RRR-α-tocopheryl succinate induces apoptosis in human gastric cancer cells via the NF-κB signaling pathway

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Abstract. To investigate the effects of the nuclear factor (NF)-κB signaling pathway on the induction of apoptosis by vitamin E succinate (RRR-α-tocopheryl succinate; VES) in human gastric carcinoma cells. Human gastric carcinoma SGC-7901 cells were treated with temperate concentrations of VES and pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB. Cell viability and apoptosis were respectively estimated by methylthiazol tetrazolium (MTT) assay and the Annexin V-FITC method. Western blot analysis was used to evaluate the protein expressions of NF-κBp65 and Bcl-2 family members Bcl-2, Bax and cleavage of caspase-3, caspase-9, and poly (ADP-ribose) polymerase (PARP). The DNA-binding activity of NF-κBp65 was measured by electrophoretic mobility shift assay (EMSA). Reverse transcription and polymerase chain reaction (RT-PCR) was implemented to evaluate the transcription of inhibitor of apoptosis (IAP) genes. Apoptosis assessment showed that VES induces apoptotic cell death in human gastric carcinoma cells. In the following experiments, PDTC (100 µM) was used in cell treatment 2 h before VES. The decreased ratio of the nuclear and cytosolic NF-κBp65 protein level was induced by VES and PDTC reinforced this trend. PDTC treatment significantly enhanced the decrease of NF-κB DNA binding activity induced by VES in human gastric SGC-7901. The decrease in protein expression of Bcl-2 as well as the increase in the protein expression of Bax were induced by VES treatment. The cleavage of caspase-9, caspase-3 and PARP was induced. There was no effect on the gene transcription of c-IAP-1, c-IAP-2, and x-linked IAP (XIAP) compared with the control group, whereas mRNA levels of survivin and NAIP markedly decreased. Notably, pretreatment with PDTC reinforced all the above VES-induced effects. In conclusion, VES-induced apoptosis in SGC-7901 cells is accompanied by the inhibition of the NF-κB signaling pathway, including changes in Bcl-2 family members, cleavage of caspases and gene transcription of survivin and NAIP.

Introduction

Gastric carcinoma is one of the most common causes of cancer-related mortality worldwide; however, there is limited effective clinical treatment for this highly malignant tumor besides surgery and chemotherapy. Furthermore, the treatment outcome remains unsatisfactory as early diagnosis of gastric cancer remains difficult and most patients have already developed metastatic lesions when diagnosed (1). Therefore, it is necessary to search novel agents to treat stomach cancer patients with adverse effects.

An increasing number of publications indicate that vitamin E succinate (VES), a natural derivative of vitamin E, exhibits powerful anticancer effects in a variety of in vivo and in vitro cancer models (2-7). Our previous studies showed that VES induced apoptosis in SGC-7901 human gastric cancer cells via multiple signaling pathways, including extrinsic Fas, MAP kinase, and endoplasmic reticulum (ER) stress (8-10).

The relationship between the dysfunction of the NF-κB signaling pathway and the carcinogenesis of stomach cancer was previously reported by several research groups (11,12). NF-κB is a ubiquitous dimeric transcription factor that plays pivotal roles in regulating the expression of a multitude of critical genes that regulate cell survival, proliferation, apoptosis, immune responses, and adaptive responses to change the intracellular redox balance (13-16). The constitutive activation of NF-κB, a multi-function nuclear factor, has been suggested to be a hallmark of highly malignant tumors (17,18). Therefore, the inhibition of NF-κB activation has been reported to be a naturally useful strategy for increasing tissue sensitivity towards cytostatic drug treatment in vitro and in vivo (19).

Materials and methods

Materials and reagents. Human medium-differentiated gastric cancer SGC-7901 cells were obtained from the Cancer...
Research Institute of Beijing. VES, ethylenediaminetetraacetic acid, dimethyl sulfoxide (DMSO), MTT, Tween-20, sodium dodecyl sulfate (SDS) and PDTC were purchased from Sigma. RPMI-1640, fetal bovine serum (FBS), TRIzol reagent, an Annexin V-FITC apoptosis assay kit, and a total-RNA isolation kit were obtained from Gibco Chemical Co. (Gibco, Rockville, MD, USA). The GeneAmp RNA PCR kit was provided by Perkin-Elmer Life Sciences (Boston, MA, USA). The enhanced chemiluminescence (ECL) kit and the horse-radish peroxidase-conjugated antibody were obtained from Amersham Life Science Inc. Taq polymerase was purchased from Roche Molecular Biochemicals (Basel, Switzerland). The LightShift Chemiluminescence EMSA kit was obtained from Pierce (Rockford, IL, USA). Nuclear and cytoplasmic protein extraction kits and a BCA protein assay kit were obtained from Viagene Biotech (Ningbo, China). Rabbit polyclonal antibodies for Bcl-2, Bax, Bak, caspase-8, caspase-9, caspase-3, PARP, NF-κBp65, TATA binding protein (TBP) and β-actin were purchased from Santa Cruz Biotechnology, Inc. Gene primers of c-IAP1, c-IAP-2, NAIP, survivin, XIAP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for RT-PCR were constructed by Invitrogen.

**Cell culture.** SGC-7901 were cultured in RPMI-1640 medium supplemented with 5% FBS, 2 mM glutamine, 100 µg/ml streptomycin, 100 IU/ml penicillin, and 20 µM sodium bicarbonate at 37°C in a humidified atmosphere containing 5% CO₂. The culture medium was changed every 2 days.

**Cell viability.** Cells were plated in 96-well plates, allowed to attach overnight, and treated with various concentrations of VES (0-160 µM). Cell viability was estimated by the MTT assay. The medium was aspirated and DMSO was used to dissolve the crystals. Absorbance was measured at 570 nm using a microplate reader.

**Assessment of apoptosis.** Apoptosis was quantified using the Annexin V-FITC method. Cells were treated with VES (40 µM), and were plated at a seeding density of 10⁵ per well in 24-well plates and incubated overnight. Floating and attached cells were harvested and treated according to the manufacturer's instructions.

**Protein extraction and western blot analysis.** After treatment with or without VES or PDTC, the cells were collected and proteins were extracted. The protein concentration was determined by DC Bio-Rad assay according to the manufacturer's instructions. The NF-κBp65 combined assay was performed with a biotin-labeled oligonucleotide (5'-AGT TGA GGG GAC TTT CTG-3'). The complex was separated on 6.5% acrylamide gel in 0.5X TBE buffer at 4°C. Blots were transferred onto N+ nylon membranes according to the manufacturer's instructions.

**Evaluation of mRNA transcription by RT-PCR.** Total RNA was isolated from 10⁶ SGC-7901 cells following treatment according to the manufacturer's protocol. First-strand cDNA was synthesized using the GeneAmp RNA PCR kit. PCR analyses were performed in a final volume of 20 µl of buffer containing 1 µl of retro-transcription product, deoxyribonucleotide triphosphates (150 µM each), MgCl₂ (2 mM), 1 unit of Taq polymerase, and each primer at 1 µM. Following inactivation at 95°C for 1 min, PCR amplification was performed under the following reaction conditions: 94°C for 1 min, 50°C (c-IAP-2), 58°C (c-IAP-1, NAIP, XIAP, and GAPDH), 62°C (survivin) for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. We used 15 cycles of amplification for GAPDH and 30 cycles for the other mRNAs. All PCR products (10 µl) were analyzed by electrophoresis on 2% (w/v) agarose gel, photographed, and quantified by densitometric scanning. The sequence of primers used for RT-PCR is shown in Table I. The GAPDH gene was used as a loading control.

**Statistical analysis.** SAS statistical software was used for data analysis. The significance between the control and treated groups was assessed by Student's t-test, and P<0.01 was considered to indicate a statistically significant difference in the experiments.

**Results**

**Inhibition of growth.** The antitumor effects of VES on human gastric cancer SGC-7901 cells were evaluated by MTT assay.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
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<tr>
<td>c-IAP-1</td>
<td>S: 5'-GAAGACATCTCTCTCATCGAG-3'</td>
</tr>
<tr>
<td></td>
<td>A: 5'-CCACAGGTGTATTCATCAGAC-3'</td>
</tr>
<tr>
<td>c-IAP-2</td>
<td>S: 5'-TCCTAGCTGAGATTCTGTT-3'</td>
</tr>
<tr>
<td></td>
<td>A: 5'-GGTAACCTGGCTGGAATGG-3'</td>
</tr>
<tr>
<td>XIAP</td>
<td>S: 5'-GCACGAGCAGGTTTCTTATACTGTTG-3'</td>
</tr>
<tr>
<td></td>
<td>A: 5'-CTTCCTCAAAATACATGCGAGGGTTTCTC-3'</td>
</tr>
<tr>
<td>NAIP</td>
<td>S: 5'-CTGGGGOCTAGTGACTCAG-3'</td>
</tr>
<tr>
<td></td>
<td>A: 5'-ACGCCTCATAAGTCACAAAAGTC-3'</td>
</tr>
<tr>
<td>Survivin</td>
<td>S: 5'-TGCGCCAGCCCCTTTCTCA-3'</td>
</tr>
<tr>
<td></td>
<td>A: 5'-TGCGCCAGCCGCACTTTCTTC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>S: 5'-CATCTCCAGAGAGGAGAT-3'</td>
</tr>
<tr>
<td></td>
<td>A: 5'-GCTTCACACCCTTCTTG-3'</td>
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S, sense; A, antisense.
Various concentrations of VES (0-160 µM) were used to investigate cell viability after treatment. As depicted in Fig. 1A, a significant decrease in cell viability was observed after 24 h of treatment with 20 to 160 µM VES. Furthermore, when the concentration of VES was > 40 µM, the cells broke into pieces quickly after addition of the agent to the culture medium. Hence, in the present study, moderate concentrations 40 µM were chosen to study the antitumor effects of VES in SGC-7901 cells.

**Induction of apoptosis.** For apoptosis assessment, the exposure of phosphatidylserine on the cell surface was examined by Annexin V staining. As shown in Fig. 1B, flow cytometric analysis revealed that the percentage of Annexin V-positive cells significantly increased following treatment with VES (40 µM) for 12 h. Following longer treatment, apoptosis became much more apparent. After VES treatment for 48 h, Annexin V-positive cells accounted for nearly 50% of all cells counted. Thus, VES leads to apoptotic cell death in human gastric carcinoma cells.

**Figure 1.** VES inhibits growth and induces apoptosis in SGC-7901 cells. SGC-7901 human gastric cancer cells were treated with different concentrations of VES. (A) Cell viability was determined using MTT assay. (B) Flow cytometric analysis was performed using Annexin V-FITC. Each point represents the mean ± SD of three independent experiments. Significance was determined using the Student's t-test. *P<0.01 vs. the control group.

**Figure 2.** Nuclear translocation and DNA binding of NF-κBp65 in SGC-7901 cells. After treating SGC-7901 cells with VES (40 µM) or PDTC (100 µM) for 24 h, total, cytosolic and nuclear proteins were extracted from cells. (A) Proteins were subjected to western blot analysis. (B) Relative densitometry of protein expression was determined by normalizing the NF-κBp65 protein densitometry with β-actin or TBP. (C) Ratio of the nuclear and cytosolic NF-κBp65 protein level. The data in (B) and (C) are expressed as the mean ± SD from three individual experiments. β-actin was used as an internal loading control for the cytosolic and total preparations, while TBP was used for the nuclear preparation. (D) DNA binding of NF-κBp65 was further detected by EMSA. The results obtained are from one representative experiment of three replicates that show similar patterns. *P<0.01 compared with the control group.
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Figure 3. Expression of Bcl-2, Bax and cleavage of caspase-9, caspase-3 and PARP. (A) The samples from Fig. 2 were used to determine the protein expression of Bcl-2 and Bax, as well as caspase-9, caspase-3 and PARP by western blot analysis. (B) Relative densitometry of Bcl-2 and Bax protein expressions were determined by normalizing protein densitometry with β-actin protein densitometry. The ratio of Bcl-2/Bax is also shown in (B). Data in (A) represent three individual experiments. The data in (B) are expressed as the mean ± SD from three individual experiments. *P<0.01 vs. the control group.

in the apoptosis induced by VES in SGC-7091 cells, PDTC, a specific inhibitor of the activation of NF-κB, was used in the following experiments. Western blotting results showed that PDTC treatment did not influence the expression of total NF-κBp65 protein level (Fig. 2B). Notably, there was a distinct increase in cytosolic NF-κBp65 compared with that in the control group (Fig. 2B). However, there was an obvious decrease in the nuclear NF-κBp65 protein level. Furthermore, as shown in Fig. 2C, the decrease ratio of the nuclear and cytosolic NF-κBp65 protein level clearly indicates the possibility that VES induced the nuclear transformation of NF-κBp65 and PDTC enhanced this transformation.

To examine our hypothesis, NF-κB activity was measured by EMSA. The DNA binding of NF-κB in SGC-7901 cells presented inhibited a decreasing trend (Fig. 2D). As shown in Fig. 2D, PDTC treatment significantly enhanced the decrease of NF-κB-DNA binding activity induced by VES in human gastric SGC-7901.

Expression of Bcl-2 family proteins. The protein expression of Bcl-2 family members Bcl-2 and Bax were investigated. VES treatment decreased the protein expression of Bcl-2 and increased the protein expression of Bax. The decreased ratio of Bcl-2 and Bax is shown as in Fig. 3B. The trend became more apparent with PDTC treatment added.

Cleavage of caspase-9, caspase-3 and PARP. Cleavage of caspase-9, caspase-3 and subsequent proteolytic cleavage of PARP were assessed by western blot analysis. As illustrated in Fig. 3A, VES treatment led to cleavage of caspase-9, caspase-3 and PARP, all of which indicate induction of apoptosis. Notably, pretreatment with PDTC reinforced the VES-induced cleavage.

Transcription of IAP genes. VES or PDTC treatment had no effect on the gene transcription of c-IAP-1, c-IAP-2, and XIAP compared with the control group, whereas gene mRNA levels of survivin and NAIP markedly decreased after treatment with VES (Fig. 4). Furthermore, PDTC intensified the decrease of gene mRNA levels.

Discussion

The potential anticancer effect of VES in human gastric carcinoma cells has been indicated by our previous in vivo experiment. We found that VES induced apoptosis in SGC-7901 human gastric cancer cells via multiple signaling pathways, including extrinsic Fas, MAP kinase, endoplasmic reticulum (ER) stress, the couple of ER stress and unfolded protein response (UPR) (8-10, 20-22). In addition, VES enhanced DOXO anticancer efficiency via promotion of DOXO influx and suppression of MDR-1 mediated DOXO efflux (21). Recent studies showed that NF-κB was not only related to immune response and inflammatory reaction, but was also involved in regulating cell proliferation, apoptosis and migration (23-25). Involvement of the NF-κB signaling pathway in the genesis and progression of malignant gastric cancer led us to undertake this study to explore the mechanism of induction of apoptosis of VES as well as the relationship
between this mechanism and the NF-κB signaling pathway in human gastric carcinoma cells.

In this study, moderate concentrations of VES (10-40 μM) exhibited apparent growth inhibition effects and induced apoptosis in human gastric carcinoma SGC-7901 cells. The concentration that significantly decreased SGC-7901 cell viability and induced apoptosis was similar to that in other cancer cell cultures, such as leukemia and breast (26-28).

VES treatment caused reductions in nuclear and an increase in cytosolic NF-κBp65 protein levels accompanied by fixedness in total NF-κBp65 protein levels. These results are consistent with those of previous reports (29,30). Several mechanisms for NF-κB inhibition are available, such as inhibition of the inhibitor of NF-κB (IκB) degradation, phosphorylation of the antagonist of IκB by inhibition of IκB kinase α, and NF-κB nuclear translocation (31-34). According to our results, VES may regulate the NF-κB pathway by inhibiting the nuclear translocation of NF-κBp65 in SGC-7901 cells. With the involvement of PDTC, this hypothesis is confirmed.

Overexpression of NF-κB-responsive proteins, such as Bcl-2 family members and IAPs, resulting from the constitutive activation of NF-κB may critically contribute to the genesis and progression of cancer and represent an important cause of tumor drug resistance (35,36). As previously reported, high NF-κB expression is associated with high Bcl-2 expression in breast cancer and leukemic cells (37). Several Bcl-2 family members are known to be involved in the apoptosis-inducing effect of VES in tumor cells in vitro (38,39). We further verified that the intrinsic pathway of cell death is mediated by Bcl-2 family proteins and the balance between pro- and anti-apoptotic Bcl-2 family proteins. This balance may terminally determine mitochondrial disruption, cytochrome c release, and caspase activation until apoptosis. Only this ratio dictates whether or not a cell responds to a proximal apoptotic stimulus (40). Caspases are known to be important mediators of apoptosis and contribute to the overall apoptotic morphology by cleaving various cellular substrates (41,42). Activation of caspases is the terminal phase of programmed cell death, and this step is contracted by anti-apoptotic molecules of the Bcl-2 family (43). Our results confirm that VES-mediated changes in Bcl-2 family members trigger the release of mitochondrial cytochrome c, which, in turn, accounts for the cleavage of caspase-9, caspase-3 and PARP and ultimately contributes to cell death. Furthermore, this effect is enhanced by PDTC, an inhibitor of NF-κB.

IAP family proteins play a role in oncogenesis via their effective suppression of apoptosis (44). NF-κB is known to regulate the expression of anti-apoptotic genes such as IAP-1, IAP-2, XIAP, NAIP and survivin (45). Thus, in this study, whether or not VES treatment regulates the gene transcription of IAP family proteins was determined. Inhibition of constitutive NF-κBp65 activation could trigger decreases in survivin and NAIP mRNA levels without changing the levels of other inhibitors. Hence, only survivin and NAIP are involved in the apoptosis-inducing effects of VES, which are caused by the inhibition of constitutive NF-κBp65 activation in VES-treated human gastric carcinoma cells. In contrast to our previous studies carried out using other types of human cancer cells (46). This result indicated that survivin and NAIP may be involved in the VES-induced apoptosis in SGC-7901.

However, the inhibition of nuclear translocation of NF-κBp65 did not downregulate the expression of all anti-apoptotic genes. There may be other nuclear translocation factors which can regulate the translocation of IAP family genes.

In conclusion, our results demonstrate that VES-induced apoptosis in human gastric carcinoma SGC-7901 cells is accompanied by changes in Bcl-2 family members, cleavage of caspases, and inhibition of the NF-κB signaling pathway. Nuclear translocation of NF-κBp65 is markedly inhibited after treatment of SGC-7901 cells with VES. Additional studies are required to understand the biochemical mechanisms involved in the induction of apoptosis caused by treatment with VES in human gastric cancer cells. We believe that NF-κB may be a favorable candidate protein for developing new molecular-targeted therapies against human gastric cancer.

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References