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γ-Tocotrienol induces mitochondria-mediated apoptosis in human gastric adenocarcinoma SGC-7901 cells

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Abstract

Tocotrienols are naturally occurring isoprenoid compounds highly enriched in palm oil, rice bran, oat, wheat germ, barley and rye. Tocotrienols have antioxidant properties as well as potent anticancer properties. In this study, the mechanisms underlying the apoptosis of γ-tocotrienol on human gastric adenocarcinoma SGC-7901 cells were further studied, especially in correlation with the involvement of the apoptotic pathway. γ-Tocotrienol inhibited SGC-7901 cell growth in a concentration- and time-dependent manner. The inhibitory effects of SGC-7901 cells were correlated with the DNA damage and arresting cell cycle at G_0/G_1 phase in a time-dependent manner at 60 μmol/L concentration of γ-tocotrienol. γ-Tocotrienol induced activation of caspase-3 and increased the cleavage of the downstream substrate poly (ADP-ribose) polymerase. Furthermore, γ-tocotrienol-induced apoptosis on SGC-7901 cells was mediated by activation of caspase-9. The data in this study suggested that γ-tocotrienol could induce the apoptosis on human gastric cancer SGC-7901 cells via mitochondria-dependent apoptosis pathway. Thus, our findings revealed γ-tocotrienol as a potential, new chemopreventive agent for human gastric cancer.

Keywords: γ-Tocotrienol; Apoptosis; Caspases; PARP; SGC-7901 cells

1. Introduction

Cancer chemoprevention has received increasing attention in recent years. Many agents, including naturally occurring and synthetic compounds, have been demonstrated to exhibit cancer preventive activity [1]. Of particular significance is the realization that many dietary components have cancer chemopreventive activity [1]. An alternative strategy to reducing the risk of cancer is through dietary modification. It has been reported that more than 30% of human cancers could be prevented by an alternative strategy of appropriate dietary modification [2,3]. Epidemiological studies, including case–control and cohort studies, have consistently shown that regular consumption of fruits, vegetables and whole grains is associated with a markedly reduced risk of developing cancer [4]. It has been suggested that isoprenoids, the bioactive plant phytochemicals of vegetables, fruits and whole grains, are thought to be primary contributors to these health benefits in the prevention of cancers [5,6]. The compounds of vitamin E family are divided into two subgroups called tocopherols and tocotrienols. Both tocopherols and tocotrienols exist as α, β, γ and δ forms. The two differ structurally in that tocopherols contain a saturated phytyl chain, and tocotrienols possess an unsaturated side chain attached to 1-position on the chroman ring. The source of these vitamins also differs; tocopherols are components of nuts and common vegetable oils, and tocotrienols are primarily derived from oat, wheat germ, barley, rye, rice bran and palm oil [7].

The tocotrienols are also found among the estimated 22,000 isoprenoid products of secondary plant metabolism that share a common precursor, mevalonic acid. The tocotrienols, the less potent of the vitamin-E-active tocols,

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represent a group of mixed isoprenoids with only a part of the molecule being derived via the isoprenoid pathway. Tocotrienols modified the 3-hydroxy-3-methylglutaryl coenzyme A reductase activity to suppress the growth of implanted tumors [5,6]. However, some evidences have shown that the anti-carcinoma properties of tocotrienols and their homologous compounds are independent of the antioxidant activity [8]. In previous studies, the data demonstrated that tocotrienols and tocotrienol-rich fraction (TRF) of palm oil inhibited the growth of breast [9–13], colon [14], prostate [15], liver [16] and gastric cancer cells [17] in a dose-dependent manner, affected the cell cycle and altered the expression of proteins to induce apoptosis of cancer cells.

Apoptosis plays an important role in the maintenance of homeostasis and in eliminating damaged cells [18]. It is well known that many chemopreventive agents take effect by inducing cancer cell apoptosis [14,15,19–23]. Apoptosis may be initiated through the stimulation of death receptors located on the cell surface or through an intrinsic pathway involving the release of apoptotic signals from mitochondria [24,25]. Both signals converge on a cascade of cysteine proteases known as caspases, which are central to the initiation, and execution leads to the activation of apoptosis. Caspase-3, in particular, is an essential player in the DNA fragmentation process and other morphological changes associated with apoptosis [26,27]. Poly(ADP-ribose) polymerase (PARP) is a zinc-finger DNA-binding protein that is implicated in the maintenance of genomic stability and the DNA-damage-triggered signaling cascade. PARP can be selectively cleaved by caspase-3 during apoptosis and become incapable of responding to DNA damage. Thus, PARP cleavage has been widely used as a hallmark of cell apoptosis [28]. Studies have shown that γ-tocotrienol and TRF of palm-oil-induced apoptosis in breast and liver cancer cells have been accompanied by activation of caspase-3 [13,16]. Our study has shown that γ-tocotrienol induced activation of caspase-3 in SGC-7901 cells in a concentration-dependent manner [17]. However, the apoptotic pathway, which is involved in apoptosis of gastric tumor cells, has not been investigated.

Therefore, the objective of this study is to explore whether death receptor and/or mitochondrial stress-mediated signaling pathways are involved in γ-tocotrienol-induced apoptosis by investigating the effects of γ-tocotrienol on cell viability and apoptosis in human gastric cancer SGC-7901 cells.

2. Materials and methods

2.1. Chemicals and reagents

Low melting point agarose, ethylenediaminetetraacetic acid disodium salt, ethidium bromide (EB) and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) were purchased from Sigma (St. Louis, MO). Purified γ-tocotrienol was purchased from Davos (Singapore). Mouse monoclonal antibody specific to β-actin was from Sigma. Rabbit polyclonal antibodies specific to caspase-3 (sc-7148), caspase-8 (sc-5263), caspase-9 (sc-8355) and PARP (sc-7150) were bought from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit (w3960) secondary antibodies were purchased from Promega, USA.

2.2. Cell culture

Human gastric adenocarcinoma SGC-7901 cell line was obtained from the Cancer Institute of Chinese Academy of Medical Science. SGC-7901 cells were cultured in RPMI 1640 (GIBCO) containing 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Material Co., Ltd., P.R. China), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2 and 95% air. Cells were plated at a density of 1×10^6 cells per 100-mm culture dish and allowed to grow to approximately 70% confluence before experimentation.

2.3. Cell growth and viability assay

The effect of γ-tocotrienol on the viability of cells was determined by the MTT assay. Briefly, SGC-7901 cells were plated at 1×10^4 cells per well in 96-well microtiter plates. After 20 h, cells were treated with 200 μl of complete culture medium containing 10, 20, 30, 40, 50, 60 and 80 μmol/L of γ-tocotrienol and with a negative control. γ-Tocotrienol stock solutions were prepared in dehydrated alcohol at 1×10^5 μmol/L concentration and mixed with fresh RPMI 1640 medium to achieve the desired final concentrations. Alcohol was added to all treatment media such that the final alcohol concentration was the same in all groups within a given experiment and always less than 0.1%. Each concentration of γ-tocotrienol was repeated in four wells. After incubation for 0, 24, 48, 72 and 96 h, cell viability was determined. We added 20 μl MTT [10 mg/ml in phosphate-buffered saline (PBS) stock, diluted to a working concentration of 1.0 mg/ml with media] to each well and incubated them for 4 h. After careful removal of the medium, 200 μl dimethyl sulfoxide (DMSO) was added to each well and shaken carefully. The absorbance was recorded on the Microplate Reader (EL_X 800, BIO-TEK Instruments, Inc.) at a wavelength of 570 nm. The effect of γ-tocotrienol on cell growth inhibition was assessed as percent cell viability where vehicle-treated cells were taken as 100% viable.

2.4. Alkaline single-cell gel electrophoresis (SCGE) assay

SCGE was performed as previously described [29]. Briefly, cells (5×10^5) were pelleted and resuspended in 1 ml of ice-cold PBS. From this suspension, 50 μl was mixed with 0.5 ml of prewarmed 0.6% low melting point agarose. We then loaded 90 μl of this mixture onto a fully frosted slide that was precoated with 0.8% normal agarose. A very thin layer was formed quickly by rapid application of a coverslip. The slide was kept at 4°C for 10 min, and then the coverslip

was removed carefully. The slides were then submerged in prechilled lysis solution [89% (1% *N*-laurylsarcosine, 2.5 mol/L NaCl and 10 mmol/L EDTA), 1% Triton X-100 and 10% DMSO, pH 10] for 1 h at 4°C. After washing with electrophoresis (0.3 mol/L NaOH and 1 mmol/L EDTA, pH 13), slides were then subjected to electrophoresis for 15 min at 2 V/cm. After electrophoresis, slides were stained by EB for 1 min and then washed four times with deionized water. The nuclei were visualized under a fluorescence microscope (IX 70, Olympus), and the images were captured by a camera (PM-C35, Olympus).

2.5. Transmission electron microscopy

SGC-7901 cells from the negative control and γ-tocotrienol-treated (60 μmol/L, 48 h) groups were harvested and washed with PBS at 4°C; then, cells were fixed with 4% glutaraldehyde in phosphate buffer overnight at 4°C. After fixation with 1% OsO_4 in cacodylate buffer for 1 h at 4°C, the pellets were dehydrated in graded ethanol solutions and embedded in Epon 812. Ultrathin sections of pellet were counterstained with uranyl acetate and lead citrate and observed under transmission electron microscope.

2.6. DNA damage assay

SGC-7901 cells were grown to about 70% confluence and treated with 60 μmol/L of γ-tocotrienol at 0, 24, 36 and 48 h. Following these treatments, the cells were washed twice with PBS (10 mmol/L Tris, pH 7.5, 150 mmol/L NaCl and 5 mmol/L $MgCl_2$) containing 0.5% Triton X-100, incubated on ice for 15 min and pelleted by centrifugation (12,000 rpm) at 4°C. The pellets were incubated with DNA lysis buffer (10 mmol/L Tris, pH 7.5, 400 mmol/L NaCl, 1 mmol/L EDTA and 1% Triton X-100) for 30 min on ice and then centrifuged (12,000 rpm) at 4°C. The supernatant obtained was incubated overnight with RNase (0.2 mg/ml) at 4°C and then with proteinase K (0.1 mg/ml) for 2 h at 37°C. DNA was extracted using phenol:chloroform (1:1) and precipitated with 95% ethanol overnight at −20°C. The DNA precipitate was centrifuged at 12,000 rpm at 4°C for 15 min. Then, the pellet was air dried and dissolved in 20 μl of Tris–EDTA buffer (10 mmol/L Tris–HCl, pH 8.0, and 1 mmol/L EDTA). Total amount of DNA was running in 1.2% agarose gel, containing 0.3 μg/ml EB in 0.5×TBE buffer (pH 8.3, 89 mmol/L Tris, 89 mmol/L boric acid and 2 mmol/L EDTA). The bands were visualized under a UV transilluminator followed by Polaroid photography.

2.7. Flow cytometric analysis

SGC-7901 cells were seeded in 24-well plates and then treated with 60 μmol/L concentration of γ-tocotrienol for 0, 24 and 48 h. After treatments, the percentages of cells in the different phases of the cell cycle were evaluated by determining the DNA content after propidium iodide staining. Briefly, cells were washed with PBS, trypsinized and centrifuged at 1000 rpm at 4°C for 10 min. Pellets were fixed and resuspended in 70% ice-cold ethanol for 1 h at 4°C, then centrifuged again and incubated in PBS containing 4 mg/ml RNase (type II-A) for 15 min at room temperature. Finally, samples were stained with 1.8 mg/ml propidium iodide for 30 min at 4°C. Data acquisition was done on a FACScan Flow Cytometer (Becton and Dickinson, Mountain View, CA) and analyzed by MultiCycle software (BD Biosciences, USA).

2.8. Western blotting analysis

SGC-7901 cells in various treatment groups were detached by 0.02% EDTA and washed three times with PBS. Whole-cell lysates obtained from the different treatment groups were isolated by lysing in 20 mmol/L Tris–HCl, pH 7.5, 2% SDS (w/v), 2 mmol/L benzamidine, 0.2 mmol/L phenyl-methanesulphonyl fluoride. The total protein concentrations of each sample were determined using the nucleic acid and protein analyzer (DU 640, BECKMAN, USA) according to the manufacturer's directions. For Western blotting analysis, 50–80 μg protein was separated from 10% or 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked in blocking buffer (1% BSA, 1% Tween 20 in 20 mmol/L TBS, pH 7.6) for 1 h at 37°C in a hybridization oven (Amersham, Life Science), incubated with appropriate monoclonal or polyclonal primary antibody in blocking buffer for 2 h at 37°C or overnight at 4°C. The membrane was washed by TBST three times (5 min each time) followed by incubation with anti-mouse or anti-rabbit secondary antibody at 37°C for 1 h. The membrane was washed twice 5 min each time with TBST and then washed with TBS once. The membrane was then incubated with alkaline phosphatase until an appropriate signal level was obtained. The protein bands were detected by FluorChem Imaging Systems (Alpha Innotech).

2.9. Statistical analysis

Differences between means were analyzed for significance using the one-way ANOVA test with the Bonferroni post hoc multiple comparisons of SPSS 13.0, used to assess the difference between independent groups. All values were expressed as means±S.D., and differences were considered significant at $P<05$.

3. Results

3.1. The viability and proliferation of SGC-7901 cells induced by γ-tocotrienol

To investigate the inhibitory effect of γ-tocotrienol on the cell proliferation of SGC-7901 cells, we determined the cell viability by MTT assay. The cells were treated with various concentrations (10, 20, 30, 40, 50, 60 and 80 μmol/L) of γ-tocotrienol for 24, 48, 72 and 96 h. As shown in Fig. 1, cell viabilities of SGC-7901 cells were significantly inhibited in a time- and dose-dependent response by γ-tocotrienol. The median effective concentrations (EC_{50}) of γ-tocotrienol for

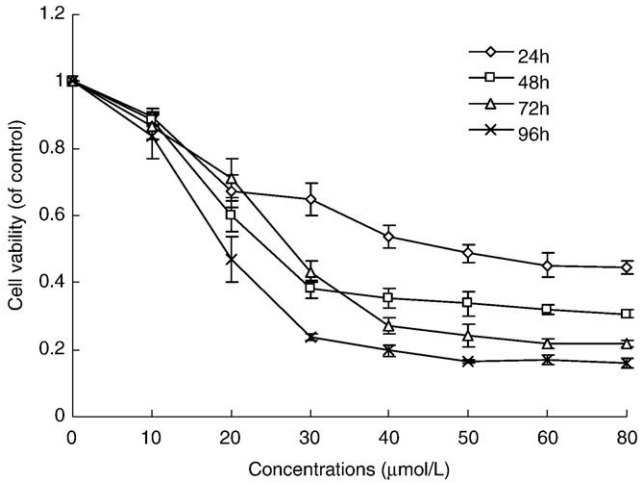

Fig. 1. Effects of γ-tocotrienol on cell viability in human gastric adenocarcinoma SGC-7901 cells determined by MTT assay. SGC-7901 cells were seeded in 96-well plates (1×10^4 per well) and treated with different concentrations of γ-tocotrienol for 24, 48, 72 and 96 h. Each concentration was repeated in four wells. The cell viability values were expressed relative to the negative control group. The results represent the mean of at least three independent experiments.

inhibition of SGC-7901 cell viability were 61.88±6.23, 38.02±3.68, 32.62±4.35 and 23.20±1.98 μmol/L at 24, 48, 76 and 96 h, respectively.

3.2. The DNA damage of SGC-7901 cells induced by γ-tocotrienol

DNA damage in SGC-7901 cells treated with γ-tocotrienol was determined by the SCGE assay. Chromosomal DNA strand breaks were measured using the incidence of comet cell and tail length. The DNA damage increased in SGC-7901 cells exposed to various concentrations of γ-tocotrienol (15, 30 and 60 μmol/L) in comparison with the negative control group (Fig. 2). There was a significant increase in tail length and incidence of comet cell in the γ-tocotrienol-treated groups compared to the negative control group ($P<01$) in a dose-dependent manner. The results indicated that γ-tocotrienol could efficiently induce the chromosomal DNA strand breaks.

3.3. Apoptosis on SGC-7901 cells induced by γ-tocotrienol

Morphological changes were observed by transmission electron microscope in SGC-7901 cells treated with 60 μmol/L γ-tocotrienol for 48 h. The cells showed obvious characteristic changes of apoptosis, including cytoskeletal disruption, cell shrinkage, chromatin condensation and margination of nucleus, cell blebbing, formation of apoptotic body and mitochondrial denaturation such as swelling and disappearance of mitochondrial cristae in the γ-tocotrienol-treated cells (Fig. 3C and D). In addition, cells in the control group had the clear cell organs in cytoplasm, and mitochondrial cristae were also observed clearly (Fig. 3A and B). Ladder patterns of discontinuous DNA fragments were detected in SGC-7901 cells treated with γ-tocotrienol at 60 μmol/L for 24, 36 and 48 h. No DNA fragments were observed in the control group (Fig. 4).

3.4. γ-Tocotrienol-arrested cell cycle of SGC-7901 cells at G_0/G_1 phase

The cell cycle distribution of SGC-7901 cells treated with γ-tocotrienol was determined by flow cytometry. As

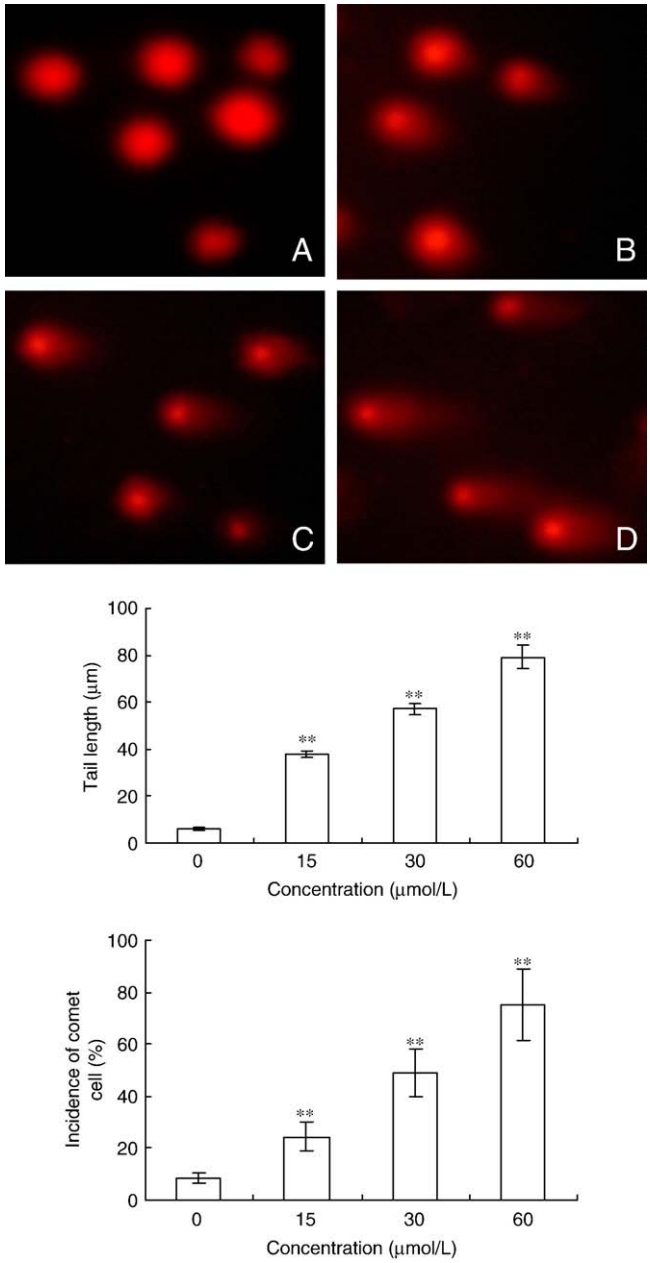

Fig. 2. DNA damage was induced in SGC-7901 cells treated with γ-tocotrienol for 48 h. DNA damage in SGC-7901 cells was measured using the comet assay (0, 15, 30 or 60 μmol/L for Panels A, B, C or D, respectively). The tail of the comet was measured in each cell under a microscope and expressed. Values are expressed means with standard deviations (n=3). Mean values of at least 100 cells calculated in each dose are shown. $^{**}P<01$, compared to the negative control group.

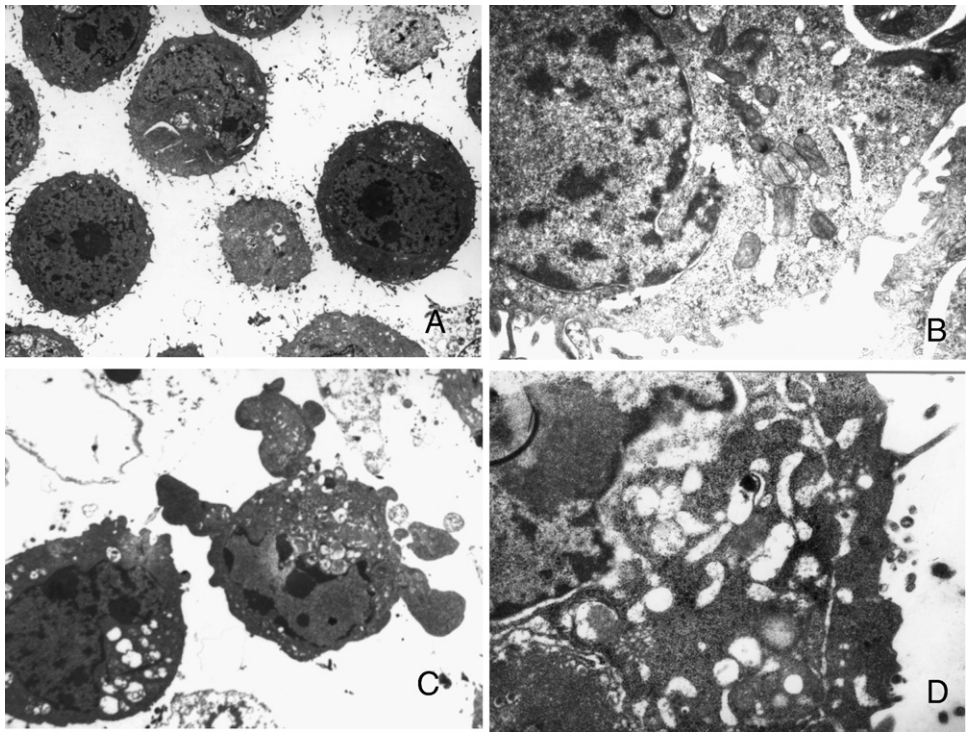

Fig. 3. Morphological changes of apoptosis were observed in SGC-7901 cells by transmission electron microscopy. Characteristics of apoptosis observed were chromatin condensation and margination, cell blebbing and vacuoles after SGC-7901 cells treated with 60 μmol/L γ-tocotrienol for 48 h (C and D) compared to the control cells (A and B). Magnification: (A and C) ×3000 and (B and D) ×15,000.

shown in Fig. 5, γ-tocotrienol increased the ratio of G_0/G_1 phase from 53.06% in the control group to 65.14% in the γ-tocotrienol-treated groups at 48 h ($P<.01$). The G_2/M values were decreased from 13.25% in the control group to 4.16% ($P<.01$), and the S phase was not changed. In addition, the expression of cyclin D1 was also measured in SGC-7901 cells treated with 60 μmol/L of γ-tocotrienol for 24 and 48 h. The expression of cyclin D1 in the 60-μmol/L γ-tocotrienol group was significantly decreased compared to the negative control group in a time-dependent manner ($P<.05$) (Fig. 6).

3.5. Caspase-3 activation of γ-tocotrienol-induced apoptosis in SGC-7901 cells

To investigate the molecular mechanism of γ-tocotrienol-induced apoptosis, we examined the expression levels of caspase-3 and PARP cleavage in SGC-7901 cells by Western blotting. In response to apoptotic stimuli, procaspase-3 is cleaved into a 20-kDa fragment, and the subsequent autocatalytic reaction leads to the formation of the active 17-kDa fragment. The expression of cleaved caspase-3 in SGC-7901 cells treated with 60 μmol/L γ-tocotrienol from 12 to 60 h showed an increased trend and the highest expression at 48 h. An increase of the 85-kDa cleavage form of PARP, an apoptosis marker generated by the caspase-3 activation cascade, was also observed in the γ-tocotrienol-treated cells compared to the negative control group (Fig. 7).

3.6. Apoptosis of SGC-7901 cells induced by γ-tocotrienol via mitochondrial pathway

To explore the apoptotic pathway of SGC-7901 cells induced by γ-tocotrienol, the activation of caspase-8 and -9, up-regulators of caspase-3 activation related to mitochondrial pathway, was investigated by Western blotting. The

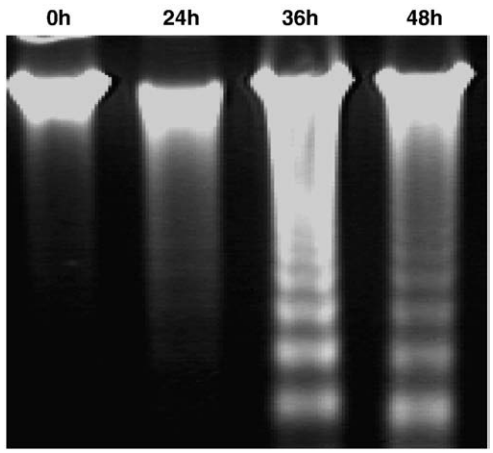

Fig. 4. γ-Tocotrienol-induced apoptosis in SGC-7901 cells. SGC-7901 cells were treated with 60 μmol/L γ-tocotrienol for 24, 36 and 48 h. DNA was isolated and subjected to 1.2% agarose gel electrophoresis, followed by visualization of bands and photography. DNA fragments were observed in the γ-tocotrienol-treated groups ($n=3$).

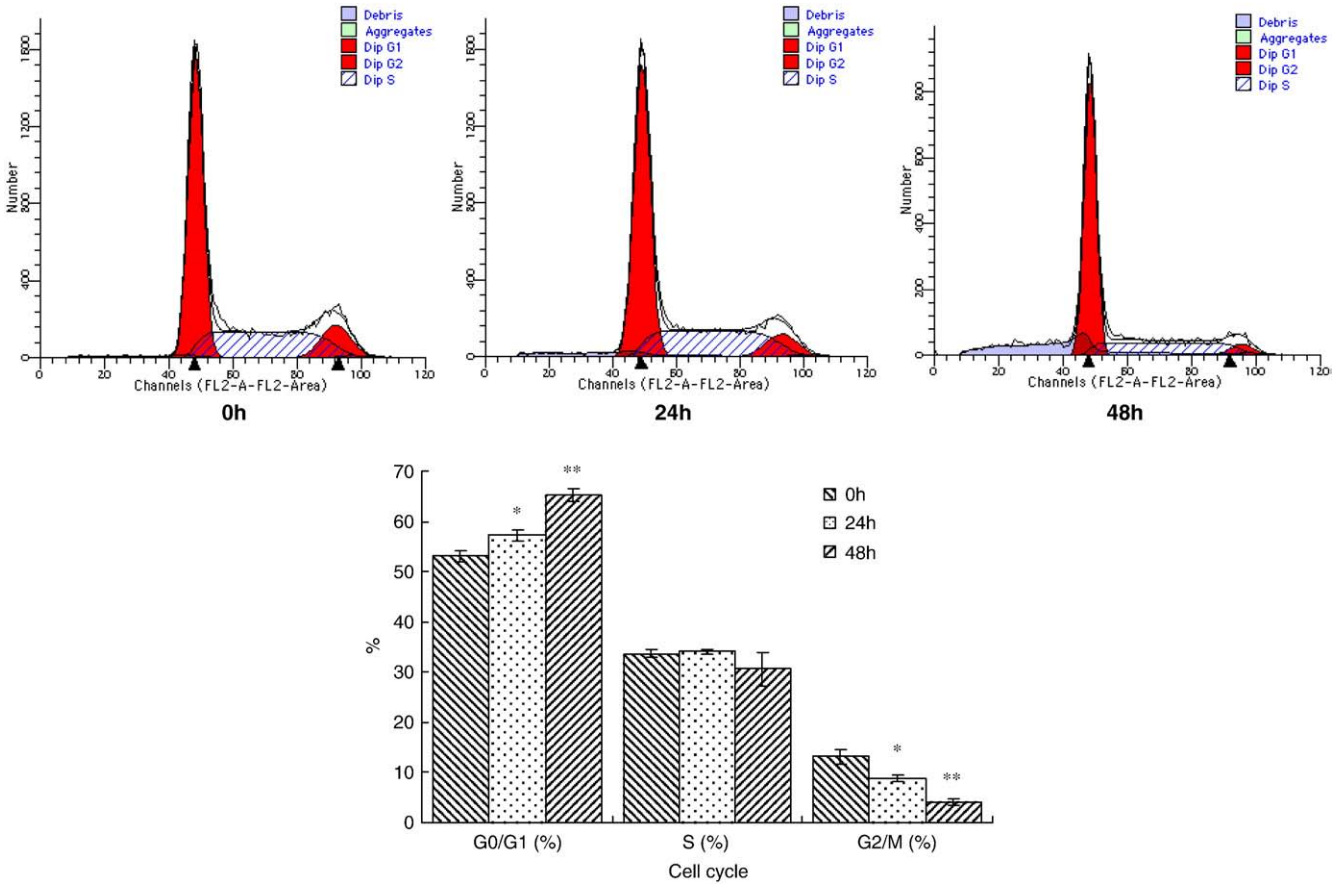

Fig. 5. Cell cycle distribution in SGC-7901 cells treated with 60 μmol/L γ-tocotrienol for 24 and 48 h was analyzed by flow cytometry. *P<05, **P<01, compared to the negative control group (n=3).

results as shown in Fig. 8; the expression of activated caspase-8 and caspase-9 and cleaved caspase-9 was induced in SGC-7901 cells treated with 60 μmol/L γ-tocotrienol.

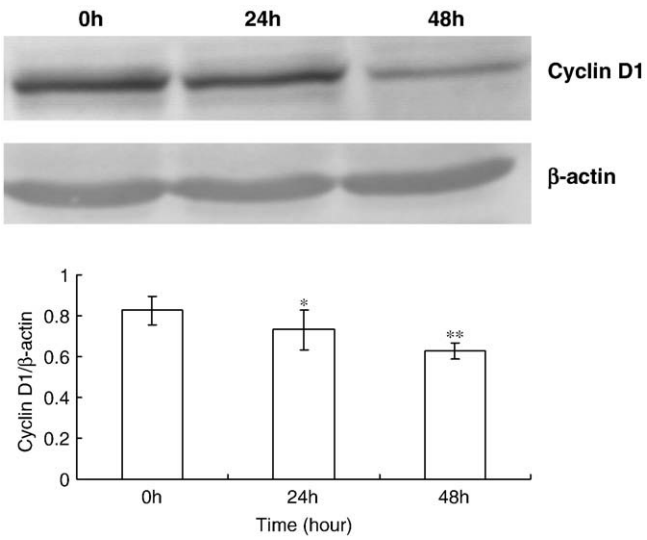

Fig. 6. The expression of cyclin D1 in SGC-7901 cells induced by 60 μmol/L of γ-tocotrienol for 24 and 48 h. *P<05, **P<01, compared to the negative control group (n=3).

4. Discussion

Apoptosis plays an important role in the maintenance of homeostasis and in eliminating damaged cells. It is well known that many chemopreventive agents take effect by inducing cancer cell apoptosis [1]. Previous studies have shown that γ-tocotrienol and TRF of palm oil have a potent growth inhibition in many tumor cell lines [7,13–16]. In our previous studies, γ-tocotrienol exerted a strong inhibitory effect in SGC-7901 cells in a dose-dependent manner [17].

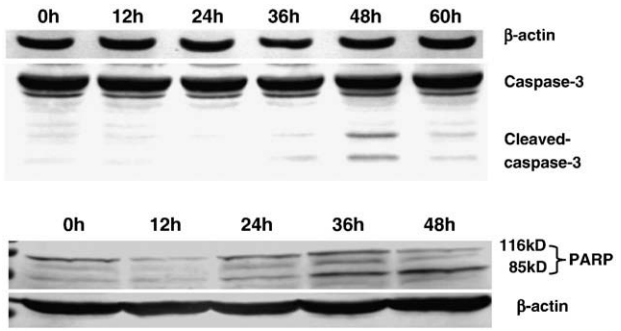

Fig. 7. The expressions of caspase-3, cleaved caspase-3 and PARP in SGC-7901 cells treated with 60 μmol/L γ-tocotrienol for 12, 24, 36, 48 or 60 h.

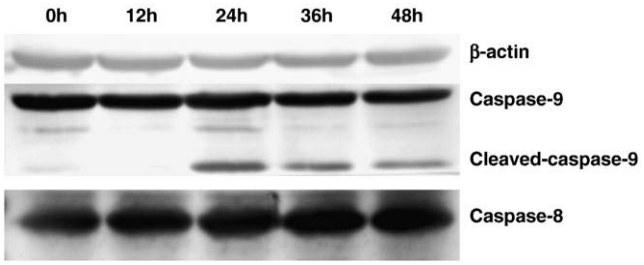

Fig. 8. The expressions of caspase-8, caspase-9 and cleaved caspase-9 in SGC-7901 cells treated with 60 μmol/L γ-tocotrienol for 12, 24, 36 and 48 h.

The inhibitory action mechanism of γ-tocotrienol-induced cell proliferation on SGC-7901 cells has been elucidated to decrease the expression of Bcl-2 and to enhance the expression of Bax in a dose-dependent manner. In addition, γ-tocotrienol could also regulate the Raf–Erk signaling transduction pathway to attenuate the expression of c-Myc [17]. The c-myc oncogene is a downstream target gene of the MAPK pathway and is among the most commonly over-expressed genes in human cancer [30]. Its down-regulation has reduced progression of cancer cell cycle and influenced apoptosis [31,32]. Thus, targeting of c-Myc by means of the MEK/ERK inhibitor can be tested as a promising strategy in anticancer therapy [33].

In recent years, many chemotherapeutic and chemopreventive agents have been shown to impart antiproliferative effects via arrest cell division at certain checkpoints in the cell cycle [15,34]. The control of cell cycle progression in cancer cells is considered to be a potentially effective strategy for the control of tumor growth [35,36]. In the present study, γ-tocotrienol inhibited the cell growth of SGC-7901 cells. The EC_{50} values were 61.88±6.23, 38.02±3.68, 32.62±4.35 and 23.20±1.98 μmol/L at 24, 48, 72 and 96 h, respectively. γ-Tocotrienol also caused the DNA damage in SGC-7901 cells induced by 60 μmol/L of γ-tocotrienol for 48 h. These data suggested that γ-tocotrienol may be further examined as an effective chemopreventive agent against human gastric cancer. Characteristic apoptotic events such as morphological changes, including chromatin condensation, cell shrinkage, nuclear fragmentation, blebbing and formation of apoptotic bodies, and DNA fragmentation were also detected in SGC-7901 cells treated with γ-tocotrienol. It has been recognized that control of cell cycle progression in cancer cells is an effective strategy to halt tumor growth [35,37], as the molecular analyses of human cancers have revealed that cell cycle regulators are frequently deregulated in most of the common malignancies [38,39]. To elucidate the mechanism of inhibitory growth, we treated SGC-7901 cells with 60 μmol/L of γ-tocotrienol for 24 and 48 h. The results showed that γ-tocotrienol arrested the cell cycle of SGC-7901 cells in the G_0/G_1 phase. The ratio of G_0/G_1 phase significantly increased in the γ-tocotrienol-treated groups compared to the negative control group ($P<01$) and was accompanied by a decrease in cell population in the G_2/M phase of the cell cycle in SGC-7901 cells. Our in vitro data demonstrate that treatment of SGC-7901 cells with γ-tocotrienol arrested cell cycle of SGC-7901 cells at G_0/G_1 phase. It indicates that one of the mechanisms by which γ-tocotrienol inhibits the proliferation of SGC-7901 cells is by arresting cell cycle progression. This study also demonstrated a marked decrease in the expression of cyclin D1 in SGC-7901 cells in a time-dependent manner. It has been shown that γ-tocotrienol arrests the cell cycle as a result of the DNA damage and the down-regulation of cyclin D1. These data indicate that γ-tocotrienol-induced inhibitory cell growth is likely to involve the modulation of cell cycle progression arrest in SGC-7901 cells.

Caspases, a family of aspartate-specific cysteinyl proteases, play a pivotal role in the execution of apoptosis [18]. There are at least two major apoptotic pathways such as the extrinsic pathways (death receptors) and intrinsic pathways (mitochondria), which are initiated by caspase-8 and caspase-9, respectively [40,41]. The stimulation of the death receptor pathway caspase-8 follows the recruitment of the procaspase to the death-inducing signaling complex. In contrast, the mitochondrial pathway requires the release of mitochondrial cytochrome *c* and the formation of a large multiprotein complex comprising cytochrome *c*, Apaf-1 and procaspase-9. Caspase-8 and caspase-9 will then proteolytically activate downstream caspases, in particular caspases-3 and -7, which are responsible for the apoptotic destruction of the cell. The Bcl-2 family proteins have been reported to regulate apoptosis by controlling the mitochondrial membrane permeability [42]. Bcl-2 suppresses apoptosis by stabilizing the mitochondrial membrane, while Bax and Bid induce apoptosis by enhancing mitochondrial membrane permeability, which leads to the release of cytochrome *c* from mitochondria [43]. In our previous study, γ-tocotrienol affected the expressions of Bcl-2 and Bax in SGC-7901 cells [17]. In this study, caspase-9, the apical caspase in mitochondria-mediated apoptotic pathway, and caspase-3, but not caspase-8, were activated during the process of apoptosis induced by γ-tocotrienol in SGC-7901 cells. γ-Tocotrienol treatment of SGC-7901 cells resulted in a time-dependent activation of caspase-9 and caspase-3 and cleavage of PARP. These results indicated for the first time in human gastric cancer cells that γ-tocotrienol-induced apoptosis was caspase dependent and suggested that mitochondria were involved in apoptosis induction. An increase in the ratio of Bax/Bcl-2 stimulates the release of cytochrome *c* from mitochondria into the cytosol. The cytosolic cytochrome *c* interacts with Apaf-1, and ATP forms a complex with procaspase-9 (apoptosome), leading to activation of procaspase-9 and caspase-3 [44]. Activated caspase-3 is the key executioner of apoptosis, and cleaved caspase-3 leads to cleavage and inactivation of key cellular proteins, such as PARP [14,44–46]. Hence, we presume that γ-tocotrienol induces the expression of Bcl-2 family proteins and increases the release of cytochrome *c*, which then leads to

activation of procaspase-9 and caspase-3 to induce fragmentation of PARP.

In conclusion, the present study indicated that γ-tocotrienol inhibited cell proliferation of SGC-7901 cells by apoptosis initiation. The induction of apoptosis was partly regulated to arrest cell cycle G_0/G_1 phase and mitochondrial pathway such as activation caspase pathway as well as cleavage of PARP. The exact mechanism needs to be further studied.

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