries such as DNA damage. Cytotoxic stress affect the pro-apoptotic members of the Bcl-2 family such as Bax to translocate from the cytosol to mitochondria, leading to the release of cytochrome c into the cytosol. The other apoptotic signaling pathway was known to be triggered by the death-receptor superfamily members through the activation of caspase-8. This directly activate the downstream caspases leading to the cellular degradation.

Reactive oxygen species (ROS) were known to be involved in the induction of apoptosis mediated through mitochondria [5] and senescence [7]. Abundant ROS representatives in the living cell include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl anion radical ($OH^·$) [13,26]. ROS were generated from the interaction of ionizing radiation with biological molecules, the byproducts of cellular respiration, and as the second messengers in various signal transduction pathways [32].

Vitamin E, an essential fat-soluble vitamin, is an antioxidant involved in the metabolism of the cells. It protects vitamin A and essential fatty acids from the oxidation in the cells and prevents the breakdown of body tissues [33]. RRR-α-tocopheryl succinate (vitamin E succinate, VES, Fig. 1), a derivative of vitamin E, exhibits antioxidant properties upon hydrolysis of its succinate group by specific ester hydrolase [19]. Previous studies have demonstrated that normal cells have the differential susceptibility to the VES-triggered apoptosis as compared to that of the cancer cells possibly due to its ability to rapidly hydrolyze VES, thereby generating α-tocopherol in the cells [19,28]. The

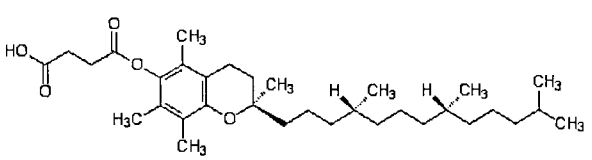


Fig. 1. The chemical structure of Vitamin E succinate (VES).

***Corresponding author**
Tel : +82-51-240-7839, Fax : +82-51-510-3024
E-mail : parkhr@pusan.ac.kr

pro-apoptotic activity of VES may result from mitochondrial destabilization, formation of ROS [12,27] as well as the release of cytochrome c and activation of multiple caspases. VES were also known to generate free radicals by alteration of mitochondrial structure and provokes relocalization of cytochrome c and activation of multiple caspases in human lymphoma Jurkat cells [27]. In addition, VES-induced apoptosis via ROS generation could be blocked by a mitochondrially targeted coenzyme Q acting as an antioxidant [1].

Leukemia is a malignant disease (cancer) of the bone marrow and the blood characterized by the uncontrolled accumulation of blood cells. Leukemia were divided into four categories, myelogenous or lymphocytic, each of which can be acute or chronic. Studies on VES were carried out with human acute myeloid leukemia HL-60 cells [3] but not with human chronic myelogenous leukemia K562 cells. In order to understand the effects of VES in chronic myelogenous leukemia and provide the detailed molecular basis for the leukemia treatment, effects of VES on the apoptosis were analyzed in this study.

Materials and Methods

Materials

RPMI 1640 media, fetal bovine serum (FBS) and antibiotic-antimycotic were purchased from GIBCO-BRL (Grand Island, NY, USA). RRR-α-tocopheryl succinate, Hoechst 33258, Proteinase K, Propidium iodide and RNase A were purchased from Sigma-Aldrich (St. Louis, MO, USA). DCFH-DA (2′,7′-dichloro- dihydrofluorescein diacetate) was purchased from Molecular Probe (Eugene, OR, USA). Protease inhibitor cocktail tablets was purchased from Roche (Nutley, NJ, USA). BCA protein assay kit was purchased from Pierce (Rockford, IL, USA). MTT assay kit was purchased from Promega Corporation (Madison, WI, USA). Enhanced chemiluminescence (ECL), anti-mouse IgG antibody and anti-rabbit IgG antibody were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL, USA). The $p21^{WAF1/CIP1}$ monoclonal antibody was purchased from BD Biosciences (San Jose, CA, USA). The NF-κB polyclonal antibody and the Bax monoclonal antibody were purchased from Oncogene (San Diego, CA, USA). The PARP polyclonal antibody and the COX-2 polyclonal antibody were purchased from Cell Signaling (Beverly, MA, USA).

Cell lines and culture conditions

Human chronic myelogenous leukemia cell line K562 cells and murine lymphoma cell line EL-4 cells, purchased from American Tissue Type Collection [ATTC] (Manassan, VA, USA) were used in this study. Cells were grown in RPMI 1640 medium supplemented with 1% antibiotic-antimycotic and 10% FBS at 37°C in a humidified atmosphere of 5% CO_2. Media were changed twice a week. The cells were passed every 2-3 days and replated in 100 mm cell culture dish at a density of 1×10^5 cells/ml media. One day before the experiments, the cells were replated in 60 mm cell culture dish at 1×10^6 cells/ml media.

Cell viability analysis

In vitro growth inhibitory effects of VES on K562 cells were determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dye absorbance by the living cells using 1×10^6 cells/ml media seeded in 96-well microtiter plates were exposed to the drug for 24 hr and 48 hr. After the addition of 15 μl MTT dye solution followed by incubation for 4 hr at 37°C, MTT stop solution was added to the well. Plates were incubated for 2 hr at room temperature followed by gentle rotation on a shaker for 10 min to fully release formazan crystals trapped within viable cells. Absorbance were determined at 490 nm in a microplate reader (Anthos 2020, Ver. 1.5).

ROS measurement

Amounts of ROS were measured using DCFH-DA dye. VES-treated cells (1×10^6) were incubated in 1 ml PBS containing 20 mM DCFH-DA for 1 hr at 37°C. The relative ROS units were determined using a fluorescence ELISA reader with 530 nm excitation and 485 nm emission. An aliquot of the cell suspension was lysed to determine protein concentration and the results were expressed as an arbitrary absorbance units per μg protein.

Western blot analysis

Harvested cells were washed twice with ice-cold PBS and lysed in 50 μl of TNES buffer containing a protease inhibitor cocktail tablet, 1 M Tris-HCl, pH 7.4, 1% NP-40, 0.5 M EDTA, and 5 M NaCl for 30 minutes. The protein concentration was determined by the BCA protein assay. Samples containing 30 μg of total or nuclear protein were subjected to a 12% or 8% SDS-PAGE gel electrophoresis. Proteins transferred onto a nitrocellulose membrane by

electroblotting were probed with the antibodies against $p21^{WAF1/CIP1}$, NF-κB, Bax, PARP, and COX-2. Antibody was detected with the respective secondary antibody, either anti-mouse IgG or anti-rabbit IgG antibodies linked to horseradish peroxidase followed by its detection using the ECL kit.

Cell cycle analysis

Cell cycle distribution was determined by staining DNA with propidium iodide (PI). Cells (1×10^6) incubated with or without VES treatment for 48 hr were washed with PBS and then fixed with 80% ice-cold ethanol for 24 hr. The cells were incubated in a PI solution containing 50 μg/ml of PI, 100 μg/ml of RNase A, and 0.1% NP-40 at 4°C for 30 min in a dark room. The percentage of the cells in the apoptotic stage of the cell cycle was measured with FACS Caliber flow cytometry (Beckman coulter) using Expo 32 ADC XL 4 color with an excitation of 488 nm and an emission of 585 nm.

DNA staining

The harvested K562 cells washed with PBS were spun down on microscope slides using Cytospin 2 from Shandon Inc. (Pittsburgh, PA, USA). The cell pellets were fixed with 4% paraformaldehyde for 15 min at room temperature followed by treatment with permeablization solution (acetone : methanol = 1:1) for 5 min at room temperature and then incubation with 1 μg/ml of Hoechst 33258. The cells were washed with PBS between the steps. Cells were viewed using a fluorescence microscope with ultraviolet (UV) excitation at 300-500 nm. The cells with nuclei containing a clearly condensed chromatin were scored as apoptotic cells.

Animal experiment

Female C57BL/6 mice were purchased from HyoChang Bioscience (Daegu, Korea). Mice maintained under the pathogen-free condition were used at 8-10 weeks of age. EL-4 cells (2.5×10^5/mouse) were injected subcutaneously and the VES was injected intraperitoneally on days 8, 11, 16 and 23 while a control group received corn oil. Tumor diameter was measured every other day using a caliper. The size of the tumor size was expressed as the tumor volume calculated from the following formula: tumor volume (mm^3) = (major axis) × (minor axis) × (height) × 0.52. Animals were killed when their tumors were grown to 20 mm in the longest dimension. At least eight mice per treatment group were examined throughout the study. Each experiment was the representative of at least three similarly performed experiments.

Statistics

Unless stated otherwise, data were given as mean ± SE of at least three independent experiments, and images shown are representative pictures of three to six independent experiments. Log-rank statistics were used to analyze the significance in long-term survival in animal experiment.

Results

Cell growth inhibition

To evaluate the effects of VES on the growth of K562 cells, cells were treated with 5, 10, 20, and 40 μg/ml of VES for 24 hr and 48 hr, respectively. The viability of the cells was measured by its ability to form formazan crystals through mitochondrial respiration. Fig. 2 showed that the viability of K562 cells was decreased as VES dosage increased (Fig. 2). It was declined to 20% upon incubation with 40 μg/ml of VES for 48 hr. Concentration of VES at 20 μg/ml showing as IC_{30}, a drug concentration that produced a 30% inhibition of cell viability, was used for the further experiments.

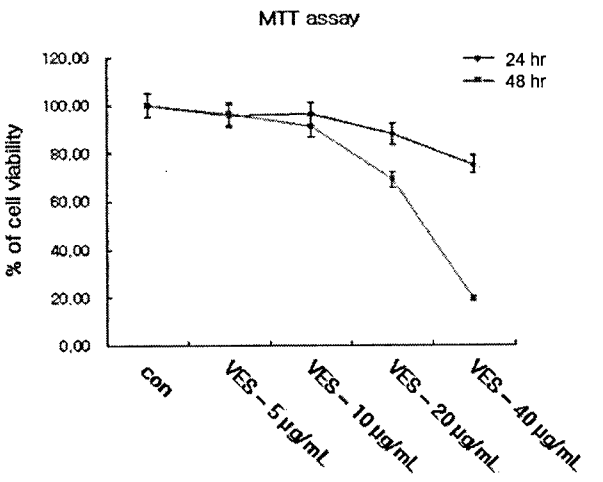
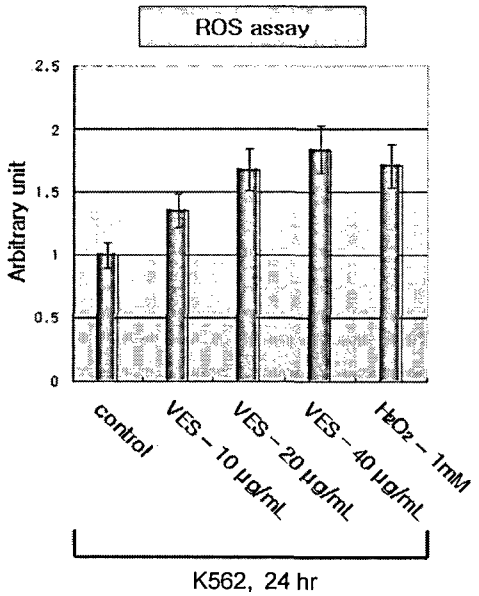


Fig. 2. Potencies and maximal cytotoxicities of VES. K562 cells were incubated with 0, 5, 10, 20, and 40 μg/ml of VES, respectively. MTT assay was performed after 24 hr and 48 hr as described in "Materials and Methods". Results were calculated as the percentage of values obtained with untreated cells and represent mean ± SD.

ROS generation

Intracellular production of ROS was measured by DCFH-DA probe which is permeable to the cell. Nonfluorescent DCFH-DA was hydrolyzed to DCFH in the cytosol which in turn yields highly fluorescent DCF in the presence of intracellular hydrogen peroxide and related peroxides. Therefore, the level of hydrogen peroxide and superoxide present in the cells could be determined from the measurement of DCF. Upon VES treatment, an increase in the level of ROS in K562 cells (Fig. 3) was observed. In particular, the level of ROS generated in the cells treated with high concentrations of VES was even higher than that of ROS generated in the presence of 1 mM H_2O_2. The result indicated that VES treatment enhanced ROS generation in K562 cells affecting the cellular oxidative state.



zFig. 3. Intracellular levels of ROS in K562 cells Cells treated with 0, 5, 10, 20, and 40 μg/ml of VES for 24 hr were subjected to ROS analysis using DCFH-DA.

Effects of VES on the expres sion of NF-κB and COX-2 in K562 cells

Expression of NF-κB, a redox sensitive nuclear transcription factor, has been known to be associated with a variety of stimuli including cytokines, MAPK signaling and ROS. Analysis of the NF-κB was carried out with K562 cells treated with 20 μg/ml of VES for 6, 12, 24 and 48 hr (Fig. 4A), respectively. The result indicated the increase of NF-κB as VES incubation time was increased. This suggested that the generation of ROS might be a controller for the activation of NF-κB in the cell. NF-κB has been shown to be a positive regulator of COX-2 expression in macrophages [8] and cancer cell lines [20]. Our result also showed the increase in the level of COX-2 expression upon VES treatment. This confirms the role of NF-κB as a potential regulator of COX-2 in K562 cells.

Effect of VES treatment on the expression of $p21^{WAF1/CIP1}$ and Bax in K562 cells

ROS was known to affect the cell cycle progression through the several redox-dependent changes in cell cycle-related proteins. This was exemplified in the p53-independent accumulation of $p21^{WAF1/CIP1}$ requiring the integrity of the ras-MAPK pathway. Activation of $p21^{WAF1/CIP1}$ by VES treatment has been reported in various cell lines [23]. To determine whether the increased levels of ROS cause the activation of $p21^{WAF1/CIP1}$ in K562 cells, its level in the cells treated with VES were analyzed (Fig. 4B). The re-

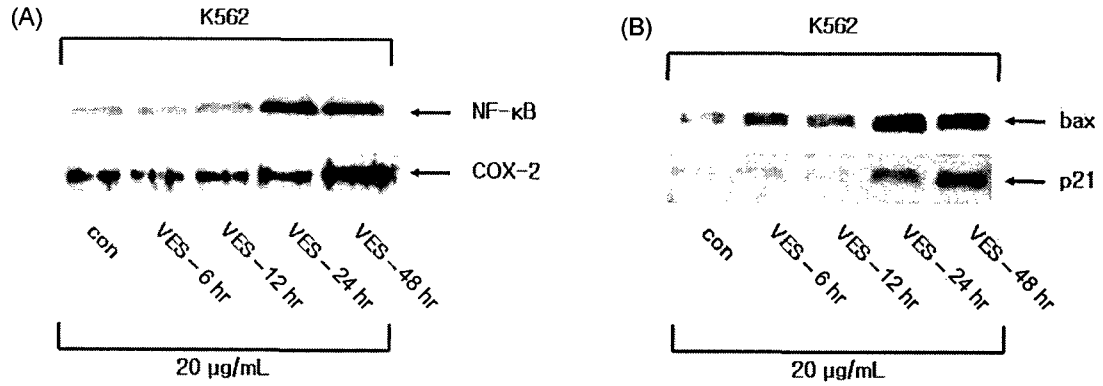


Fig. 4. Effects of VES on induced expression of NF-κB, COX-2, Bax and $p21^{WAF1/CIP1}$. (A) Nuclear(top), cytoplasmic(bottom), and (B) total extracts from cells treated with VES (20 μg/ml) for the designated times. Western blot analysis was carried out with an antibody against the proteins indicated.

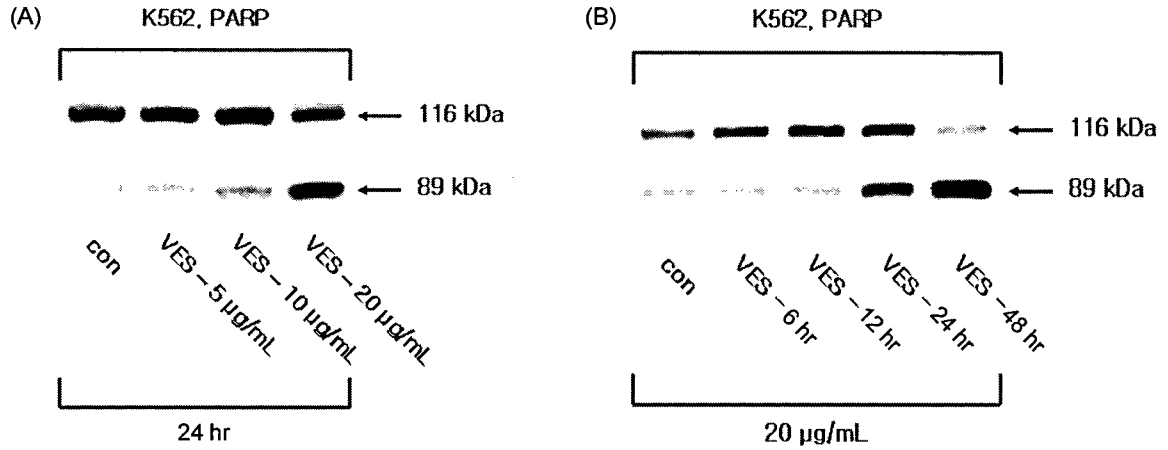


Fig. 5. Stimulation of PARP cleavage after addition of VES. (A) Cells treated with various concentrations of VES for 24 hr were subjected to the analysis. (B) Total cellular proteins were prepared from the cells treated with 20 μg/ml of VES for the times indicated. Western blot analysis as carried out with an antibody against PARP.

sult showed an increase of p21 in the cells upon incubation with VES.

Bax, one of the pro-apoptotic proteins, is a key regulator in the apoptotic pathway. It was known to be activated during an interruption of the mitochondrial pathway and play a key role in releasing cytochrome *c* from mitochondria. Analysis of the Bax protein (Fig. 4B) in K562 cells showed an increase upon VES treatment, hence provoking an apoptosis in the cells. In eukaryotic cells, execution of apoptosis depends on the activation of a family of cysteine proteases, caspases [21] which was known to be initiated by poly (ADP-ribose) polymerase, PARP, a substrate for caspase-3. Therefore, the formation of a 89 kDa C-terminal fragment resulting from the cleavage of PARP by caspase-3 has been used as an apoptotic marker. Analysis of the VES treatment effects on the apoptosis indicated that the level of PARP was increased as VES concentraion increased (Fig. 5A). It was also noticed that the cleavage of PARP into the 89 kDa fragment in K562 cells treated with 20 μg/ml of VES was increased as the exposure time increased (Fig. 5B).

Cell cycle analysis

In order to determine the phase of the cell cycle at which VES exerts its growth inhibitory effects, flow cytometry analysis was carried out with K562 cells treated with various concentrations of VES for 48 hr (Fig. 6). The result indicated the increase of sub-G1 peak in the cells incubated with 10, 20, and 40 μg/ml of VES for 48 h probing its role in apoptosis in K562 cells.

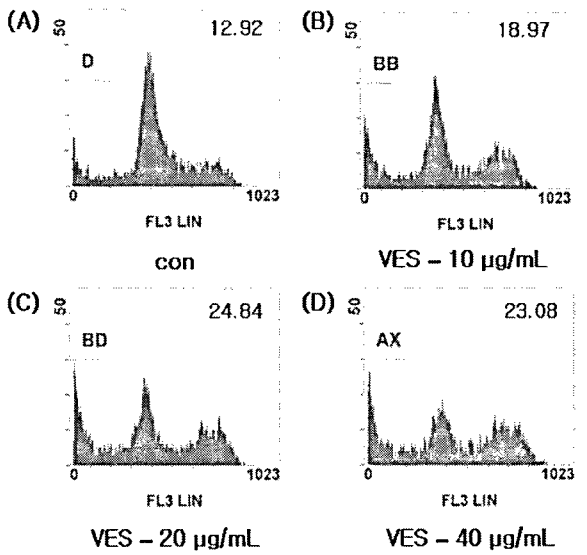


Fig. 6. Cell cycle analysis of VES-treated K562 cells. K562 cells were treated with 0 μg/ml (A), 10 μg/ml (B), 20 μg/ml(C), and 40 μg/ml (D) of VES for 48 hr. Harvested cells were subjected to cell cycle analysis upon staining of DNA with PI followed by flow cytometry. Numbers indicate the percentage of cells in the sub-G1 phase of the cell cycle.

To further examine the VES-induced apoptosis, cells were stained with Hoechst 33258 for DNA morphology analysis using a fluorescent microscope (Fig. 7). A large number of cells treated with VES displayed morphological changes such as cell shrinkage and condensed chromatin which were known to be the characteristic of apoptosis. All the results demonstrated that VES is a potent inducer of apoptosis in K562 cells.

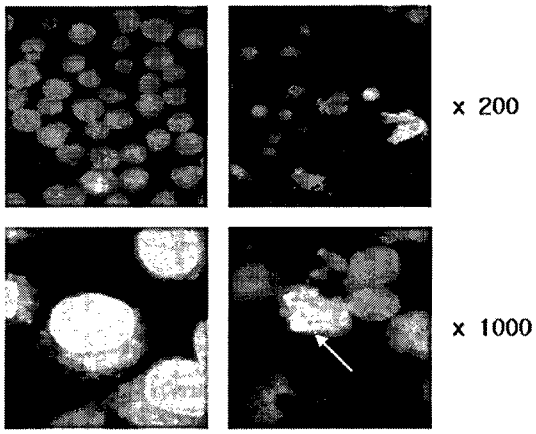


Fig. 7. Flurorescent microscopy of K562 cells stained with Hoechst 33258. K562 cells treated with VES (20 μg/ml) for 72 hr were subjected to the analysis. Arrows indicates the condensed chromatin in the K562 cells.

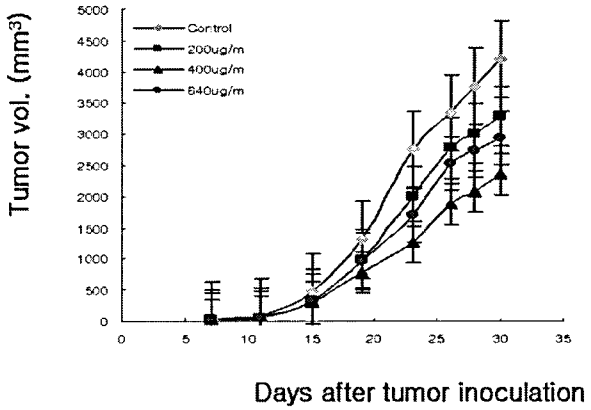


Fig. 8. Effect of VES concentration on EL-4 tumor growth in C57BL/6 mice. Mice injected with EL-4 cells (2.5×10^5/mouse) were administered intraperitoneally with various concentrations of VES as described in the Method. Tumor volume was calculated from the diameters of the tumor. Animals were killed when their tumors were 20 mm in the longest dimension.

Effects of VES treatment of the inhibition of EL-4 tumor growth *in vivo*

To explore the effects of VES treatment on the tumor cells growth, mice with EL-4 cell-derived xenograft were treated with equimolar dose of VES. Mice injected with 400 μg of VES/mouse weighing approximately 20g showed a marked retardation of tumor growth as compared to that of the control mice (Fig. 8). Treatment with VES resulted in an inhibition of tumor volume growth by 50%. VES also prolonged survival (P = 0.028) rate of the mice. This was shown by that 63% of the survival in the mice with 400 μg /mouse treatment until day 38 while 100% of control vehicle treated-mice died at the day.

Discussion

VES was known to enhance the immune response and induce cellular differentiation and growth inhibition [10, 36]. It has also been implicated in the growth inhibition and apoptosis induction in a variety of human tumor cells [29]. Inhibitory role of VES on the proliferation in BT-20 human breast cancer cells was mediated by decreasing the level of E2F-1 phosphorylation [35] or by enhancing the expression of $p21^{WAF1/CIP1}$, a cell cycle checkpoint protein. Induction of an apoptosis was known to be mediated by an activation of protein phosphatase 2A, leading to inhibition of protein kinase C activity, or by regulating TGF-signaling pathway in RL human B lymphoma cells [34]. VES induced apoptosis in K562 cells was assessed by chromatin analysis in this study. DNA content analysis showed that a low molecular weight DNA was lost from apoptotic cells, resulting in a peak at the sub-G1 position. Cells treated with VES showed apoptotic morphological characteristics including a small condensed body chromatins which were fragmented and packed into compact membrane-bound bodies together with randomly distributed cell organelles.

Oxidative stress plays a key role in apoptosis through the generation of ROS [11]. It causes a wide range of adaptive cellular responses ranging from transient growth arrest to permanent growth arrest, apoptosis or necrosis depending on the species and the level. Earlier studies were accomplished for the ROS-generating ability of cancer cells in response to VES and the intervention of ROS in apoptosis [17]. VES may generate different types of ROS depending on the species or tissue of the origin and play a role in inducing apoptosis generally in cancer cells including HL-60 [36].

Numerous genes and signal transduction pathways including NF-κB, HIF-1α, COX-2, p53, and $p21^{WAF1/CIP1}$ have been implicated in ROS signaling [30]. NF-κB is a redox-sensitive nuclear transcription factor composed of a heterodimer of p50 and p65 protein subunits. It has been found to play an active role in inflammatory responses, cellular growth, and apoptosis as present in diseases such as cancer, arthritis, and asthma [4]. NF-κB was known to be activated by a variety of stimuli including cytokines,

MAPK signaling and ROS. The activation complex for NF-κB is located in the cell cytoplasm where it is bound to I-κB in its inactive form. Activation of the NF-κB signaling cascade results in the complete degradation of I-κB or partial degradation of the carboxyl termini of the p105 and p100 precursors, allowing the translocation of the NF-κB complexes into the nucleus. The levels of NF-κB was correlated with the dosage of VES treatment and ROS level in K562 cells. This seems to be due to cellular stress responsive gens inducing apoptosis as some of the NF-κB target genes, such as p53 [37] and COX-2 [22], were known to be implicated in an apoptosis. Our result also supported the role of NF-κB in VES-induced apoptosis in K562 cells.

VES treatment increased the expression of COX-2 (Fig. 4) which was known to be regulated by NF-κB through its 5'-promoter region containing two putative NF-κB binding sites. Therefore, NF-κB has been recognized as a positive regulator of COX-2 expression. The expression of COX-2 was also known to be regulated by ROS without its transcriptional activation by NF-κB [25]. Our result indicated that COX-2 play a role in the VES-induced apoptosis in K562 cells although the pathway involved in the up-regulation of COX-2 was not confirmed.

A cyclin dependent kinase (CDK) inhibitor, $p21^{WAF1/CIP1}$ is a key component in the ROS signaling affecting the cell cycle progression. It belongs to the family of Cip/Kip proteins which contains a conserved sequence at the terminus required for the inhibition of cyclin/CDK complexes. Various levels of $p21^{WAF1/CIP1}$ was detected depending on the cell lines and responses to different drugs. In general, $p21^{WAF1/CIP1}$ was known to be down-regulated in an apoptotic process [31]. Earlier study have shown the results in which $p21^{WAF1/CIP1}$ was up-regulated in VES-induced apoptosis in leukemia [20]. Expressions of $p21^{WAF1/CIP1}$ was increased in K562 cells by VES treatment in a dosage-dependent manner. VES-induced expression of $p21^{WAF1/CIP1}$ appears not to be directly associated with apoptosis. ROS could be a potential regulator of $p21^{WAF1/CIP1}$ in K562 cells treated with VES.

Bax, a pro-apoptotic member of Bcl-2 family proteins, was known to form channels in the synthetic membrane [2] and induce cytochrome *c* release from the isolated mitochondria [16]. Bax was known to contain the hydrophobic membrane-anchoring domain at its C-terminus although the N-terminal domain repress its anchoring potential allowing the Bax to be distributed typically in the cytosol [14]. Upon apoptotic stimulation, Bax was inserted into mitochondria membrane for its function [15]. It was noticed that the cells treated with VES showed an altered mitochondrial structure, generation of free radicals, relocalization of cytochrome *c* and activation of multiple caspases. Our result showed an increased expression of Bax in K562 cells upon VES treatment as consistent with the previous result.

PARP is a nuclear enzyme that detects and binds DNA strand breaks produced by various genotoxic agents [9]. The function of PARP was known to be related with the transcription and DNA repair, hence essential for the maintenance of the genomic integrity [24]. VES treatment in K562 cells showed the stimulation of the cleavage of the PARP into 89 kDa form, an early marker of apoptosis [9]. Our study indicated that VES made PARP fail to repair DNA of K562 cells.

Tumor suppressing activity of the VES was investigated to evaluate the effects of VES *in vivo*. Various concentration of VES were tested to find an optimum dosage of VES that can suppress the tumor growth in mice. VES treatment to the mice bearing s.c. tumors resulted in the retardation of tumor growth although all mice eventually had progressive tumors. Mice injected intraperitoneally with VES showed a prolonged survival in comparison to the control without VES injection. EL-4 tumor-bearing mice treated with VES extended lives more than 10 days against the control 25 days after the injection. Mice treated with 400 μg of VES prolonged the survival rate by 60% after 35 days after the inoculation. The dual mode of anti-tumor activity and survival rate suggest that the efficacy of VES to tumor regression in the established EL-4 murine lymphoma model.

In this study, effects of VES on the generation of ROS and apoptosis in the chronic myelgenous leukemia K562 cells. The VES treatment affect the expression of multiple proteins including NF-κB, COX-2 and $p21^{WAF1/CIP1}$ in K562 cells. Moreover, during induction of apoptosis, VES triggered translocation of cytosolic Bax to the mitochondria followed by cleavages of PARP. Schematic representation of the suggested effects of VES on the apoptosis in K562 cells was shown in Fig. 9. Significant number of clinically useful anticancer agents have been shown to induce apoptosis in tumor cells [18] by affecting the pathway. Since the successful development of the chemotherapeutic drugs/agents is largely dependent on their ability to trigger cell

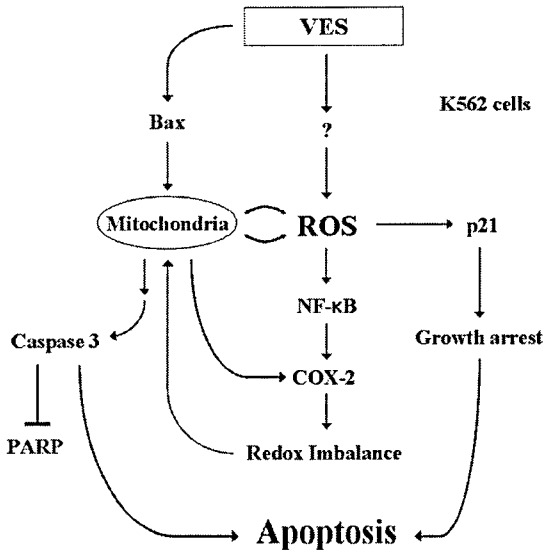


Fig. 9. Schematic representation of the suggested effects of VES on the apoptotic pathway. VES induces apoptosis mediated with NF-κB by generation of ROS in K562 cells.

death in tumor cells, our study on VES in K562 cells will provide a new therapeutic approach for finding an anti-cancer reagent for leukemia.

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초록 : 인간 만성백혈병 세포주에서의 Vitamin E Succinate에 의한 세포사멸 유도

장창덕 · 김종명[1] · 안원근[2] · 박혜련*
(부산대학교 치과대학, [1]부경대학교 수산과학대학, [2]부산대학교 나노과학대학)

비타민 E 유도체인 RRR-α-tocopheryl succinate (vitamin E succinate, VES)는 만성골수성 백혈병 세포인 K562 세포에서 apoptosis를 유도하였다. VES의 처리에 의해 apoptosis가 유도되는 과정에서 K562 세포 내의 ROS의 생성이 증가되었으며, ROS와 관련된 NF-κB, COX-2 그리고 $p21^{WAF1/CIP1}$ 등의 유전자가 활성화되었다. 뿐만 아니라, apoptosis의 과정 중 중요한 역할을 하는 Bax의 발현증가 및 손상된 DNA의 회복에 중심적 기능을 하는 PARP의 분열이 야기되었다. VES를 처리한 세포의 세포주기 분석에서는 apoptotic phase인 sub-G1 phase에서 세포사멸이 증가되고, 형태적으로는 염색질의 응축이 일어나는 결과로 미루어볼 때 VES는 K562 세포의 apoptosis를 유도한 것을 알 수 있다. C57BL/C의 림프종 이종이식을 통한 VES의 항암활성 실험 결과, C57BL/C의 대조군에 비하여 종양의 성장억제를 확인하였으며 높은 생존율을 확인하였다. 이러한 결과는 백혈병 치료에 대한 분자적 기초를 제공하였으며 동물실험을 통하여 보다 실질적인 백혈병 치료의 가능성을 보여주었다.