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Bid integrates intrinsic and extrinsic signaling in apoptosis induced by α -tocopheryl succinate in human gastric carcinoma cells

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ABSTRACT

The underlying mechanisms of α -tocopheryl succinate (α -TOS)-mediated apoptosis are not understood in detail, although the redox-silent vitamin E analog is a potent apoptogen and anti-cancer agent. Our previous studies showed the important role of Fas signaling in apoptosis induced by the mitocan. The objective of the present study was to investigate whether apoptosis triggered by α -TOS in gastric carcinomas cells involves both mitochondria- and death receptor-dependent pathways. α -TOS induced apoptosis and mitochondrial permeability transition in a concentration- and time-dependent manner. As a consequence, cytochrome c and the apoptosis-inducing factor were released and caspases were activated. Bax was translocated from the cytosol to mitochondria and Bid was cleaved into its truncated form, tBid. Knocking down Bid by RNAi and Fas antisense oligodeoxynucleotides resulted in a decreased release and cleavage. The results imply that Bid may serve as a critical integrating factor of the death receptor and mitochondrial pathway in α -TOS-mediated apoptosis.

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1. Introduction

Gastric cancer ranks as the second most common cause of death from cancer worldwide [1,2]. Current therapies are limited due to considerable side effects. It is therefore necessary to search for novel agents to treat stomach cancer patients with less adverse effects. Great interest has recently focused on the potential use of vitamin E (VE) analogs as anti-cancer drugs and adjuvants, in particular due to the unique selectivity of α -tocopheryl succinate (α -TOS), a redox-silent analog of VE that kills tumor cells without harming normal tissues and cells [3,4]. α -TOS

has been proved an efficient and selective inducer of apoptosis in a variety of tumor cells [5]. VE analogs represent a novel group of selective anti-cancer agents, mitocans, acting by apoptosis induction by way of mitochondrial destabilization [5]. Recent advancement in understanding the apoptotic signaling pathways has provided the basis for novel targeted therapies that can induce cancer cells death and/or sensitize them to established cytotoxic agents and radiation therapy [6].

Although the precise details are not known, agents that target mitochondria use two apoptotic pathways. The extrinsic pathway is initiated by death receptors (e.g. Fas, TNFR1) with sequential activation of the initiator caspase-8 and of the effector caspase-3. In most cases, the intrinsic mitochondria-dependent pathway is required for the amplification of the caspase cascade and the demise of the cell [7–9]. Mitochondria are important organelles in mammalian cells not only due to their essential role in

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energy metabolism and homeostasis, but also due to their critical involvement in programmed cell death [10–12]. In the initiation phase of apoptosis transmitted via mitochondria, the opening of the permeability transition pore in the mitochondrial membrane elicits the mitochondrial permeability transition (MPT) in response to apoptotic stimuli [13,14]. MPT causes dissipation of the mitochondrial inner trans-membrane potential, with the consequent outer membrane 'rupture' and the release of pro-apoptotic proteins, including cytochrome *c*, Apaf-1, Smac/Diablo or the apoptosis-inducing factor (AIF), promoting cell death via both caspase-dependent and -independent mechanisms in the execution phase [15,16]. Mitochondria-dependent apoptosis is regulated positively and negatively by the Bcl-2 family members, which include three subfamilies based on the structural and functional characteristics [17–19]. The anti-apoptotic subfamily comprises proteins such as Bcl-2, Bcl-x_L and Mcl-1, with four Bcl-2 homology (BH) domains. The pro-apoptotic subfamily with the Bax, Bak and Bok proteins, capable of forming a pore in the mitochondrial outer membrane, contains BHL-3 domains. And finally the BH3 only group, includes proteins like Bid that modulate the activity of the other Bcl-2 family proteins. Bid is known to interact with both Bcl-2 and Bax through its BH3 domain and trigger cytochrome *c* release from mitochondria upon activation [20].

Our previous studies showed that α -TOS inhibited proliferation and induced apoptosis in human gastric carcinoma cells via the extrinsic Fas-dependent pathway, in which Fas activates caspase-8, following recruitment of the Fas-associated death domain (FADD), also involving the MAP kinase pathway [21–23]. In the present study, the role of mitochondria and the relationship between endogenous and exogenous signaling pathways in α -TOS-mediated apoptosis were explored. The results show that α -TOS induced apoptosis via the mitochondrial pathway involving MPT, translocation of Bax, cytochrome *c* and AIF, and the cleavage of Bid and caspase activation. We also show that Bid is crucial for the extrinsic 'component' of the process, which points to the protein as a point of convergence of the intrinsic and extrinsic pathways.

2. Materials and methods

2.1. Cell culture and treatment

SGC-7901 human gastric cancer cells were maintained in the RPMI-1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 μ g/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 treated with α -TOS (Sigma) at 5, 10 or 20 μ g/ml (equivalent to 9.4, 18.8 or 37.7 μ mol/L, respectively) in 0.1% ethanol for the indicated time periods. Equal amount of ethanol was used as a solvent control.

2.2. Detection of apoptosis

Apoptosis was assessed on the bases of changes in the nuclear morphology by staining the cells with the fluores-

cent DNA dye Hoechst 33342 (Sigma). Briefly, cells were exposed to the compounds for 24 h and then incubated with 1 μ g/ml Hoechst 33342 for 30 min at 37 °C. Apoptosis was observed using an Olympus fluorescence microscope. Cells with nuclei that contained condensed chromatin or fragmented nuclei were scored as apoptotic.

2.3. Measurement of MPT

Mitochondrial function and integrity are dependent on maintenance of the mitochondrial membrane potential. MPT was examined by staining cells with the cell-permeable mitochondria-sensitive fluorochrome MitoTracker Red CMXRos (Molecular Probes) that is concentrated and retained in energized mitochondria. Cells were incubated with MitoTracker Red CMXRos at the final concentration of 50 pg/ml for 30 min, washed with PBS and visualized using a confocal microscope (Nikon EZ-C1).

2.4. Determination of AIF translocation

Cells were washed twice with PBS and fixed with 4% formaldehyde for 30 min at room temperature and permeabilized with Triton X-100 for 5 min. Following blocking with 1% bovine serum albumin in PBS for 1 h, cells were incubated with AIF antibody (Cell Signaling) overnight at 4 °C. Cells were immunostained with FITC-conjugated secondary antibody for 1 h at room temperature in dark, counterstained with PI and then visualized under a multi-channel confocal microscope (Nikon EZ-C1).

2.5. Preparation of cell lysate and subcellular fractionation

Cells were washed with PBS and incubated in the lysis buffer (150 mM NaCl, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, 1 mM DTT, 5 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml trypsin inhibitor, 10 μ g/ml aprotinin, 5 μ g/ml leupeptin; pH 7.4). The lysate was centrifuged at 12,000 g for 30 min at 4 °C and the supernatant was collected as whole cell lysate. The cytosolic and mitochondrial fractions were prepared using the Mitochondria/Cytosol Fractionation Kit (BioVision). In brief, cells were centrifuged at 600 g for 5 min at 4 °C, re-suspended in the extraction buffer containing DTT and protease inhibitors, incubated on ice for 10 min and then homogenized with an ice-cold Dounce tissue grinder. Unbroken cells and nuclei were pelleted by centrifugation at 700 g for 10 min at 4 °C. The supernatant was centrifuged at 10,000 g for 30 min to yield a cytosol-enriched fraction. The resulting pellet was re-suspended in the mitochondrial extraction buffer and represented the mitochondrial fraction. The nucleic fractions were prepared using Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Biotech). Cells were mixed with the cytoplasmic extraction buffer on ice for 15 min. Suspension was shaken vigorously and centrifuged at 12,000 g for 5 min at 4 °C. The supernatant was discarded and the pellet was re-suspended in nuclear extraction buffer on ice for 30 min. The resulting supernatant was used as nucleic fractions following centrifuge at 12,000 g for 10 min. All subcellular fractions were stored at –80 °C.

2.6. Immunoblotting

Cell lysate as well as the cytosolic and mitochondrial fractions prepared as described above were used for western blotting. Equivalent amounts of protein were separated by SDS–PAGE and transferred onto a nitrocellulose membrane. Immunoblotting was performed using Bax, Bcl-2, caspase-3 and β -actin antibodies (Santa Cruz), anti-cytochrome *c* and Bid antibodies (BD Pharmingen), AIF, caspase-9 and PARP antibodies (Cell Signaling), and the anti-cytochrome *c* oxidase, subunit IV antibody (Cox IV; Molecular Probes). 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 Chemilmager 4000 instrument (Alpha Innotech).

2.7. Transient transfections

The Bid protein was downregulated using small interfering RNA (siRNA) with the target sequence 5'-UGC GGU UGC CAU CAG UCU GCA GCU C-3'; as a negative control, a non-silencing (NS) RNA was used (both Stealth siRNAs from Invitrogen). Fas suppression was accomplished by

transfection with sense or antisense oligonucleotides (AS ODNs) synthesised as described previously [21]. Cells were transfected with siRNA or ODNs using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instruction. Protein expression was then evaluated by western blotting.

2.8. Statistical analysis

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

3. Results

3.1. α -TOS Induced apoptosis and MPT

SGC-7901 cells were treated with α -TOS at 5, 10 and 20 μ g/ml for 24 h and apoptosis was evaluated following Hoechst 33342 staining using fluorescence microscopy. Compared with the control cells, \sim 30% of α -TOS-treated cells exhibited morphological changes with typical characteristics of apoptotic cell death, including cell shrinkage, chromatin condensation, DNA fragmentation and apoptotic body formation, when exposed to 20 μ g/ml of α -TOS. α -TOS-induced shrinkage of SGC-7901 cells was also observed using a light microscope (Fig. 1A and B).

α -TOS caused a dose- and time-dependent opening of the permeability transition pore, as suggested by the loss of the MitoTracker Red CMX-Ros fluorescence assessed by confocal microscopy. The intensity of the

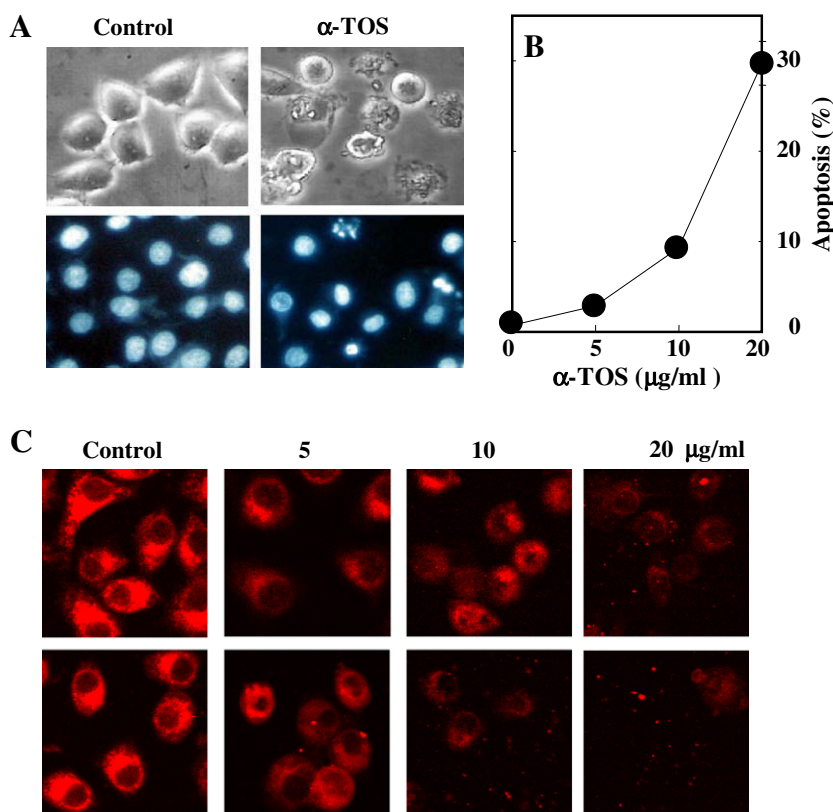


Fig. 1. α -TOS induces apoptosis and MPT in human stomach cancer cells. (A) SGC-7901 cells were treated with 20 μ g/ml α -TOS for 24 h. The cells were photographed under a light microscope (upper panel) or the cells were stained with Hoechst 33342 and visualized under a fluorescence microscope (lower panel). (B) The level of apoptosis was estimated by scoring apoptotic cells as indicated in panel A. Data shown are mean values \pm SD. (C) Cells were exposed to α -TOS at 5, 10 and 20 μ g/ml for 12 h (upper panel) and 24 h (lower panel). MPT was examined by staining the cells with the fluorochrome MitoTracker Red CMXRos and retention of the dye was observed using a confocal microscope.

dye was clearly reduced by α -TOS at 10 and 20 μ g/ml when compared to the control. When the cells were exposed to 20 μ g/ml α -TOS for 24 h, the MitoTracker Red CMXRos fluorescence positively related to the membrane potential was significantly lower than at 12 h, suggesting a progressive effect (Fig. 1C).

3.2. Translocation of proteins between mitochondria and cytosol initiated by α -TOS in association with MPT

As a consequence of MPT, small pro-apoptotic proteins are released from mitochondria into the cytosol. α -TOS had no significant effect on the level of the cytochrome *c* protein expression in the whole cell lysate (Fig. 2A). However, at 20 μ g/ml, the VE analog caused a decrease in the levels of the mitochondrial pools of cytochrome *c* and AIF, while their levels in the cytosol increased (Fig. 2B). β -Actin and Cox IV were used as loading controls for the cytosolic and mitochondrial fractions, respectively. α -TOS then initiated translocation of AIF into nucleus with the increase in protein level (Fig. 2B) and the colocalization of immunofluorescence (Fig. 2C). 20 μ g/ml α -TOS caused the merging immunofluorescence of green and red, while green and red fluorescence was separate in the cytoplasm and nucleus in the case of control.

The role of the Bcl-2 family proteins in α -TOS-induced apoptosis was assessed by western blotting. α -TOS upregulated the level of the pro-apoptotic Bax, while it did not change the level of the anti-apoptotic Bcl-2 protein in the whole cell lysate of the SGC-7901 cells (Fig. 3A). Optical densitometric analysis revealed 2- and 3.3-fold increase in the ratio of Bax to Bcl-2 protein expression at 10 and 20 μ g/ml α -TOS, respectively (Fig. 3A). Bax protein level in the cytosol was reduced by 47% and the level in the mitochondria was elevated by twofold, implying that Bax efficiently relocated from the cytosolic to the mitochondrial compartment (Fig. 3B). α -TOS did not significantly alter the level of the full-length Bid protein, an important BH3-only pro-apoptotic factor. The VE analog did, however, cause cleavage of Bid to its truncated, pro-apoptotic form (tBid) in a dose- and time-dependent manner (Fig. 3C).

We next explored the apoptogenic events induced by α -TOS downstream of mitochondria. Activation of caspase-9, caspase-3 and cleavage of the PARP protein in SGC-7901 cells treated with α -TOS for 24 h were detected by immunoblotting. α -TOS caused cleavage of pro-caspase-9 after 6 h (Fig. 4A), while pro-caspase-3 was cleaved into p21 and p17 fragments and the full length 116 kDa PARP into a smaller protein after 24 h (Fig. 4B).

3.3. Link between α -TOS-mediated death receptor and mitochondrial pathways

The potential role of Bid in connecting the extrinsic and intrinsic pathways was investigated next. When the cells were transfected with Bid siRNA, the protein was downregulated, which was not observed in the negative control using NS siRNA, indicating high efficacy of this approach (Fig. 5A). We then found that downregulation of the Bid protein counteracted the effect of α -TOS on the mitochondrial membrane potential; on the other hand, the NS siRNA negative control did not alter the effects of α -TOS on the mitochondrial potential (Fig. 5B). Bid downregulation also reversed α -TOS-induced release of cytochrome *c* into the cytosol and the cleavage of PARP (Fig. 5C).

Finally, we suppressed the expression of the Fas protein using Fas-specific AS ODNs, which was verified by western blotting (Fig. 5D). We found that the gastric carcinoma cells with downregulated Fas failed to efficiently cleave Bid to tBid as well as to activate caspase-9 (Fig. 5D).

4. Discussion

Apoptosis or programmed cell death, is essential for maintenance of development and homeostasis of multicellular organisms by eliminating superfluous or unwanted cells [24]. Inefficient apoptosis is considered one of the hallmarks of tumorigenicity [25–27]. Moreover, induction of apoptosis is an important target for cancer therapy [28]. In the present report we investigated the intrinsic pathway of apoptosis induced by α -TOS and its interaction with the extrinsic pathway in human gastric carcinoma cell line SGC-7901. The results demonstrate that: (i) α -

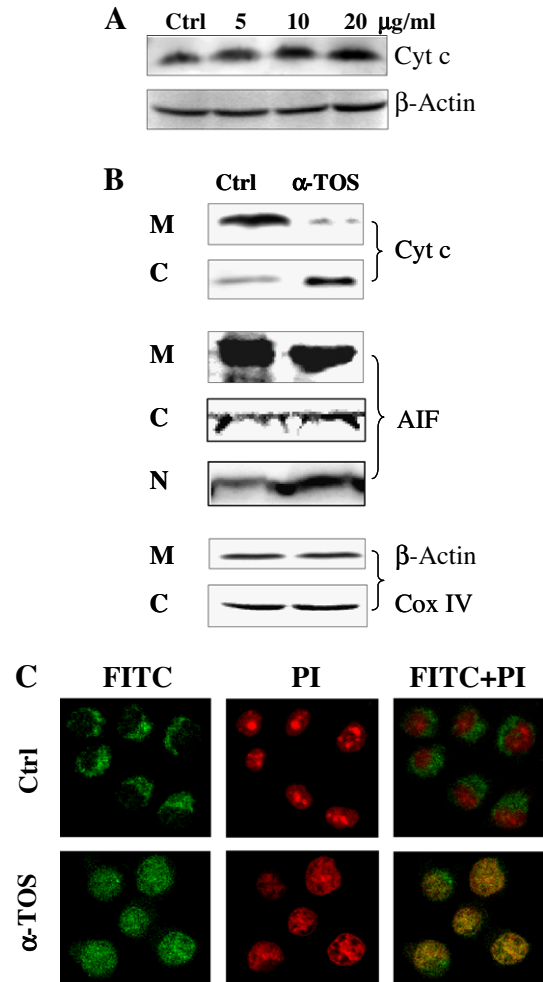


Fig. 2. α -TOS induces release of cytochrome *c* and AIF from mitochondria. (A) SGC-7901 cells were treated with 5, 10 and 20 μ g/ml α -TOS for 24 h. Cell lysate was subjected to western blotting using anti-cytochrome *c* IgG. (B) SGC-7901 cells were treated with 20 μ g/ml α -TOS for 24 h. Mitochondrial (M), cytosolic (C) and nuclear (N) fractions were assayed to examine cytochrome *c* and AIF by western blotting as described in Section 2. β -Actin and Cox IV were used as loading controls for the cytosolic (or cell lysate) and mitochondrial fractions, respectively. (C) Cells were exposed to the vehicle (upper panel) or 20 μ g/ml α -TOS (lower panel) for 24 h and subjected to immunostaining with AIF antibody followed by FITC-conjugated secondary antibody; nuclei were detected with PI. AIF translocation was determined by confocal microscopy.

TOS induced MPT as an early event of apoptosis; (ii) cytochrome *c* and AIF were translocated from mitochondria into the cytosol and nucleus, respectively, caspase-9 and caspase-3 were activated and PARP was cleaved as a consequence of MPT in response to α -TOS exposure, and these events were counteracted by Bid downregulation; (iii) Bax was mobilized from the cytosol to mitochondria and the ratio of Bax to Bcl-2 increased; and (iv) Bid was cleaved into tBid, which was blocked by Fas AS ODN, suggesting a link between death receptor- and mitochondria-mediated apoptotic pathways.

α -TOS has been the focus of recent research, which convincingly documented that the VE analog features signifi-

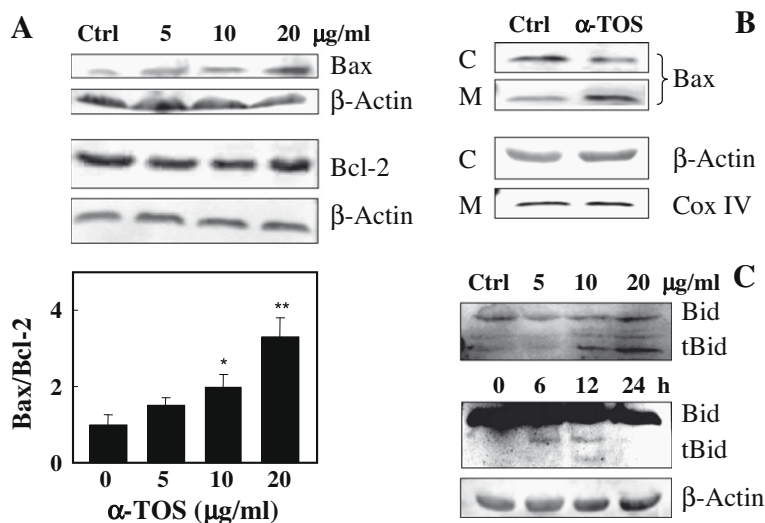


Fig. 3. Bcl-2 family proteins are involved in α -TOS-induced apoptosis in SGC-7901 cells. (A) SGC-7901 cells were treated with 5, 10 and 20 μ g/ml α -TOS for 24 h. Cell lysates were subjected to western blotting using anti-Bax and anti-Bcl-2 IgG. Bottom graph shows the ratio of relative expression of Bax to Bcl-2. Data shown represents mean values \pm SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$. (B) Cytosolic (C) and mitochondrial (M) fractions isolated from the cells exposed to 20 μ g/ml α -TOS for 24 h were assessed for Bax protein expression by western blotting. (C) SGC-7901 cells were treated with α -TOS at 5, 10 and 20 μ g/ml for 24 h or at 20 μ g/ml for 6, 12 and 24 h. Immunoblotting was used to analyze Bid protein expression in the cell lysate. β -Actin and Cox IV were used as a loading control for the cytosolic (or cell lysate) and mitochondrial fractions, respectively.

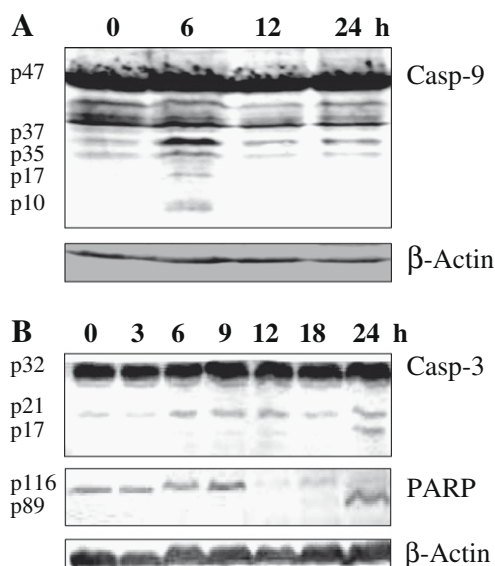


Fig. 4. α -TOS causes caspases cascade at the execution phase of apoptosis. SGC-7901 cells were treated with 20 μ g/ml α -TOS for the indicated time points. Caspase-9 (A), and caspase-3 and PARP (B) protein expression was analyzed by immunoblotting. β -Actin was used as a loading control.

cant pro-apoptotic activity toward malignant cells *in vitro* and *in vivo* in diverse species and in different tissue types [3–5,29–34]. In the present study, α -TOS efficiently induced apoptosis in a dose-dependent manner in human gastric cancer cells. Some 30% of the cells underwent apoptosis when exposed to α -TOS at 20 μ g/ml for 24 h, indicating that α -TOS is a potent apoptogen in this cell type. This

is consistent with the general picture of α -TOS as well as other apoptogenic VE analogs as very efficient and selective anti-cancer drugs [35–37]. Our work now extends the potential use of α -TOS against stomach cancer.

Mitochondria are increasingly recognized as the bioenergetic and metabolic centers essential to both life and death [38–40]. Our study revealed a dose- and time-dependent dissipation of the mitochondrial trans-membrane potential, as documented by the loss of the Mito-Tracker Red CMXRos fluorescence as a result of exposure of the cells to α -TOS. Consequently, formation of the MPT brought about a leakage of apoptogenic proteins such as cytochrome c and AIF from mitochondria, culminating in caspase-dependent and independent cell death. α -TOS treatment thus resulted in the activation of caspase-9, most likely by the formation of the apoptosome complex with cytoplasmic cytochrome c and Apaf-1 followed by the cleavage of pro-caspase-3 into the active form and a cascade of subsequent changes responsible for the execution of apoptosis, exemplified by the fragmentation of PARP. AIF, a phylogenetically conserved flavoprotein, translocates to the cytosol as well as the nucleus, where it contributes to peripheral chromatin condensation, a large-scale DNA fragmentation and apoptosis. It is of note that AIF functions independently of caspases [41,42]. Mobilization of AIF to the nucleus in response to α -TOS exposure was observed before for breast cancer cells [31]. Therefore, the VE analog could efficiently kill cancer cells also in the case of compromised caspase cascade.

Bcl-2 family proteins are structurally related molecules, which positively or negatively modulate apoptosis. The relative equilibrium of various anti- and pro-apoptotic Bcl-2 family members is a critical determinant of cellular homeostasis [43,44]. α -TOS raised the levels of Bax and

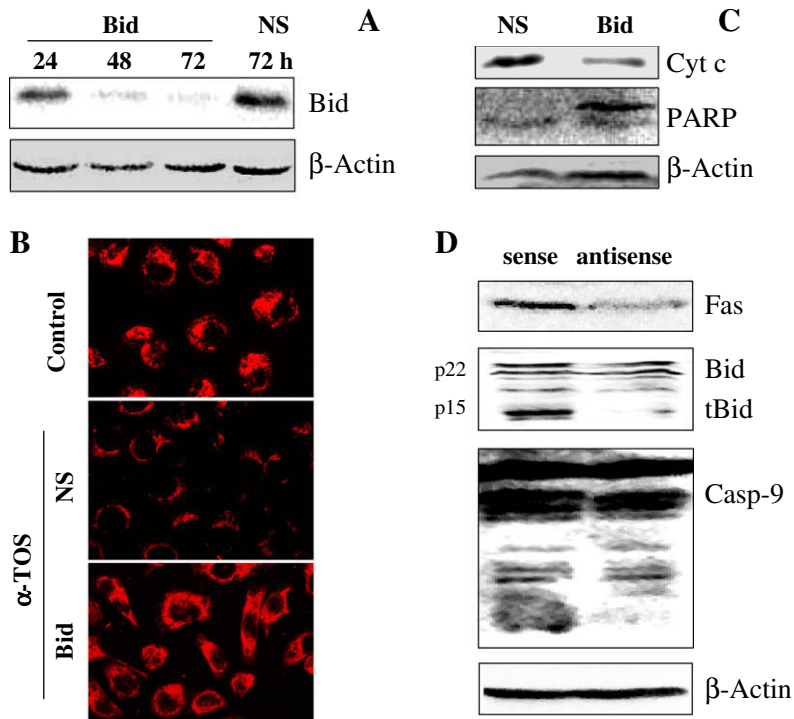


Fig. 5. Bid plays a critical role in connection of mitochondrial signaling pathway with death receptor pathway. (A) Cells were transfected with Bid siRNA for 24, 48 and 72 h or NS siRNA for 72 h as delineated in Section 2. The protein level of Bid was examined by western blotting. (B) Following 72 h transfection with Bid siRNA or NS siRNA and exposure of the cells to 20 $\mu\text{g/ml}$ $\alpha\text{-TOS}$ for 24 h, MPT was detected by confocal microscopy. (C) Cytochrome *c* and PARP protein levels were assessed by western blotting in the cells treated as described in panel B. (D) Cells were transiently transfected with Fas sense or antisense oligonucleotides. The protein levels of Fas, Bid and caspase-9 were examined by immunoblotting in control cells or cells exposed for 24 h to $\alpha\text{-TOS}$ at 20 $\mu\text{g/ml}$. $\beta\text{-Actin}$ was used as loading control.

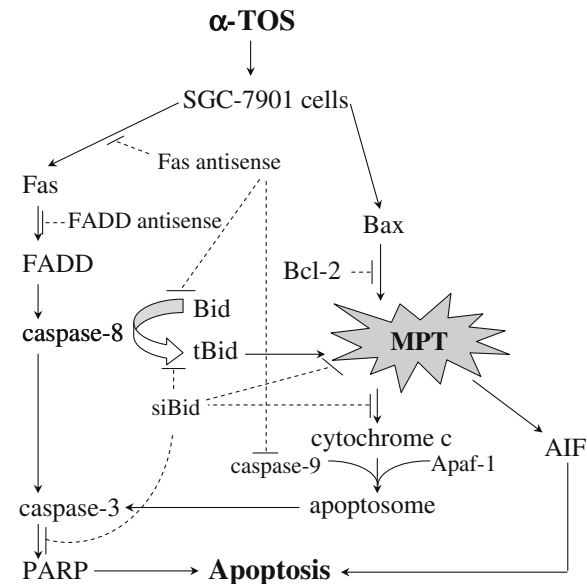


Fig. 6. A scheme of proposed mechanisms of $\alpha\text{-TOS}$ -induced apoptosis in the SGC-7901 cells depicting the integration of the extrinsic and intrinsic pathways.

the ratio of Bax to Bcl-2 in the gastric carcinoma cells in a dose-dependent manner, which was accompanied by

translocation of Bax from the cytosol into mitochondria. In the presence of death stimuli, the pro-apoptotic monomeric Bax in the cytoplasm undergoes a conformational change and contributes to the formation of the MPT [45,46]. A recent publication indicates that, interestingly, $\alpha\text{-TOS}$ may induce apoptosis in prostate cancer cells through inhibition of the binding of the pro-apoptotic Bax via its BH3 domain to Bcl-2 and Bcl-x_L, acting as a BH3 mimetic [47]. Knocking out the Bax protein breast cancer cells suppressed $\alpha\text{-TOS}$ -induced apoptosis [31]. Exactly how $\alpha\text{-TOS}$ disrupts mitochondrial integrity by targeting the Bcl-2 family proteins is not well characterized. This may be explained, at least in part, by the notion that $\alpha\text{-TOS}$ triggers apoptosis via the production of reactive oxygen species that could result in Bax- and Bak-dependent formation of the mitochondrial outer membrane pore [31,49,50].

Several excellent review papers suggest the paradigm that extrinsic and intrinsic pathways are not separated from each other, suggesting a crosstalk between these pathways mediated by the protein Bid [51,52]. However, not much is known about the role of Bid in the interaction of extrinsic with intrinsic signals, resulting in apoptosis. We report here that Bid was cleaved into its truncated product in cells exposed to $\alpha\text{-TOS}$ in a dose- and time-

dependent manner, and tBid is known to facilitate translocation of Bax to mitochondria, triggering the release of cytochrome *c*. That Bid plays a major role in apoptosis progression in gastric carcinoma cells in response to α -TOS is documented in this study by lack of loss of the mitochondrial trans-membrane potential and no discernible caspase activation and PARP cleavage in cells with downregulated Bid using the RNA interference technology. In previous studies we demonstrated the important roles of Fas signaling in α -TOS-induced apoptosis [21]. However, it has been unclear whether there is any connection between extrinsic and intrinsic pathways during the process. Data presented here show that transfection with Fas antisense oligonucleotides repressed the cleavage of Bid and pro-caspase-9 triggered by α -TOS, implying that there exists a crosstalk between the death receptor and the mitochondrial apoptotic pathways. Based on our results, we suggest that Bid serves as a 'bridge' between the two major apoptotic pathways in α -TOS-mediated apoptosis, as shown here for gastric carcinoma cells. To the best of our knowledge, this is the first study documenting that Bid is the point of convergence of apoptotic signaling in programmed cell death induced by a mitocan [5,50], epitomized here by α -TOS.

In conclusion, the current study provides evidence that mitochondria are critically involved in α -TOS-induced apoptosis by inducing of MPT and the ensuing relocation of Bax and cytochrome *c* in human gastric cancer SGC-7901 cells, and, in particular, that there is a crosstalk between death receptor- and mitochondria-related pathways via the signal-intergrating protein Bid, as depicted in Fig. 6. These findings shed insights into the diverse caveats of the molecular mechanism of α -TOS-induced apoptosis. They also indicate the possibility that the VE analog may contribute to not only tumor suppression by direct activation of apoptosis in cancer cells, but also to the immune tumor surveillance [53,54].

Conflict of interest

There is no potential conflict of interest or financial dependence regarding this publication for any of the authors.

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