Endoplasmic reticulum stress contributes to vitamin E succinate-induced apoptosis in human gastric cancer SGC-7901 cells

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**A B S T R A C T**

Vitamin E succinate (RRR-α-tocopheryl succinate, VES), an efficient inducer of apoptosis, acts as a potent agent for cancer therapy. However, the mechanism by which VES mediates the effects is not yet fully understood. Here we studied the effect of endoplasmic reticulum (ER) stress and unfolded protein response (UPR) on VES-induced apoptosis of SGC-7901 human gastric cancer cells. VES caused cytopathological changes typical of apoptosis, increased ER dilation and cytosolic Ca2+ concentration. And endogenous ER stress markers, GRP78 and GRP94 were transcriptionally and translationally altered. In response to VES, induction of CHOP, activation of caspase-4 and JNK were observed. Furthermore, VES also triggered activation of UPR components, including RNA-dependent protein kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6), X-box-binding protein 1 (XBP1), and ATF4 in a concentration- and time-dependent manner. Consequently, our results suggest that VES-induced apoptosis is coupled to ER stress and UPR activation in SGC-7901 human gastric cancer cells.

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**1. Introduction**

Gastric carcinoma is a leading cause of cancer death worldwide. To date, there is little effective clinical treatment for this highly malignant tumor. Therefore, numerous approaches have been conducted to search for efficient strategies for the development of cancer chemopreventive and chemotherapeutic agents. In recent years, apoptosis has been shown to play a significant role in elimination of cancer cells [1,2]. Thus, targeting apoptosis pathways in malignant cells may be an effective method of cancer prevention and therapy. Vitamin E succinate (RRR-α-tocopheryl succinate, VES), a derivative of natural vitamin E, has been proved a potent inducer of apoptosis that selectively inhibits the growth of various cancer cell types in vitro and in vivo [3–10].

Up to now, several apoptotic pathways have been identified in cells responsive to apoptotic insult, such as the apoptosis mediated by the activation of death receptors ('extrinsic'), mitochondria-dependent signaling ('intrinsic'), and endoplasmic reticulum (ER)-induced apoptotic cell death [11–13]. Recently, numerous studies have identified the ER as a third subcellular compartment implicated in apoptotic execution [12]. The endoplasmic reticulum (ER) is an organelle responsible for the synthesis, initial post-translational modification, folding, export, and secretion of membrane proteins [14]. Several cellular stress conditions, such as nutrient deprivation, alterations in glycosylation status, hypoxia, changes in calcium homeostasis, and treatment with a variety of agents, can lead to accumulation and aggregation of unfolded and/or misfolded proteins in the endoplasmic reticulum (ER) lumen and cause so-called ER stress [15,16]. But the ER has evolved highly specific signaling pathways to ensure that its protein-folding capacity is not overwhelmed, which are collectively termed the unfolded protein response...
The activation of UPR involves the concerted action of three prototypical ER localized stress sensors: RNA-dependent protein kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol requiring enzyme 1 (IRE1). The UPR alleviates ER stress by an initial decrease in general protein synthesis, promotion of protein folding via induction of ER chaperones (e.g., GRP78) and prevention of accumulating misfolded proteins. However, if the stress is severe or prolonged, distinct death signals may be transduced during the UPR, and cells undergo apoptosis [18–20]. Various mechanisms have been suggested to play a role in ER stress-induced apoptosis. For example, the C/EBP homologous protein transcription factor (CHOP), c-Jun NH2-terminal kinase (JNK), and caspases have been implicated in mediating apoptotic signals in response to ER stress [20–22].

Our previous studies showed that VES inhibited proliferation and triggered apoptosis in human gastric cancer cells by several pathways, including the extrinsic Fas-dependent pathway and the intrinsic mitochondria-mediated apoptotic pathway, also involving the MAP kinase pathway [23–26]. In this study, we explored that ER stress played a significant role in VES-induced apoptosis of human gastric cancer cells. Then we further investigated roles of UPR signals in the regulation of apoptosis in VES-exposed cells. To our knowledge, this is the first report that ER stress-mediated apoptosis pathways are investigated in VES-induced apoptosis in carcinoma cells.

2. Materials and methods

2.1. Cell culture and treatment

SGC-7901 human gastric cancer cells were cultured in the RPMI-1640 medium containing 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM l-glutamine in a humidified atmosphere with 5% CO2 at 37°C. For experiments, the level of FBS was reduced to 2%. Exponentially growing cells were incubated for different time periods in the presence of VES (Sigma) at 5, 10 or 20 µg/ml in 0.1% ethanol. Equal amount of ethanol was used as a solvent control. Tunicamycin (Sigma), as a positive control, was dissolved in DMSO to make 5 mg/ml stock solution.

2.2. Detection of apoptosis

Apoptosis was assessed as described previously, based on changes in the nuclear morphology by staining the cells with the fluorescent DNA dye 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI). Briefly, cells were treated with VES at 5, 10 and 20 µg/ml for 24 h. And then the cells were pelleted, washed with PBS and then incubated with 2 µg/ml DAPI in methanol for 30 min at 37°C. Apoptosis was observed using an Olympus X70 fluorescence microscope with ultraviolet (UV) excitation at 300–500 nm. Cells with nuclei that contained clearly condensed chromatin or cells with fragmented nuclei were scored as apoptotic.

2.3. Measurement of intracellular Ca2+ levels

Free cytosolic calcium [Ca2+]c was measured using cell permeant Ca2+ sensitive fluorescent dye Fluo-3AM. After cells were exposed to VES with various concentrations for 24 h, the medium was removed and replaced with 5 µM Fluo-3AM diluted in the loading solution at 37°C for 45 min. Cells were then washed twice with PBS and visualized using a confocal microscope (Nikon EZ-C1). Digital image analysis from cellular fluorescence was done with Lasersharp Software (Bio-Rad) and Confocal Assistance (Free Software by Todd-Clark-Brelje).

2.4. Transmission electron microscopy

Transmission electron microscopy was performed on SGC-7901 cancer cells treated with 5 µg/ml VES for 12 h. Transmission electron microscopy of cells was done as described previously [27]. Briefly, samples were fixed with 3% glutaraldehyde dissolved in 0.1 mol/l sodium cacodylate, post-fixed in osmium tetroxide, dehydrated with graded acetone series and finally embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate, examined in a JEM 1220 transmission electron microscope operated at 80 kV.

2.5. Preparation of whole cell lysate

SGC-7901 human gastric cancer cells were washed with PBS and lysed in the lysis buffer (150 mM NaCl, 0.1% NP-40, 0.5% sodium deoxy-cholate, 0.1% SDS, 50 mM Tris, 1 mM DTT, 5 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml trypsin, 10 µg/ml aprotinin, 5 µg/ml leupeptin; pH 7.4). Lysates were centrifuged at 15,000g for 8 min at 4°C. The supernatant was collected as whole cell lysate and its protein concentration determined using the Bio-Rad DC assay.

2.6. Immunoblotting

Equivalent amounts of protein were separated on 10% SDS–PAGE and transferred to a nitrocellulose membrane. Immunoblotting was performed using JNK, p-JNK, PERK, p-PERK, β-actin antibodies (Santa Cruz), caspase 4, ATF6 antibodies (Abcam), GRP78, GRP94, CHOP antibodies (Cell Signaling). The membrane was then incubated with the secondary alkaline phosphatase-conjugated IgG and detected with the Western Blue Stabilized Substrate for alkaline phosphatase (Promega). The relative density of the individual bands was analyzed densitometrically using the Chemilumager 4000 instrument (Alpha Innotech).

2.7. Reverse transcriptase-polymerase chain reaction—RT-PCR

Total RNA was extracted from SGC-7901 human gastric cancer cells using the TRIzol reagent (Invitrogen, Carlsbad, CA). Following the manufacturer’s protocol (RNA PCR kit, TaKaRa Shuzo Co., Ltd., Japan), reverse transcription-PCR (RT-PCR) was done. RT was performed using a thermal program of 25°C for 10 min, 42°C for 30 min, and 95°C
for 5 min. Samples were subjected to 36 cycles of PCR amplification. In each cycle denaturing was at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 10 min.

Sequences of the primers are as follows: β-actin (forward: 5'-GTGGGCGGCTCTTA GCACTGAA-3'; reverse: 5'-CTTTGATGTCACGCATTTTCA-3'); GRP78 (forward: 5'-GATAATCACAACCTGTTAC-3'; reverse: 5'-GTATCTCTTTCAC CATTGGG-3'); GRP94 (forward: 5'-CAGCTGTAGATTCTTCTTGC-3'); CHOP (forward: 5'-GCACCTCCAGAG CACACCTA-3'; reverse: 5'-GTCTACTCCAACCTCTCCCTGCCG-3'); ATF4 (forward: 5'-TGACCGAGGGTCTTGTTGC-3'; reverse: 5'-TCATCTGGCATG GTTCATGC-3'). Amplified PCR products were visualized on a 1.5% agarose gel and visualized with ethidium bromide.

Amplification yielded the predicted size of the amplified fragment (β-actin: 540 bp; GRP78: 577 bp; GRP94: 274 bp; CHOP: 422 bp; ATF4: 313 bp).

To analyze the splicing of XBP1 mRNA, RT-PCR was performed with forward (5'-CCCTGTAAGAACCAGG-3') and reverse (5'-GGGGCTTCG ATATATGTGG-3'). 416 and 442 bp fragments, which represent spliced (XBP1S) and unspliced (XBP1U) XBP1 respectively, were visualized after separation on a 2% agarose gel.

### 2.8. Statistical analysis

All experiments were performed at least three times. Data are presented as mean values ± SD. Statistical differences were evaluated by one-way ANOVA, with p < 0.05 considered significant.

### 3. Results

#### 3.1. VES-induced apoptosis in SGC-7901 cells

SGC-7901 cells were treated with 20 μg/ml VES and apoptosis was investigated by DAPI staining using fluorescence microscopy. Compared with control, a large number of cells displayed morphological changes with typical characteristics of apoptotic cell death, including cell shrinkage, chromatin condensation, chromatin crescent formation/margination, DNA fragmentation and apoptotic body formation. 20 μg/ml VES induced 32% of SGC-7901 cells to undergo apoptosis for 24 h. (Fig. 1A and B).

#### 3.2. Induction of ER stress by VES

To assess the effect of VES on ER stress, we evaluated its effect on ER morphology by transmission electron microscopy (Fig. 2A). The results showed that VES increased ER dilation at 5 μg/ml for 12 h. To obtain functional evidence for ER stress, we investigated the effect of VES on cytosolic...
Ca2+ levels, which are also common consequences of ER stress [28]. The results confirmed that exposure to VES led to an increase in the cytosolic Ca2+ concentration in a dose-dependent manner (Fig. 2B and C).

Next, we examined the expression of the ER molecular chaperones GRP78/Bip and GRP94 by RT-PCR and immunoblotting analyses. In these experiments, the glycosylation inhibitor tunicamycin, known to induce ER stress and UPR, were conventionally served as positive control [29]. We found that exposure of SGC-7901 cells to VES resulted in the induction of GRP78 at the mRNA and protein levels in a time-dependent manner (Fig. 3A and C). However, the mRNA and protein levels of GRP78 increased in 20 μg/ml VES-treated cells, while a decrease was observed in cells treated with VES at 5 and 10 μg/ml for 24 h (Fig. 3B and D). Meanwhile, for another ER chaperone-GRP94, its alteration was consistent with that of GRP78 at mRNA level (Fig. 3A and B), but down-regulation of GRP94 at protein level occurred in response to treatment with 20 μg/ml VES (Fig. 3C). And the GRP94 protein expression was also decreased by VES at 5 and 10 μg/ml when compared to the control (Fig. 3D).

Fig. 2. VES increased ER dilation and cytosolic Ca2+ concentrations in SGC-7901 cells. (A) SGC-7901 cells were treated without or with 5 μg/ml for 12 h. After a series of washing, fixation, dehydration and staining, cells were visualized by electron microscopy. Arrows, individual ER. The data are representative of at least three independent experiments. (B) Confocal microscopy images of SGC-7901 cells treated with graded concentrations (a, control; b, 5 μg/ml; c, 10 μg/ml; d, 20 μg/ml) of VES were loaded with Fluo-3 AM (green fluorescence), and were excited at 488 nm. Green fluorescence shows increased [Ca2+]. (C) Free [Ca2+], was analyzed with Lasersharp Software and Confocal Assistance. Data are expressed as mean of triplicate experiments ± SD (error bars). * p ≤ 0.05 compared with control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Role of ER stress in VES-induced apoptosis of SGC-7901 cells

To examine whether VES induced-apoptosis is mediated by ER stress, the levels of essential ER-associated apoptotic molecules were explored using immunoblotting. Briefly, VES significantly induced cleavage of caspase-4, increased the expression of CHOP and phosphorylated JNK in a dose- and time-dependent manner (Fig. 4A and B). Moreover, the RT-PCR result of CHOP was not completely the same as immunoblotting. Our data showed that CHOP mRNA was increased to the maximum in cells exposed to 20 μg/ml VES for 24 h, unlike the protein level at 10 μg/ml (Fig. 4C and D).

3.4. Activation of UPR signaling by VES in ER stress-induced apoptosis

To further investigate the effects of VES on the UPR, we examined three main UPR signaling systems, including PERK, ATF6, and IRE1-XBP1 pathways. Immunoblotting analysis showed that VES induced the phosphorylation of PERK and generation of p50ATF6 in SGC-7901 cells in a dose- and time-dependent manner (Fig. 5A and B). Activated IRE1 catalyzes removal of a small intron from XBP1 mRNA, leading to production of the active transcription factor [30,31]. So the alternatively spliced forms of XBP1 mRNA were detected by RT-PCR. As shown in Fig. 5C and D, like the positive control, VES treatment at 20 μg/ml induced the spliced form of XBP1 in time-dependent manner. Meanwhile, RT-PCR analysis demonstrated that ATF4 mRNA was also up-regulated in cells exposed to VES in a dose- and time-dependent manner, which increased the transcription of CHOP in response to ER stress (Fig. 5C and D).

4. Discussion

Apoptosis is an important mechanism in both development and homeostasis in adult tissues for the removal of either superfluous, infected, transformed or damaged cells by activation of an intrinsic suicide program, such as tumor regression. Thus, initiation of apoptotic signal pathway and
induction of apoptosis is considered as an effective approach for tumor treatment [32]. It has become clear that each of the major cellular organelles can be involved in apoptotic signaling pathways, and recent advances have highlighted the role of the endoplasmic reticulum (ER) in cell death processes. In the present study we investigated the contribution of ER stress and UPR to VES-induced apoptosis of SGC-7901 human gastric cancer cells. Our results demonstrated that: (1) VES induced the expression of endogenous ER stress markers-GRP78 and GRP94 at mRNA and protein levels, increased ER dilation and cytosolic Ca²⁺ concentrations, suggesting that ER stress was triggered by VES in stomach cancer cells. (2) After exposure to VES, CHOP, caspase4 and JNK were found to be induced and activated in a dose- and time-dependent manner respectively, which were the main ER-associated proapoptotic factors. (3) PERK, ATF6, and IRE1-XBP1 pathways were activated, thus ER stress and UPR contributed to the apoptosis induced by VES.

VES has emerged as a novel target for cancer treatment, which selectively induces apoptosis of malignant cells at levels at which it exerts no toxicity to normal cells or tissues [4,8,33]. It also inhibits a wide range of tumors in experimental animals [3,6,34]. In the present report, VES at 20 μg/ml for 24 h induced 32% of SGC-7901 cells to undergo apoptosis. This result confirmed the pro-apoptotic effect of VES on gastric carcinoma, which is consistent with that of our previous studies [23–26].

The ER fulfills multiple cellular functions [35,36]. Because of its role in protein folding and transport, the ER is rich in Ca²⁺-dependent molecular chaperones, such as GRP78 and GRP94, which are generally considered as markers of ER stress [37,38]. GRP78 and GRP94 are known to act as molecular chaperones that regulate protein folding and translocation into the ER and protein secretion. It is generally accepted that GRP78 and GRP94 are increased by physiologic stress that perturbs ER function and homeostasis, protecting against tissue or organ damage, and could protect cells against death [39,40]. But reduction of GRP94 in response to honokilo exposure was observed before for various human gastric cancer cells [41]. Our study revealed that GRP78 and GRP94 are increased by physiologic stress that perturbs ER function and homeostasis, protecting against tissue or organ damage, and could protect cells against death [39,40]. But reduction of GRP94 in response to honokilo exposure was observed before for various human gastric cancer cells [41]. Our study revealed that GRP78 and GRP94 are increased by physiologic stress that perturbs ER function and homeostasis, protecting against tissue or organ damage, and could protect cells against death [39,40]. But reduction of GRP94 in response to honokilo exposure was observed before for various human gastric cancer cells [41].
of GRPs at first, and increased their levels soon. Furthermore, perturbation of ER Ca\(^{2+}\) homeostasis is one of the early events leading to disruption of ER functions. And it is known that increased cytosolic Ca\(^{2+}\) seems to be a result of ER-Ca\(^{2+}\) depletion by a process known as capacitative Ca\(^{2+}\) entry [42]. In our study, an increase in cytosolic Ca\(^{2+}\) level and ER dilation were observed in cells treated with VES. All these data provide strong evidence that ER stress is possibly induced by VES in SGC-7901 human gastric cancer cells.

On ER stress, the signaling pathways involved in the UPR are relatively well characterized, and sensed by three ER-resident transmembrane proteins [43]. Upon removal of the ER-resident chaperones, the PERK kinase phosphorylates eIF2\(\alpha\), selectively enhancing the translation of ATF4 transcription factor [36]. Activation of IRE1 by dimerization and phosphorylation causes IRE1-mediated splicing of XBP1 mRNA, leading to production of the active transcription factor [43]. Liberated ATF6 translocates from the ER to the Golgi where it is proteolytically processed to release a 50-kDa transcription factor that promotes transcription of ER-resident chaperones and folding enzymes [36]. In this report, we showed that phosphorylation of PERK, mRNA level of ATF4, and activation of ATF6 and XBP1 were all obtained as a result of exposure to VES in a dose- and time-dependent manner. Our preliminary data indicate a possibility that three UPR pathways may be activated by VES in SGC-7901 cells.

The UPR is fundamentally a cytoprotective response. However, if the adaptation does not prove to be sufficient, the apoptotic response is initiated. And the mechanism of ER stress-induced apoptosis has already been extensively studied, which is also regulated by UPR [43]. Among the ER-associated apoptotic molecules, CHOP/GADD153 and caspase-12 are apparently key pro-apoptotic factors that are closely associated with ER stress [44,45]. CHOP, known as growth-arrest and DNA-damage-inducible gene 153 (GADD153), has been shown to promote apoptosis by downregulating the expression of anti-apoptotic Bcl-2 in several contexts [45–47]. CHOP–/– MEFs are partially resistant to ER stress and have reduced ER stress-induced apoptosis [46,48]. Caspase-12, a murine protein associated with the ER membrane, is specifically activated by ER stress but not by other non-ER signals, which is further associated with activation of caspase-9 and caspase-3 [22,49]. However, it is worth noting that caspase-12 is expressed only in rodents, and its human homologue is silenced by several mutations during evolution. But it was recently shown that caspase-4 performs the function of caspase-12 in humans [44]. Another apoptotic pathway in ER stress involves the activation of the JNK pathway, which may contribute to apoptosis in response to other
stimuli [50,51]. In this study, RT-PCR and immunoblotting results showed that VES enhanced CHOP induction, caspase-4 cleavage, and JNK activation in a dose- and time-dependent manner. Furthermore, in our previous studies, we demonstrated that VES treatment resulted in the activation of caspase-9 and caspase-3, induction of pc-jun, and downregulation of Bcl-2 in SGC-7901 cells, which are downstream events of the ER-associated apoptotic molecules [25,52]. Take together, our data have confirmed the hypothesis that VES induces apoptosis in SGC-7901 cells through ER stress and leads to the activation of CHOP, caspase-4, and JNK.

In summary, the current report evidenced for the first time that VES-induced apoptosis of human gastric cancer cells is mediated, at least in part, by ER stress and UPR is implicated in the apoptotic process. Currently, one question addressed in this study is that how individual UPR plays a role in the regulation of apoptosis in VES-exposed cells. In fact, the mechanisms underlying VES-induced apoptosis are complex and remain elusive.

Conflicts of interest

None declared.

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