

## **$\gamma$ -Tocotrienol-induced apoptosis in human gastric cancer SGC-7901 cells is associated with a suppression in mitogen-activated protein kinase signalling**

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Tocotrienols have been shown to inhibit proliferation and induce apoptosis in cancer cells. However, the molecular mechanisms involved in tocotrienol-induced apoptosis are still unclear. In the present study,  $\gamma$ -tocotrienol induced apoptosis in human gastric adenocarcinoma SGC-7901 cell line through down regulation of the extracellular signal-regulated kinase (ERK) signalling pathway. Furthermore,  $\gamma$ -tocotrienol-induced apoptosis was accompanied by down regulation of Bcl-2, up regulation of Bax, activation of caspase-3, and subsequent poly (ADP-ribose) polymerase cleavage. These results indicated that up or down regulation of Bcl-2 family proteins play a major role in the initiation of  $\gamma$ -tocotrienol-induced apoptosis as an activator of caspase-3.  $\gamma$ -Tocotrienol also down regulated the activation of the Raf-ERK signalling pathway, and down regulated c-Myc by decreasing the expressions of Raf-1 and p-ERK1/2 proteins. The results suggest that key regulators in tocotrienol-induced apoptosis may be Bcl-2 families and caspase-3 in SGC-7901 cells through down regulation of the Raf-ERK signalling pathway.

 **$\gamma$ -Tocotrienol: SGC-7901 cells: Apoptosis: Poly (ADP-ribose) polymerase: Caspase-3: Extracellular signal-regulated kinase 1/2: c-Myc**

According to the world estimate of cancer incidence in the year 2002 by the International Agency for Research on Cancer, gastric cancer was the fourth most commonly occurring cancer (about 9% of all cancers) after lung, breast and colorectal cancers. Gastric cancer was also the second most common cancer-related cause of death (10% of all cancer deaths) after lung cancer. In 2002, the incidence of gastric cancer was estimated at 934 000 cases, 56% of the new cases being derived from Eastern Asia. On the whole, 65–70% of new cases and deaths from gastric cancer occur in less-developed countries<sup>(1)</sup>. In 2005, the incidence rates of gastric cancer (0.3 million deaths and 0.4 million new cases) ranked third among the most common cancers in China<sup>(2)</sup>. Presently, surgical removal with resection of adjacent lymph nodes offers the only chance for cure, which is less than 33% of patients with gastric cancer. The 5-year survival rate is only 30–40%, with a poorer prognosis for advanced tumours. Thus, there is a great need for new alternative agents for the prevention and treatment of gastric cancer.

Dietary modification is an alternative strategy for lowering cancer risk. It has been suggested that one-third of all cancer deaths could be avoided through this approach. Cancer chemoprevention has emerged as an important means of cancer control by using dietary agents or synthetic compounds that are capable of preventing cancer occurrence or delaying cancer

progression<sup>(3,4)</sup>. Natural vitamin E is the mixture of two classes of compounds, tocopherols and tocotrienols. The structural difference between tocopherols and tocotrienols lies in the phytyl chain: in tocopherols, the phytyl chain is saturated, while in tocotrienols it is unsaturated. Vitamin E, a fat-soluble vitamin, is well known for its antioxidant capacity<sup>(5,6)</sup>. Barley, rice bran and palm oil have been reported to be rich in tocotrienols<sup>(7)</sup>. Previous studies have shown that tocotrienols exert more significant neuroprotective, anti-cancer and cholesterol-lowering properties than do tocopherols<sup>(8–10)</sup>. Recently, more and more accumulating evidence has shown that tocotrienols and their homologous compounds have anti-carcinoma properties, and the anti-proliferative activity is independent of the antioxidant activity<sup>(11)</sup>. The accumulation of tocotrienols in the cells was much greater than tocopherols. One of the reasons may be that tocotrienols have more significant physical effect than tocopherols<sup>(12,13)</sup>. Tocotrienols and tocotrienol-rich fractions of palm oil led to the induction of apoptosis in malignant carcinoma cells, such as breast<sup>(14–18)</sup>, colon<sup>(19)</sup>, prostate<sup>(20)</sup> and hepatoma<sup>(21)</sup> carcinoma cells, by various inhibitory mechanisms.

Poly (ADP-ribose) polymerase (PARP) is a Zn-finger DNA-binding protein which is implicated in the maintenance of genomic stability and the DNA damage-triggered signalling cascade. PARP can be selectively cleaved by caspase-3 during apoptosis and become incapable of responding to

**Abbreviations:** EB, ethidium bromide; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH<sub>2</sub>-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, methyl ethyl ketone; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide; PARP, poly (ADP-ribose) polymerase; Tris, tri(hydroxymethyl)-aminomethane.

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DNA damage. Thus, PARP cleavage has been widely used as a hallmark of cell apoptosis<sup>(22)</sup>. Caspase-dependent PARP cleavage during tocotrienol-induced apoptosis in Hep3B cells and MDA-MB-435 human breast cancer cells has been reported<sup>(17,21)</sup>.

Mitogen-activated protein kinases (MAPK) are serine/threonine kinases activated in response to a variety of external signals. They include extracellular signal-regulated kinase (ERK), p38, and c-Jun NH<sub>2</sub>-terminal kinase (JNK). MAPK are proposed to be critical integrators of various signalling transduction systems including cell proliferation, differentiation, apoptosis and transformation<sup>(23)</sup>. ERK play a key role in a variety of human carcinomas to regulate cellular proliferation and survival. ERK are up regulated in human gastric cancer<sup>(24)</sup>. Raf-1 is the upstream signal protein of ERK1/2, and regulates cellular proliferation and survival. At present, several agents have been designed to target Raf and are involved in various stages of clinical development<sup>(25)</sup>. ERK1/2 targets transcription factors such as c-Myc, which is involved in carcinogenesis and is enhanced in several human tumours. Down regulation of c-Myc following methyl ethyl ketone (MEK)/ERK inhibition halted the proliferation of carcinoma cells<sup>(26)</sup>.

The purposes of the present study were (1) to investigate the effects of  $\gamma$ -tocotrienol on cell growth inhibition and apoptosis in SGC-7901 cells; (2) to determine the expression of ERK in SGC-7901 cells; (3) to explore the possible mechanism of regulating signals in SGC-7901 cells.

## Materials and methods

### *Chemicals and reagents*

EDTA-2 sodium, acridine orange, ethidium bromide (EB) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) were purchased from Sigma (St Louis, MO, USA). Purified  $\gamma$ -tocotrienol was purchased from Davos (Singapore). Mouse monoclonal antibody specific to  $\beta$ -actin was from Sigma. Rabbit polyclonal antibodies specific to ERK1 (sc-94), p38 (sc-535), JNK (sc-571), Bcl-2 (sc-492), caspase-3 (sc-7148) and PARP (sc-7150) were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibodies specific to Bax (sc-7480), p-ERK1 (sc-7383), p-p38 (sc-7973), p-JNK (sc-6254) and c-Myc (sc-40) were obtained from Santa Cruz Biotechnology. Goat anti-rabbit (w3960) and anti-mouse (w3950) secondary antibodies were purchased from Promega (Madison, WI, USA).

### *Cell culture*

Human gastric adenocarcinoma SGC-7901 cells were 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, Hangzhou City, China), penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml) at 37°C in a humidified incubator with 5% CO<sub>2</sub> and 95% air. Cells were plated at a density of  $1 \times 10^6$  cells per 100 mm culture dish and allowed to grow to approximately 70% confluence before experimentation.

### *Cell growth and viability assay*

The effect of  $\gamma$ -tocotrienol on the viability of cells was determined by the MTT assay. Briefly, SGC-7901 cells were plated at  $1 \times 10^4$  cells per well in ninety-six-well microtitre plates. After 24 h, cells were treated with 200  $\mu$ l complete culture medium containing  $\gamma$ -tocotrienol (10, 20, 30, 40, 50, 60 or 80  $\mu$ mol/l) or with a negative control.  $\gamma$ -Tocotrienol stock solutions were prepared in dehydrated alcohol at  $1 \times 10^5$   $\mu$ mol/l concentration and mixed with fresh medium to achieve the desired final concentration. Each concentration of  $\gamma$ -tocotrienol was repeated in five wells. After incubation for 72 h, cell viability was determined. Then 20  $\mu$ l MTT (10 mg/ml in PBS stock, diluted to working concentration of 1 mg/ml with media) was added to each well and incubated for 4 h. After careful removal of the medium, 200  $\mu$ l dimethyl sulfoxide was added to each well and shaken carefully. The absorbance was recorded on the microplate reader (EL<sub>X</sub> 800; Bio-Tek Instruments, Inc., Winooski, VT, USA) at a 570 nm wavelength. The effect of  $\gamma$ -tocotrienol on cell growth inhibition was assessed as percentage cell viability where vehicle-treated cells were taken as 100% viable.

### *Analysis of nuclear morphology*

A cell suspension was mixed with fluorescent DNA-binding dyes and examined by fluorescence microscopy. Briefly, SGC-7901 cells in various treatment groups were detached by 0.02% EDTA and centrifuged at 1000 rpm for 5 min at 4°C. The pellets were re-suspended with 50  $\mu$ l, then stained by acridine orange (2  $\mu$ g/ml) and EB (2  $\mu$ g/ml). Fluorescence was visualised immediately with a fluorescence microscope. The normal cells were stained uniformly green and early apoptotic cells were shown green and contained bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells were stained by the EB and therefore appeared orange with obvious condensation, whereas those with red nuclei without nuclear condensation were considered necrotic. Some apoptotic bodies emerged and the nucleus was counterstained by acridine orange and EB.

### *DNA fragmentation assay*

The SGC-7901 cells were grown to about 70% confluence and treated with  $\gamma$ -tocotrienol (60  $\mu$ mol/l) at 48, 72 and 96 h. Following these treatments, the cells were washed twice with PBS (10 mM-tri(hydroxymethyl)-aminomethane (Tris), pH 7.5, 150 mM-NaCl, 5 mM-MgCl<sub>2</sub>) containing 0.5% Triton X-100, left on ice for 15 min, and pelleted by centrifugation (12 000 rpm) at 4°C. The pellets were incubated with DNA lysis buffer (10 mM-Tris, pH 7.5, 400 mM-NaCl, 1 mM-EDTA and 1% Triton X-100) for 30 min on ice and then centrifuged (12 000 rpm) at 4°C. The supernatant fraction 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, v/v) 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  $\mu$ l of Tris-EDTA buffer (10 mM-Tris-HCl, pH 8.0, and 1 mM-EDTA).

Total amount of DNA was resolved over 1.2% agarose gel, containing EB (0.3  $\mu$ g/ml) in 0.5  $\times$  TBE buffer (pH 8.3, 89 mM-Tris, 89 mM-boric acid and 2 mM-EDTA). The bands were visualised under a UV transilluminator followed by Polaroid photography.

#### Western blot 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 mM-Tris-HCl, pH 7.5, 2% SDS (w/v), 2 mM-benzamide and 0.2 mM-phenylmethanesulfonyl fluoride. The protein concentrations of each sample were determined using the nucleic acid and protein analyser (DU<sup>®</sup> 640; Beckman Coulter, Inc., Fullerton, CA, USA) according to the manufacturer's directions. For Western blot analysis, 50–80  $\mu$ g protein was resolved over 10 or 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked in blocking buffer (1% bovine serum albumin, 1% Tween 20 in 20 mM-Tris-buffered saline (TBST), pH 7.6) for 1 h at 37°C in a hybridisation oven (Amersham Life Science, Little Chalfont, Bucks, UK), 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 with 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 for 5 min two times with TBST, and then washed with TBS one time. The membrane was then incubated with alkaline phosphatase until an appropriate signal level was obtained. The protein bands were detected by FluorChem<sup>®</sup> Imaging Systems (Alpha Innotech, Corp., San Leandro, CA, USA).

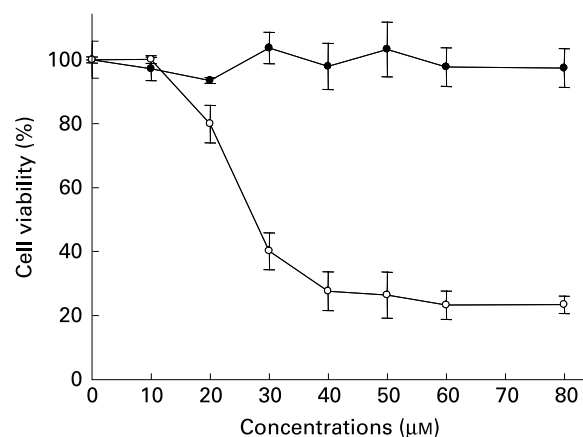
#### Statistical analysis

Differences between means were analysed for significance using the one-way ANOVA test with the Bonferroni *post hoc* multiple comparisons, used to assess the difference between independent groups. All values are expressed as mean values and standard deviations. Differences were considered significant at  $P < 0.05$ .

## Results

#### Effect of $\gamma$ -tocotrienol on viability of SGC-7901 cells

The anti-viability of  $\gamma$ -tocotrienol toward human gastric cancer SGC-7901 cells was tested. As shown in Fig. 1, SGC-7901 cell viability was significantly inhibited in a dose-dependent manner by  $\gamma$ -tocotrienol above 30  $\mu$ mol/l ( $P < 0.05$ ). There was no significant difference between solvent control cells and cells treated with  $\gamma$ -tocotrienol at 10 and 20  $\mu$ mol/l. At the dose of 40  $\mu$ mol/l, the inhibition rate was approximately 72.4 (SD 6.05) % ( $P < 0.01$ ). The median effective concentration (EC<sub>50</sub>) of  $\gamma$ -tocotrienol for inhibition of SGC-7901 cell viability was 30.02 (SD 4.68)  $\mu$ mol/l.



**Fig. 1.** The effect of  $\gamma$ -tocotrienol (○) on the viability of SGC-7901 cells compared with an ethanol control (●). Actively dividing cells were treated for 72 h with different concentrations of  $\gamma$ -tocotrienol varying from 10 to 80  $\mu$ mol/l. Cell viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide. Values are means, with standard deviations represented by vertical bars. Each point on the graph represents the mean of five samples.

#### Effect of $\gamma$ -tocotrienol on nuclear morphology of SGC-7901 cells

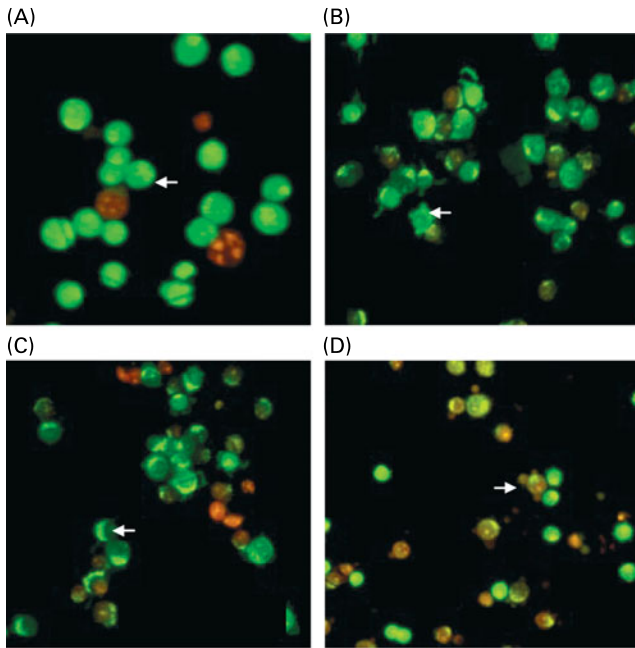
To determine whether  $\gamma$ -tocotrienol may induce apoptosis, the nuclear morphological alteration of SGC-7901 cells was further studied. SGC-7901 cells stained by acridine orange and EB showed some typical apoptotic features such as membrane blebbing, and chromatin condensation as half-moon at late apoptosis (Fig. 2). Apoptotic bodies were apparent and the nucleus showed counterstaining.

#### Effect of $\gamma$ -tocotrienol on DNA damage at different time points

The effect of  $\gamma$ -tocotrienol treatment on apoptosis in SGC-7901 cells was further assessed by the DNA fragmentation assay. The cells were treated with a 60  $\mu$ mol/l concentration of  $\gamma$ -tocotrienol at different time points. As shown in Fig. 3,  $\gamma$ -tocotrienol treatment resulted in the formation of DNA fragments in SGC-7901 cells at 48 h, and the DNA ladder bands were not visible at 72 and 96 h compared with the negative control group.

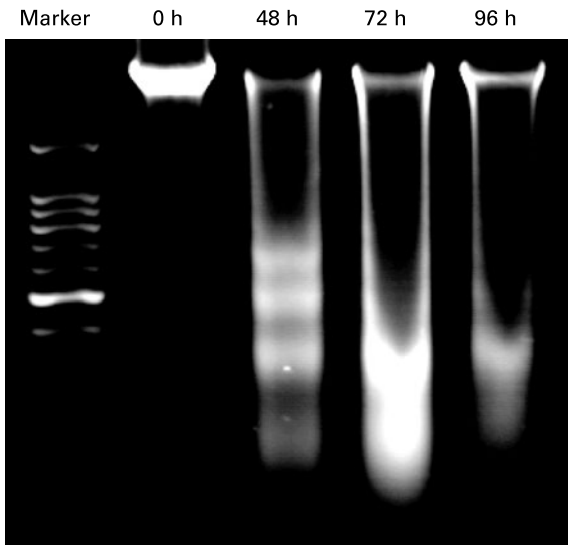
#### Apoptotic effect of $\gamma$ -tocotrienol treatment in SGC-7901 cells

In an effort to elucidate the mechanism of  $\gamma$ -tocotrienol-induced apoptosis in SGC-7901 cells, expression levels of apoptotic-regulation proteins such as Bcl-2, Bax and caspase-3 were evaluated. SGC-7901 cells were treated with different concentrations of  $\gamma$ -tocotrienol at 15, 30, 45 and 60  $\mu$ mol/l for 48 h. As shown in Fig. 4, the expression levels of Bax and Bcl-2 were not changed in comparison with the negative control group when the cells were treated with  $\gamma$ -tocotrienol at 15  $\mu$ mol/l for 48 h. There were significant differences in the expressions of Bax and Bcl-2 in SGC-7901 cells treated with  $\gamma$ -tocotrienol at the doses of 30 and 60  $\mu$ mol/l in comparison with the negative control group ( $P < 0.05$  and  $P < 0.01$ ).

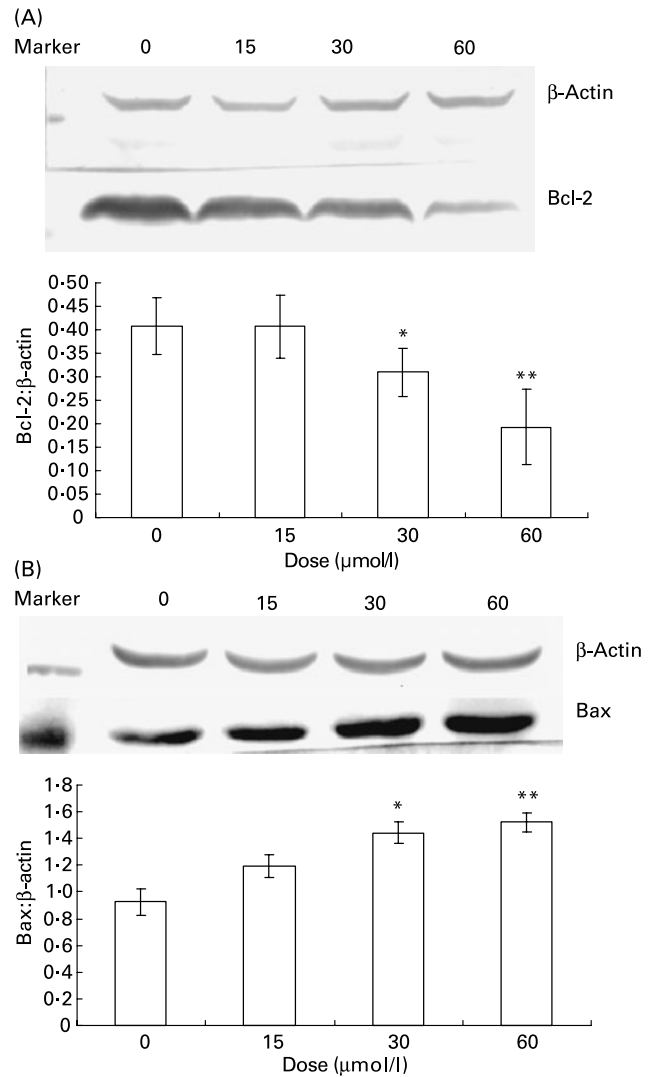


**Fig. 2.** The morphological changes of SGC-7901 cells were examined by acridine orange–ethidium bromide staining. (A) Negative control group; (B)  $\gamma$ -tocotrienol at 15  $\mu\text{mol/l}$ ; (C),  $\gamma$ -tocotrienol at 30  $\mu\text{mol/l}$ ; (D)  $\gamma$ -tocotrienol at 60  $\mu\text{mol/l}$ . (A) Karyotin of the control cells was uniform and showed green fluorescence. (B, C, D) Typical apoptotic features shown, such as pullulation and frothing-up of perinuclear membrane, and chromatin condensation as half-moon at late apoptosis. Apoptotic bodies were apparent and the nucleus showed counterstaining.

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. When the caspase-3 is activated, PARP is cleaved. Thus cleavage of PARP is used as an indicator of apoptosis. In order to obtain direct evidence showing the relationship of caspase



**Fig. 3.** The SGC-7901 cells were treated with  $\gamma$ -tocotrienol at the dose of 60  $\mu\text{mol/l}$  for 48, 72 and 96 h. DNA was isolated and subjected to 1.2% agarose gel electrophoresis, followed by visualisation of bands and photography.



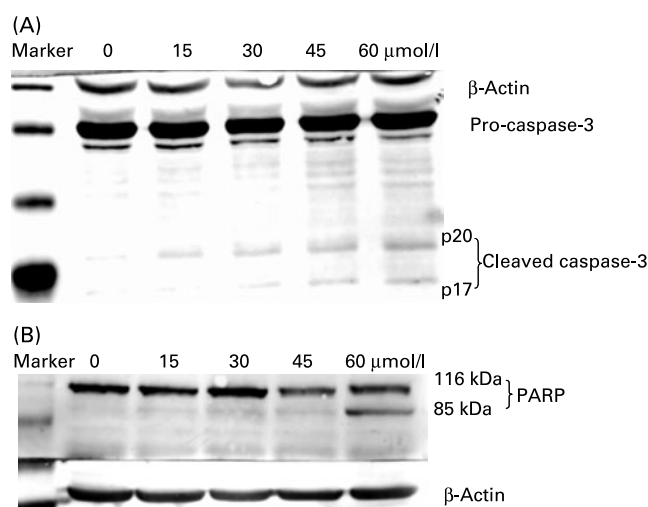
**Fig. 4.** The expression of  $\beta$ -actin and Bax (A) and  $\beta$ -actin and Bcl-2 (B) in SGC-7901 cells treated by  $\gamma$ -tocotrienol for 48 h. The cell lysates were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membrane and probed with anti- $\beta$ -actin, anti-Bax and anti-Bcl-2. Protein contents were normalised by probing the same membrane with anti- $\beta$ -actin. Values are means ( $n$  3), with standard deviations represented by vertical bars. Mean value was significantly different from that of the negative control group: \* $P$  < 0.05, \*\* $P$  < 0.01.

activation and apoptosis, procaspase-3 cleavage and PARP were examined in SGC-7901 cells after  $\gamma$ -tocotrienol treatment. As shown in Fig. 5,  $\gamma$ -tocotrienol induced the cleavage of 32 kDa procaspase-3 into its active 17 kDa form and cleavage of PARP appeared in SGC-7901 cells.

*Effect of  $\gamma$ -tocotrienol treatment on extracellular signal-regulated kinase–mitogen-activated protein kinase signalling transduction pathway*

Activation of the MAPK signalling cascade, which regulates cell growth and differentiation, is an important signalling transduction pathway in SGC-7901 cells. The effects of  $\gamma$ -tocotrienol on the ERK–MAPK signalling cascade were evaluated in SGC-7901 cells. The expression levels of total





**Fig. 5.** The expression of caspase-3 (A), poly (ADP-ribose) polymerase (PARP) (B) and  $\beta$ -actin (A, B) in SGC-7901 cells treated with  $\gamma$ -tocotrienol for 48 h. The cell lysates were separated on 8 and 12% SDS-PAGE gel, transferred to nitrocellulose membrane and probed with anti- $\beta$ -actin, anti-caspase-3 and anti-PARP antibodies. Protein contents were normalised by probing the same membrane with anti- $\beta$ -actin.

ERK1/2 and phosphorylated ERK1/2 (p-ERK1/2) proteins in SGC-7901 cells are shown in Fig. 6. There was no change in the expression of total ERK1/2 protein in SGC-7901 cells treated with  $\gamma$ -tocotrienol at doses of 15, 30, 45 and 60  $\mu\text{mol/l}$  for 48 h. There were significant differences in the expression of p-ERK1/2 and Raf-1 in SGC-7901 cells treated with  $\gamma$ -tocotrienol compared with the negative control group ( $P < 0.01$ ). A dose response was observed.

There was no change in the expression of phosphorylated p38 and JNK proteins in SGC-7901 cells treated with  $\gamma$ -tocotrienol at doses of 15, 30, 45 and 60  $\mu\text{mol/l}$  for 48 h (Fig. 7).

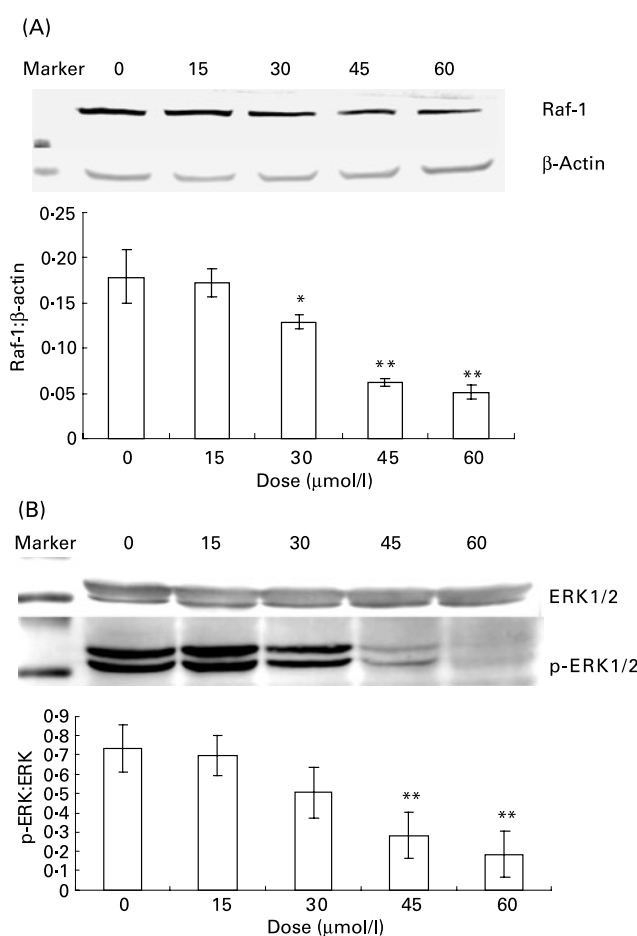
#### Effect of $\gamma$ -tocotrienol treatment on c-Myc

The proto-oncogene c-Myc is over-expressed in a wide range of human cancers, and is a downstream target of the ERK–MAPK signalling transduction pathway, which promotes c-Myc stability by enhancing c-Myc expression and activity. There was significant difference in the expression of c-Myc in SGC-7901 cells induced by different concentrations of  $\gamma$ -tocotrienol in comparison with the negative control group ( $P < 0.05$ ) in a dosage-independent manner (Fig. 8).

#### Discussion

Epidemiological studies have shown that the consumption of vegetables, fruits and grains is associated with a low risk of cancer<sup>(27)</sup>. Many natural dietary phytochemicals found in fruits, vegetables, spices and tea have been shown to be protective against cancer in various animal models<sup>(28,29)</sup>.

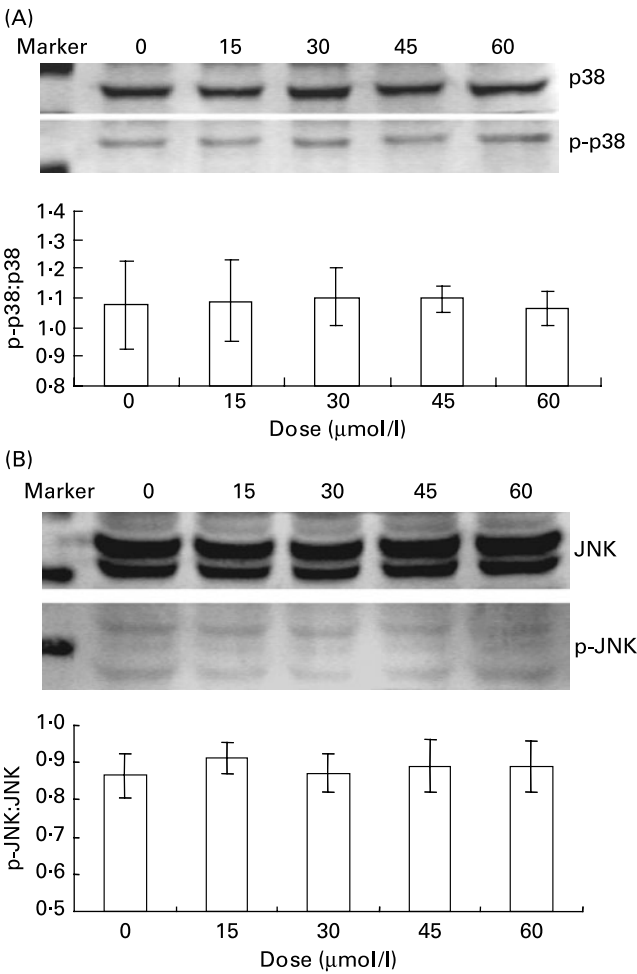
A study from eight adult healthy male volunteers showed that maximum concentrations of  $\gamma$ -tocotrienol were 5.20 (SD 1.67) and 1.41 (SD 0.70)  $\mu\text{mol/l}$  in the plasma of volunteers with or without food, respectively, when they were orally administered 300 mg of mixed tocotrienols (containing 166.4 mg  $\gamma$ -tocotrienol)<sup>(30)</sup>. Tocotrienols have been shown to possess



**Fig. 6.** The expression of Raf-1 and  $\beta$ -actin (A) and extracellular signal-regulated kinase (ERK)1/2 and p-ERK1/2 (B) in SGC-7901 cells treated with different concentrations of  $\gamma$ -tocotrienol for 48 h. The cell lysates were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membrane and probed with anti- $\beta$ -actin, anti-Raf-1, anti-p-ERK1/2 and anti-ERK1/2 antibodies. Protein contents were normalised by probing the same membrane with anti- $\beta$ -actin, or with anti-ERK1/2. Values are means ( $n$  3), with standard deviations represented by vertical bars. Mean value was significantly different from that of the negative control group: \* $P < 0.05$ , \*\* $P < 0.01$ .

biological activity besides antioxidant activity, such as anticarcinogenic properties, which are evidenced mainly by studies from *in vivo* and *in vitro* trials. The anti-carcinoma effects studied in different cancer cell lines demonstrated that tocotrienols repressed cell proliferation and induced apoptosis. Tocotrienols acted through different mechanisms: down regulation of the PI3P/PDK/AKT signalling transduction pathway, induction of procaspase-3 cleavage to p17 (cleaved caspase-3)<sup>(31)</sup> and stimulation of the expression of the anti-tumorigenic proteins Bax and p53<sup>(19)</sup> in several cancer cell lines.

However, the molecular mechanisms responsible for the anti-proliferative action of tocotrienols are not entirely understood, especially in digestive tract tumours. In our studies,  $\gamma$ -tocotrienol exerted a strong inhibitory effect in SGC-7901 cells in a dosage-dependent manner.  $\gamma$ -Tocotrienol treatment resulted in the formation of DNA fragments in SGC-7901 cells at 48 h, suggesting  $\gamma$ -tocotrienol inhibited SGC-7901 cells by induction of apoptosis. However, apoptosis was not observed when SGC-7901 cells were treated with  $\gamma$ -tocotrienol at the dose of 60  $\mu\text{mol/l}$  for 72 and 96 h. This indicates

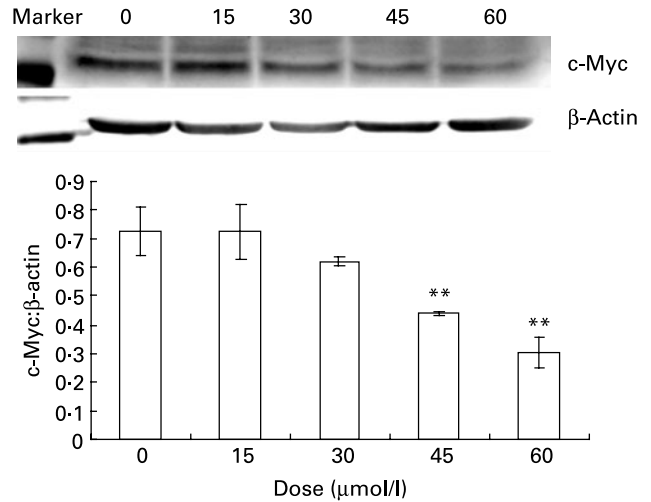


**Fig. 7.** The expressions of phosphorylated p38 (p-p38) (A) and c-Jun NH<sub>2</sub>-terminal kinase (JNK) (B) in SGC-7901 cells induced by  $\gamma$ -tocotrienol. Cells were treated by different concentrations of  $\gamma$ -tocotrienol for 48 h. The cell lysates were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membrane and probed with anti-p38 and anti-JNK antibodies. Values are means (*n* 3), with standard deviations represented by vertical bars. There were no significant differences.

that  $\gamma$ -tocotrienol induces apoptosis of SGC-7901 cells within 48 h (Fig. 3).

Active caspases play an important role in the induction of apoptosis. When caspase-3 was activated, PARP is cleaved late. Usually the cleavage of PARP was used as an indicator of apoptosis. In the present study, we found  $\gamma$ -tocotrienol at 60  $\mu$ .mol/l activated caspase-3 to produce cleaved caspase-3 (p17 and p20) fragments in SGC-7901 cells at 48 h. At the same time, the cleavage of PARP was also detected. The results indicate that  $\gamma$ -tocotrienol induced fragmentation of PARP as well as increased caspase-3 activity in SGC-7901 cells.

The Bcl-2 family proteins have been reported to regulate apoptosis by controlling the mitochondrial membrane permeability<sup>(32)</sup>. Bcl-2 suppresses apoptosis by stabilising the mitochondrial membrane, while Bax and Bid induce apoptosis by enhancing mitochondrial membrane permeability, which leads to the release of cytochrome c from mitochondria<sup>(33)</sup>.  $\gamma$ -Tocotrienol up regulated the expression of Bax and down regulated the expression of Bcl-2 in SGC-7901 cells. Similarly, alteration of the Bax : Bcl-2 ratio in favour of apoptosis



**Fig. 8.** The expression of c-Myc in SGC-7901 cells induced by  $\gamma$ -tocotrienol. Cells were treated by different concentrations of  $\gamma$ -tocotrienol for 48 h. The cell lysates were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membrane and probed with anti- $\beta$ -actin and anti-c-Myc antibodies. Values are means (*n* 3), with standard deviations represented by vertical bars. \*\* Mean value was significantly different from that of the negative control group ( $P < 0.01$ ).

has been reported in human colon carcinoma RKO cells treated with the tocotrienol-rich fraction of palm oil<sup>(19)</sup>. These results suggest that Bcl-2 and Bax participated in the regulation of apoptosis induction in SGC-7901 cells by  $\gamma$ -tocotrienol.

Proliferation of cancer cells is often associated with ERK activation, whereas proliferation inhibition may probably be induced through down regulation of the ERK1/2 signalling pathway<sup>(34,35)</sup>. Pharmacological inhibitors of the Raf/MEK/ERK pathway have been proposed as anticancer drugs<sup>(36,37)</sup>. At present, chemopreventors in food, such as conjugated linoleic acid<sup>(38)</sup> and  $\beta$ -ionone<sup>(39,40)</sup>, were found to halt the Raf/MEK/ERK signalling pathway and induce apoptosis<sup>(41)</sup>. The tocotrienol-rich fraction of palm oil suppressed pre-neoplastic mammary epithelial cell proliferation via inhibiting the expression of p-ERK<sup>(34)</sup>. In the present study,  $\gamma$ -tocotrienol down regulated the expression of Raf-1, down regulated the phosphorylation of ERK1/2 and caused the attenuated expression of c-Myc, a downstream target protein of the signalling pathway. Thus we presume the induced apoptosis of SGC-7901 cells by  $\gamma$ -tocotrienol might be related to the deregulation of Raf-1, triggering a cascade of reactions. The c-Myc oncogene is a downstream target gene of the MAPK pathway and is among the most commonly overexpressed genes in human cancer. Targeting of c-Myc by means of the MEK/ERK inhibitor can be tested as a promising strategy in anticancer therapy<sup>(26)</sup>. Its down regulation could reduce cell cycle progression and influence apoptosis. Repression of a pathway ending in c-Myc activation could represent one mechanism by which  $\gamma$ -tocotrienol exerts its biological activities.

JNK (also known as stress-activated protein kinases) are ubiquitously expressed. The JNK stress pathways participate in many different intracellular signalling pathways that control a spectrum of cellular processes, including cell growth, differentiation, transformation and apoptosis<sup>(23,42-44)</sup>. p38-MAPK signalling pathways are involved in a variety of cellular

responses, and the outcomes of cellular response are varied and complicated. Similar to JNK pathways, the involvement of p38-MAPK in apoptosis is also diverse. It has been shown that p38-MAPK signalling promotes cell death<sup>(45,46)</sup>, whereas it has also been shown that p38-MAPK cascades enhance survival<sup>(47)</sup>, cell growth<sup>(48)</sup>, and differentiation<sup>(49)</sup>. Our data showed that various concentrations of  $\gamma$ -tocotrienol did not affect JNK and p38 activity in SGC-7901 at 48 h. At present, there are no data to show whether or not tocotrienols affected JNK and/or p38 activity in other carcinoma cell lines. The results with SGC-7901 cells support the notion that JNK and p38 activity may not be necessary for  $\gamma$ -tocotrienol-induced apoptosis.

In summary,  $\gamma$ -tocotrienol induced apoptotic effects of human gastric adenocarcinoma cells via up or down regulation of the expression of Bcl-2 family proteins. The results also showed that  $\gamma$ -tocotrienol affected the Raf-ERK signalling transduction pathway. Thus the ERK signalling transduction pathway may be targeted by  $\gamma$ -tocotrienol and be related to apoptosis of SGC-7901 cells. Given the known role of MAPK in signalling transduction and the regulated mechanism of  $\gamma$ -tocotrienol on cell survival and apoptosis, the present studies offer a critical contribution to elucidation of whether the inhibition of MAPK signalling may represent the molecular basis underlying the preventive effects of  $\gamma$ -tocotrienol in human gastric cancer.

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