Alpha-tocopheryl succinate enhances doxorubicin-induced apoptosis in human gastric cancer cells via promotion of doxorubicin influx and suppression of doxorubicin efflux

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Abstract
Doxorubicin (DOXO), a chemotherapy drug, is widely used in clinic for treating a variety of cancers. However, the treatment eventually fails due to drug resistance and toxicity. Therefore, a combination strategy is needed to increase efficacy and reduce toxicity of DOXO. alpha-tocopheryl succinate (α-TOS) exhibits anticancer actions in vitro and in vivo. Here, we reported that combination of DOXO + α-TOS cooperatively acted to induce apoptosis in SGC-7901 cells. α-TOS enhanced cellular level of DOXO via promotion of DOXO influx and suppression of DOXO efflux. DOXO induced MDR1 mRNA and protein expression and α-TOS inhibited this event, indicating that α-TOS suppressed DOXO efflux via inhibition of MDR1. Furthermore, combination of DOXO + α-TOS induced increased levels of Fas and Bax protein expression and cleavage of caspase-8 and caspase-9, suggesting that combination treatment induced Fas/caspase-8 and Bax mediated mitochondria dependent apoptosis. Taken together, our results demonstrated that α-TOS enhanced DOXO anticancer efficiency via promotion of DOXO influx and suppression of MDR-1 mediated DOXO efflux.

1. Introduction
Gastric cancer is the most commonly diagnosed cancer in the world. The clinical treatment of gastric cancer includes surgery and chemotherapy. The commonly used chemotherapeutic drugs are 5-fluorouracil, doxorubicin (DOXO), and cisplatin. However, chemotherapy eventually fails due to drug resistance and toxicity [1–4]. Therefore, new treatments for overcoming drug resistance and toxicity in gastric cancer are badly needed.

DOXO, a DNA damage drug, exerts its anticancer actions via inhibiting growth and inducing apoptosis of tumor cells. The mechanisms of DOXO anticancer actions involve inhibition of topoisomerase II, RNA polymerase and cytochrome c oxidase, intercalation between DNA base pairs [5] and formation of free radicals [6].

Like other chemotherapy drugs, drug resistance to tumor cells and toxicity to normal cells are two major limitations of DOXO for successful application in clinic. A major cause for acquired DOXO resistance is that the cancer cells express increased level of multi-drug resistance (MDR) protein, leading to decreased levels of cellular DOXO. MDR protein is a subfamily of ATP-binding cassette (ABC) transporters which transport various molecules across extra- and intra-cellular membranes. Members of the MDR protein subfamily are ATP-dependent drug efflux pump for xenobiotic compounds with broad substrate specificity and responsible for decreased drug accumulation in MDR
cells [7]. P-glycoprotein (Pgp) is a membrane transporter encoded by the MDR1 gene and responsible for the efflux of DOXO [8,9]. Thus, targeting MDR1 is a promising approach to reduce drug resistance. In addition, DOXO not only targets tumor cells but also normal cells, which can cause serious systemic side effects such as cardiomyopathy. Therefore, a strategy that is capable of reducing both DOXO induced systemic cytotoxic effects on normal tissues and expression of MDR1 will greatly promote DOXO clinical application.

α-TOS, a derivative of vitamin E, is used as a vitamin E supplement and exhibits potent in vitro and in vivo anticancer actions in variety of cancer types including gastric cancer with less or no toxicity to normal cells and tissues [10–17]. The anticancer actions of α-TOS include inhibition of proliferation and induction of differentiation and apoptosis of cancer cells [10–17]. α-TOS shows to enhance the growth inhibitory effects of ionizing radiation, chemotherapeutic drugs, and hyperthermia on tumor cells and importantly, to protect the chemotherapeutic drug-induced toxicity on normal cells [18–22]. Our previous studies showed that α-TOS induced apoptosis in SGC-7901 human gastric cancer cells via multiple signaling pathways, including extrinsic Fas, MAP kinase, and endoplasmic reticulum (ER) stress [23–25]. In this paper, we investigated the effect of α-TOS on DOXO induced apoptosis in SGC-7901 cancer cells and studied the mechanisms of action.

2. Materials and methods

2.1. Cell culture

SGC-7901 human gastric cancer cells were grown as a monolayer 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, FBS was reduced to 2%. α-TOS (Sigma) at 20 mM was dissolved in ethanol as stock solution. Equivalent levels of ethanol were used as a solvent control for α-TOS. DOXO (National Institute for Control of Pharmaceutic. 97.2% purity) was dissolved in H2O.

2.2. Cell morphology

SGC-7901 cells at 1.0 × 10^5 cells/6 well plate were treated with either α-TOS at 10 µM or DOXO at 1.5 µM alone or in combination for 6, 12, 24 h. Inverted microscope was used to detect cell morphology.

2.3. Quantification of apoptosis

Apoptosis was assessed as described previously [26]. Briefly, cells were treated with α-TOS at 10 µM, DOXO at 1.5 µM alone or combination of α-TOS + DOXO for 6, 12, 24 h. The cells were collected, washed and stained with Annexin V/FITC apoptotic detection kit (BD Biosciences). Fluorescence was measured by Fluorescence Activated Cell Sorter (FACS) analyses with a FACSCalibur flow cytometer, and data were analyzed using CellQuest software (BD Biosciences, San Jose, CA).

2.4. Detection of cellular levels of DOXO by confocal microscope

Confocal laser scanning microscope (nikon eclipse te2000-e) was used to determine the internal fluorescence of DOXO in cells with different treatments as relatively cellular levels of DOXO.

2.5. Quantification of cellular levels of DOXO by HPLC

Alternatively, cellular levels of DOXO were determined by HPLC (Waters 2690 Separations Module and Waters 474 Scanning Fluorescence Detector). DOXO from 1 × 10^5 cells/sample were extracted using 1 ml of chloroform/methanol (10:1) mixture and 200 µl of phosphate buffer (0.1 M, pH 9.8). The levels of DOXO were determined using fluorescence detector at Ex = 246 nm Em = 255.

2.6. Detection of cellular influx of DOXO by confocal microscope

Influx of DOXO was determined by confocal laser scanning microscope. Cells at 1.0 × 10^5 cells/6 well plate were treated with either DOX at 1.5 µM alone or DOXO (1.5 µM) + α-TOS (10 µM) for 30, 45, 60 min. Internal fluorescence of DOXO was detected by confocal laser scanning microscope.

![Fig. 1. DOXO and α-TOS induced apoptosis in SGC-7901 cells. (A and B) SGC-7901 human gastric cancer cells were treated with different concentrations of α-TOS or DOXO. Annexin V/FITC-FACS assay was used to determine the percentage of apoptosis. Data are expressed as mean ± S.D from three individual experiments. * means p < 0.05 compared with control.](image-url)
2.7. Western blot analyses

Equivalent amounts of protein were separated on 10% SDS–PAGE and transferred to a nitrocellulose membrane. Western blot analyses were performed using PARP, MDR-1, Fas, Bax, Bid and β-actin antibodies (Santa Cruz), Caspase-8 and caspase-9 (Cell Signaling Technology). The membrane was then incubated with the secondary alkaline phosphatase-conjugated IgG and detected with the Western Blue Stabilized Substrate for alkaline phosphatase (Promega). Bands was analyzed using the Chemilumager 4000 instrument (Alpha Innotech).

Fig. 2. α-TOS cooperated with DOXO to induce apoptosis in human gastric cancer cells. (A) SGC-7901 human gastric cancer cells were treated with either α-TOS at 10 µM and DOXO at 1.5 µM alone or in combination for 6, 12, 24 h. Cell morphology was detected by microscope. (B and C) Annexin V/FITC-FACS assay was used to determine the percentage of apoptosis. Data in (C) is expressed as mean ± S.D from three individual experiments. * means p < 0.05 compared with control.
2.8. 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). Reverse transcription was performed following the manufacturer’s protocol (RNA PCR kit, TaKaRa Shuzo Co., Ltd, Japan). cDNAs were amplified by PCR using thermal cycler under the following conditions: 94 °C for 5 min for initial denaturation, 36 cycles (94 °C/30 s, 56 °C/30 s, 72 °C/30 s), and 72 °C for 10 min. PCR fragments were separated by electrophoresis on a 1.5% agarose gel and visualized by staining with ethidium bromide. Sequences of the primer for β-actin (256 bp) are: forward: 5’-GTGGGC CGCTCTAGGCACCAA-3’; reverse: 5’-CTCTTTGATGTCACGATTTC-3’. and for MDR1 (262 bp) are; forward: 5’-TGCTGCTCAAGTAAAGG-3’; reverse: 5’-AACAACGGTTCGG AAGT-3’.

2.9. Statistical analysis

Apoptosis data were analyzed using a one-way analysis of variance followed by ANOVA Tukey test with P < 0.05 as significance.

3. Results

3.1. DOXO and α-TOS induced apoptosis of SGC-7901 cells

The pro-apoptotic property of α-TOS and DOXO in SGC-7901 cells was evaluated to determine the doses used in combination by Annexin V/FITC and FACS. Data showed that both α-TOS and DOXO induced dose-dependent apoptosis in SGC-7901 cells at 24 h (Fig. 1A and B).

3.2. Combination of α-TOS + DOXO cooperatively acted to induced apoptosis in SGC-7901 cells

Based on the data from Fig. 1A and B, the sub-apoptotic doses of DOXO at 1.5 µM and α-TOS at 10 µM were chosen to test if combination of them cooperatively induced apoptosis. Data showed that
combination treatment induced increased numbers of dead cells detected by morphological changes showing that cells became rounded and disattached (Fig. 2A). Alternatively, combination treatment of DOXO and α-TOS induced 4.09%, 11.53% and 39.9% apoptosis at 6, 12, and 24 h, respectively, detected by annexin V/FITC/FACS assay, (Fig. 2B and C), indicating a time-dependent manner of apoptotic induction starting at 12 h. While the same concentrations of α-TOS and DOXO alone induced low levels of apoptosis with no time-dependent manner. These data demonstrated that α-TOS could cooperate with DOXO to induce apoptosis in SGC-7901 cells, suggesting that α-TOS enhanced DOXO anticancer efficacy.

3.3. α-TOS enhanced cellular levels of DOXO

How α-TOS enhances DOXO anticancer efficacy is not known. One possibility is that α-TOS may enhance cellular levels of DOXO. To test this possibility we examined the cellular levels of DOXO treated with DOXO or DOXO + α-TOS. Since DOXO possesses autofluorescence with the wave-length as the same as Propidium Iodide (PI), we determined the expression of internal fluorescence of DOXO in SGC-7901 cells by confocal laser scanning microscope. Compared with the cells treated with DOXO alone, the cells treated with α-TOS + DOXO exhibited significantly enhanced levels of internal fluorescence (Fig. 3A), suggesting that α-TOS enhanced cellular levels of DOXO. To further confirm this data, we determined cellular DOXO concentration in DOXO and DOXO + α-TOS treated cells using HPLC analyses. Data showed that the levels of DOXO in DOXO + α-TOS treated cells were significantly higher than in DOXO treated cells (Fig. 3B and C), further confirming that α-TOS enhanced cellular levels of DOXO.

3.4. α-TOS enhanced DOXO influx

To understand how α-TOS enhances DOXO cellular levels, we tested if α-TOS enhanced DOXO influx by confocal microscope. Since DOXO increase in MDR1 mRNA and protein expression occurred after 6 h (Fig. 5), we assumed that α-TOS-induced increase in cellular accumulation of DOXO before 6 h was not due to MDR1 mediated efflux, but influx. To see if α-TOS had effect on DOXO influx, we checked cells at 1.0 × 10^5 cells/6 well plate treated with either DOX at 1.5 μM alone or DOXO (1.5 μM) + α-TOS (10 μM) for 30, 45, 60 min. Data showed that there was obvious change in 60 min. (Fig. 4). These data indicated that α-TOS enhanced DOXO influx.

3.5. α-TOS suppressed DOXO induced up-regulation of MDR1

Another possibility for α-TOS to enhance DOXO cellular level is to reduce efflux of DOXO. Since MDR1 plays important role in promoting efflux [7–9] and DOXO has been reported to induce MDR1 [27], we examined if α-TOS could reduce DOXO-induced MDR1 expression. Data showed that DOXO induced increased expression of MDR1 in both mRNA and protein levels (Fig. 5A and B) at 12 and 24 h. Although α-TOS did not reduce basal levels of MDR1 mRNA and protein, it blocked the ability of
DOXO to induce increased levels of MDR1 mRNA (Fig. 5A) and protein expression at 12 and 24 h (Fig. 5B and C). These data suggested that α-TOS enhanced DOXO cellular level partially via inhibiting MDR1-mediated DOXO efflux.

3.6. α-TOS cooperated with DOXO to induce increased levels of Fas and Bax, as well as cleavage of Bid, caspase-8 and caspase-9

To see if combination treatment induces apoptosis via triggering death receptor-mediated pro-apoptotic signaling, we examined the expression of death receptor Fas and cleavage of caspase-8, a death receptor-mediated initiation caspase. Data showed that combination of DOXO + α-TOS enhanced Fas death receptor protein expression and cleavage of caspase-8 at 12 and 24 h (Fig. 6), suggesting that combination treatment induced death receptor-mediated apoptosis. Data also showed that combination of DOXO + α-TOS enhanced cleavage of caspase-9, a mitochondria-derived initiation caspase, suggesting that combination treatment induced mitochondria-dependent apoptosis. Furthermore, data showed that combination of DOXO + α-TOS enhanced Bax protein expression and decreased Bid protein expression, indicating Bid cleavage (Fig. 6), suggesting that combination treatment induced mitochondria-dependent apoptosis via the activation of Bax and Fas/caspase-8/Bid.

4. Discussion

Accumulating data demonstrated that α-TOS is a potent anticancer agent in vitro and in vivo in variety of cancer types including gastric cancer with less or no toxicity in most normal cells and tissues. Anticancer actions of α-TOS include inhibition of proliferation and induction of differentiation and apoptosis [10–17]. In addition, α-TOS also shows to cooperate with some standard and experimental cancer therapeutic agents to inhibit cancer cell growth in one hand, and to protect from drug induced toxicity in normal cells in other hand [18–22]. The unique dual functions of α-TOS make it an idea nutrient-based agent for cancer therapy in combination with other chemotherapeutic agents. Here, we demonstrated that α-TOS cooperates with DOXO to induce apoptosis in gastric cancer cells, indicating that combination treatment of DOXO + α-TOS is a potent novel strategy for gastric cancer therapy.

The cytotoxicity of DOXO is largely determined by its cellular level [4]. Our data demonstrated that α-TOS enhances cellular levels of DOXO at 24 h in gastric cancer cells evidenced by increased fluorescence intensity of DOXO detected using confocal microscope and quantitative analyses by HPLC (Fig. 3), suggesting that α-TOS enhanced increase in cellular level of DOXO is one of the mechanisms for α-TOS to enhance DOXO anticancer actions. Furthermore, we found that α-TOS enhanced DOXO influx evidenced by increased DOXO uptake at 45 and 60 min in α-TOS + DOXO treatment in comparison with DOXO treatment alone (Fig. 4). The data showed that α-TOS inhibited DOXO induced increase in MDR-1 mRNA and protein expression suggested that α-TOS also enhances DOXO influx. Therefore, α-TOS enhances cellular DOXO levels via both promotion of DOXO influx and suppression of DOXO efflux.

Studies have been focused on improving DOXO influx to enhance DOXO toxicity in cancer cells. Data showed that DOXO combined with Polymer-Lipid Hybrid Nanoparticle or liposomes could increased cellular uptake of DOXO. DOXO physically associated with these compounds could
go through the membrane easier than free drug [28,29].

Data from Cao N et al. showed that conjugated D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS) with DOXO enhanced DOXO uptake in tumor cells and reduced DOXO level in normal tissues such as heart, indicating reduced side effects [30]. How α-TOS enhances DOXO cellular influx in our study is not known. One possibility is that α-TOS may physically associate with DOXO to carry it into cells. If this hypothesis is true there is a potential for α-TOS to reduce DOXO side effects when combined with DOXO via reducing DOXO levels in normal tissues because α-TOS has been reported to be selectively uptaken by cancer cells [31]. Thus, it is greatly interesting to further address this issue.

The big barrier to maintain DOXO cellular level is DOXO induced increase in DOXO efflux via expression of MDR, an ATP-dependent drug efflux pump, which is the main cause for DOXO acquired resistance. Thus, studies have been focused on targeting MDR protein to inhibit DOXO efflux. Several agents such as 5-Bromotetrandrine, guggulsterone, verapamil and CJ have shown the ability to inhibit DOXO efflux via reversal of MDR [32–35]. How α-TOS blocks DOXO induced MDR1 is not known. The etiology of MDR is complex. Akt and NF-kB [36] have been reported to positively regulate MDR1 [37,38] and DOXO can activate them [39,40]. Previous data published from our and other labs have shown that α-TOS can suppress Akt and NF-kB [41,42]. Therefore, α-TOS suppression of Akt and NF-kB may be the mechanisms to block DOXO induced MDR1. Further study will be conducted to address the possibility.

Our data showed that combination of α-TOS + DOXO induced increased Fas and Bax protein expression and triggered Fas/casapse-8/Bid and Bax dependent mitochondria apoptotic cascade. p53 family members, including p53, p63 and p73 are activated upon DNA damage and play critical roles in DNA damage induced apoptosis [43,44]. Both p53 and p73 induce apoptosis via regulating p53-dependent apoptosis related genes, such as Fas and Bax [44]. p53 only exert it’s anticancer functions in p53 wild type cancer cells. Whereas, p73, which is rarely mutant in cancer cells exert it’s anticancer functions in both p53 wild type and p53 mutant cancer cells. Since SGC-7901 human gastric cancer cells express mutant p53 [43], other p53 family members, such as p73, may contribute to the combination treatment mediated Fas and Bax up-regulation.

In summary, our data, for the first time, demonstrated that α-TOS cooperates with DOXO to induce apoptosis of human gastric cancer cells via enhanced cellular level of DOXO, which involves promotion of DOXO influx and suppression of MDR-1 mediated DOXO efflux. Data suggested that Fas/casapse-8/Bid and Bax mediated mitochondria-dependent apoptotic pathways were involved in this event. Since α-TOS has been shown to be a low toxicity to normal cells and to possess ability to protect normal cells from chemotherapeutic drug induced toxicity, combination of α-TOS + DOXO maybe a potential strategy to enhance DOXO antitumor efficiency and to reduce its toxicity to normal cells and tissue. Further studies are needed to confirm the in vitro combinational anticancer actions using in vivo model.

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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