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Inhibitory effects of γ-tocotrienol on invasion and metastasis of human gastric adenocarcinoma SGC-7901 cells☆,☆☆

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Abstract

Natural vitamin E is a mixture of two classes of compounds, tocopherols and tocotrienols. Recent research has revealed that tocotrienols, especially γ-tocotrienol, exhibit not only the same antioxidant ability as tocopherols, but also remarkable anticancer capacity in cancer cell lines. In this study, the invasion and metastatic capacities of gastric adenocarcinoma SGC-7901 cells and the correlation with antimetastasis mechanisms induced by γ-tocotrienol were explored. The results showed the inhibitory effects of γ-tocotrienol at doses of 15, 30, 45 and 60 μmol/L for 48 h on cell migration and cell matrigel invasion; activities of matrix metalloproteinase (MMPs) increased in SGC-7901 cells when compared to the control group ($P<.05$ or $P<.01$). An increasing trend in the chemotactic responses to fibronectin (FN) in SGC-7901 cells was found in the γ-tocotrienol treatments. SGC-7901 cell attachment decreased in the γ-tocotrienol-treated groups in comparison with the control group ($P<.01$). The mRNA expressions of MMP-2 and MMP-9 showed that γ-tocotrienol significantly reduced the matrigel invasion capability through down-regulation of the mRNA expressions of MMP-2 and MMP-9 ($P<.01$), and up-regulation of tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2 in SGC-7901 cells by treatment with γ-tocotrienol for 48 h ($P<.05$). γ-Tocotrienol also significantly increased the mRNA expression of nm23-H1 in SGC-7901 cells ($P<.01$). These findings suggest a potential mechanism of γ-tocotrienol-mediated antitumor metastasis activity and indicate the role of vitamin E as potential chemopreventative agents against gastric cancer.

Keywords: γ-Tocotrienol; Invasion; Metastasis; SGC-7901 cells; MMPs

1. Introduction

Gastric cancer is a serous public health problem although its incidence rate declined in the past several decades [1]. According to the recent estimates of global cancer incidence, gastric cancer is the fourth most common cancer with 934,000 new cases per year. Even though incidence and mortality due to gastric cancer are declining worldwide, the rate of recurrence metastasis after surgery for advanced gastric cancer is as high as 44.2% [2]. The wide spatial and temporal variations in incidence and mortality suggest an important role of environmental factors in gastric cancer risk. The consumption of fruits and vegetables has been suggested to be inversely associated with the risk of human gastric cancer [3–5]. The various carotenoids and antioxidant vitamins found in these foods may contribute to this effect [6].

Chemoprevention, as a therapy method, is applied extensively to deal with preventive cancers especially in preventing metastasis, which proves to be capable of avoiding one-third of cancer deaths [7,8]. Natural vitamin E is the name given to two classes of compounds, tocopherols and tocotrienols. In contrast to considerable studies on tocopherol, little is known about tocotrienol. Tocopherols are components of nuts and common vegetable oils, and tocotrienols are primarily derived from oat, barley, rye, rice bran and palm oil (Table 1) [9–11]. Nevertheless, previous studies showed that tocotrienols could exert more significant effects on neuroprotection, anticancer activity and cholesterol lowering than tocopherols do [12–14]. Tocotrienol, or palm oil which contains tocotrienol fractions, is reported to lead to the induction of apoptosis in many different kinds of malignant carcinoma cells, such as breast [15–19], colon [20,21], prostate [22] and hepatoma carcinoma [23] cells, by various inhibitory mechanisms.

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Table 1
Vitamin E content (mg/100 g product) of selected oils

Source	Tocotrienols (mg)				Tocopherol α
	α	β	γ	δ	
Palm oil	14.6	3.2	29.7	8.0	15.0
Rice bran	23.6	NA	34.9	–	32.4
Wheat germ	2.6	18.1	NA	NA	133.0
Coconut	0.5	0.1	–	–	0.5
Soybean	0.2	0.1	0	0	7.5
Olive	0	0	0	0	11.9

NA: not analyzed.

In our previous study, γ-tocotrienol induced apoptosis in SGC-7901 cell apoptosis through mitochondria-dependent apoptosis pathway and was associated with down-regulation of the Raf-ERK signaling pathway [24,25]. However, γ-tocotrienol's capacity to inhibit the invasion and metastasis of malignant carcinoma is still unknown. Thus, the objectives of the present study were to determine whether γ-tocotrienol inhibited the invasion and metastasis in human gastric adenocarcinoma SGC-7901 cells and to further explain its possible mechanism as a chemoprevention agent.

2. Materials and methods

2.1. Chemicals and reagents

Purified γ-tocotrienol (purity 99%) was purchased from Davos (Singapore), dissolved in absolute alcohol and stored at −20°C. Fibronectin (FN) and Matrigel were purchased from Sigma (St. Louis, MO, USA). Laminin (LN) was purchased from BD Bioscience. 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.

2.2. Cell culture

Human gastric adenocarcinoma SGC-7901 cell line was obtained from the Cancer Institute of the Chinese Academy of Medical Science (Beijing, China). SGC-7901 cells were cultured in RPMI-1640 containing 10% fetal bovine serum (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD, USA), 2 mmol/L L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. The cells were passaged with 0.02% EDTA (Sigma).

2.3. Cell growth and viability assay

The influence of γ-tocotrienol on the cell viability was determined according to MTT assay. Briefly, cells were seeded in a 24-well plate at 1.0×10^5 cells/well, which, after 24 h of incubation, were transferred to other culture medium containing 0, 15, 30, 45 and 60 μmol/L γ-tocotrienol. For each concentration four replicates were employed. The cells were separately incubated for 0, 24, 48 and 72 h. At different intervals, one plate was tested. Fifty microliters of 5 mg/ml MTT solution in phosphate-buffered saline (PBS) was added to each well, with cells continuing to incubate for 4 h. After careful removal of the medium, 500 μl of dimethyl sulfoxide (DMSO) was added to each well, and the plate was then shaken until the crystals were solubilized. The absorbance was then measured at 570 nm (Elx800 Universal Microplate Reader, Bio-Tek Instruments).

The inhibitory rate was calculated using the following equation:

$$\text{Inhibitory rate } (\%) = (\text{OD}_{\text{control}} - \text{OD}_{\text{treatment}} \times 100\%$$

2.4. Scratch-wound healing assay

The effect of γ-tocotrienol on migration in SGC-7901 cells was studied through modification of the wound closure assay described by Li et al. [26]. Cells were seeded in 70-mm culture dishes and grown to 80% confluence. The cells were then treated with culture medium containing different concentrations (0, 15, 30, 45 and 60 μmol/L) of γ-tocotrienol and incubated for 24 h. A wound track (approximately 5 mm in size) was scored in each dish with a plastic scraper. After being washed with PBS, the cells continued to incubate for 48 h with serum-free medium. After being washed again with PBS, the number of cells having crossed the wound margin was counted in eight different areas, which were chosen randomly under light microscope. Experiments were carried out in triplicate.

2.5. Chemotaxis motion for FN

The trans-well cell culture chambers (Corning Costar 3422, Corning, Cambridge, MA, USA) were used for selection of cells responding chemotactically or failing to respond to FN as described by Li et al. [27]. The trans-well microporous membrane was coated with 10 μg of FN (F0895, Sigma) on the lower surface and kept at 4°C overnight before use. RPMI-1640 medium supplemented with 0.1% BSA was placed in the lower chamber. SGC-7901 cells were then treated with 0, 15, 30, 45 and 60 μmol/L γ-tocotrienol for 48 h and split (5×10^4 vital cells per well) in the upper chamber in 0.1 ml of serum-free medium. After 4 h of incubation at 37°C, cells invaded the lower surface of the filter. Cells on the upper surface of the filter were removed with cotton swabs, and those on the lower side were fixed in methanol for 15 min and then stained with hematoxylin and eosin. The stained cells were observed through a phase-contrast microscope (Nikon Company, Japan) and counted from more than 10 fields of view at ×200 magnification. The mobility of cancer cells was expressed as the mean number of cells that had invaded to the lower side of the filter. Values for motion were determined by calculating the average number of migrated cells per square millimeter over three fields and expressed as an average of three determinations.

2.6. Matrigel invasion assays

The invasion assay was also carried out using trans-well cell culture chambers as described by Li et al. [26] and Chen et al. [28]. Briefly, the microporous membrane was coated with 5 μg matrigel (E1270, Sigma) on the upper surface and 5 μg FN on the lower surface to simulate the basal membrane, and kept at 4°C overnight before use. SGC-7901 cells were treated with 0, 15, 30, 45 and 60 μmol/L γ-tocotrienol for 48 h. The cells were then harvested with 0.02% EDTA and suspended in RPMI-1640 serum-free medium. Cell suspension (5×10^4 vital cells per well) was added to the upper compartment of the chamber, and 0.1% BSA-RPMI-1640 culture media was placed in the lower compartment of the chemotaxis chamber as a chemoattractant source. After incubation at 37°C for 4 h, the succeeding processes were the same as those described in Section 2.5.

2.7. Attachment assay

Cell adhesion experiments were performed as described by Li et al. [27] and Chen et al. [28]. A 96-well microplate was coated with 2 μg matrigel or FN or LN (BD Bioscience) per well and kept at 4°C overnight before use. Each well was added with 20 μl 2% BSA and then washed with PBS after 1 h of incubation at 37°C. SGC-7901 cells were preincubated with 0, 15, 30, 45 and 60 μmol/L γ-tocotrienol for 48 h. SGC-7901 cells suspended in serum-free medium were then added to each well (5.0×10^5 vital cells/ml, 50 μl/well). After 1 h of incubation at 37°C, the plates were washed three times using PBS in order to remove unattached cells. The remaining adherent cells were reacted with 5 mg/ml MTT solution medium at 37°C for 4 h, then crystals were solubilized with 100 μl of DMSO. Optical density was measured at 570 nm. Results were expressed as the percentage of total cells assuming that the adhesion of cells in the control was 100%. All the experiments were carried out in triplicate, and three independent assays were conducted.

2.8. Zymographic assay for metalloproteinase

Zymography was used to determine the effect of γ-tocotrienol on matrix metalloproteinase-9 (MMP-9) (92 kDa) and MMP-2 (72 kDa) activity after the SGC-7901 cells were treated with serum-free medium with 0, 15, 30, 45 and 60 μmol/L γ-tocotrienol for 48 h. After incubation, the supernatant was collected for zymography analysis as described by Chen et al. [28] and Cho et al. [29]. Briefly, the collected cell culture medium was mixed with 4× SDS sample buffer (without β-mercaptoethanol) for electrophoresis. The sample was loaded onto the SDS-PAGE in 10% polyacrylamide gel containing 0.1% gelatin and electrophoresed. After electrophoresis, the gels were washed three times with washing buffer I for 30 min at room temperature to remove SDS. The gels were then washed three times with washing buffer II for 20 min at room temperature. The buffer was replaced with zymography development buffer and incubation continued overnight on a rotary shaker at 37°C. Gels were stained with 0.25% Coomassie brilliant blue R250. Proteolytic activity was visible in the form of clear bands against the blue background of the stained gelatin. The amplified products were visualized under visible light. The strength of each band was analyzed using the ChemiImager 5500 digital system (Flour Chem, Alpha Innotech Corp., USA).Three independent experiments were performed in this study.

2.9. Reverse transcription-polymerase chain reaction

SGC-7901 cells were incubated with various concentrations of γ-tocotrienol (0, 15, 30, 45 and 60 μmol/L) for 48 h. Total RNA was isolated from cancer cells using total RNA extraction kit (BSC52S2, Bloer Technology, China). For reverse transcription reaction, cDNA was synthesized from total RNA according to the manufacturer's instructions (DRR019A, TaKaRa Biotechnology Co., Ltd., Japan). The polymerase chain reaction (PCR) primer sequences are described in Table 2.

Table 2
The sequence of primers, annealing temperature and number of cycle

Primer		Sequence	Length (bp)	Annealing temperature (°C)	Number of cycle
MMP-2	Sense	5′ tcttcaaggaccggttcatttg 3′	750	59	35
	Antisense	5′ gatgcttccaaacttcacgctc 3′			
MMP-9	Sense	5′ accctgccagtttccattcatc 3′	500	58	35
	Antisense	5′ cggcactgaggaatgatctaag 3′			
TIMP-1	Sense	5′ cttccacaggtcccacaacc 3′	304	58	35
	Antisense	5′ cagccctggctcccgaggc 3′			
TIMP-2	Sense	5′ gagcaccacccagaagaagag 3′	339	58	35
	Antisense	5′ ccaggaagggatgtcagagc 3′			
nm23-H1	Sense	5′ atggccaactgtgagcgta 3′	468	58	35
	Antisense	5′ gccctcctgtcattcatag 3′			
β-actin	Sense	5′ tcatgccatcctgcgtctg 3′	256	50	30
	Antisense	5′ gcatcggaaccgctcat 3′			

The conditions for all PCR amplifications were as follows: predenaturation for 2 min at 94°C; 35 PCR cycles (β-actin for 30 cycles); denaturation at 94°C for 30 s; annealing temperature for 30 s and extension at 72°C for 60 s in a PTC-0220 thermocycler (Bio-RAD, USA). PCR products were isolated electrophoretically on a 1.2% (w/v) agarose gel with 0.5 nmol/L EB. The expression of targeted genes in each sample was normalized to β-actin expression. The amplified products were visualized under ultraviolet light. The strength of each band was analyzed using the ChemiImager TM 5500 digital system. All samples were analyzed four times.

2.10. Statistical analysis

Data were expressed as mean±S.D. Each value is the mean of at least three repetitive experiments in each group. Differences were analyzed for significance using the one-way ANOVA test with the Bonferroni post hoc multiple comparisons, used to assess the difference between independent groups. Statistical significance was set at $P<.05$ and $P<.01$, and all P values were unadjusted for multiple comparisons.

3. Results

3.1. γ-Tocotrienol inhibited SGC-7901 cell growth

The cell viability was determined using the MTT assay. SGC-7901 cell viability was significantly inhibited in a time- and dose-dependent response by γ-tocotrienol (Fig. 1). Compared to the control group the cell inhibitory frequency was 2.15±3.8, 12.36±5.9, 30.64±1.6, 54.84±7.1 at 24 h; 2.7±3.0, 21.62±6.2, 57.66±6.6, 66.21±2.2 at 48 h; and 11.36±3.4, 38.46±3.6, 71.06±1.9 and 74.73±2.0 at 72 h, for 15, 30, 45 and 60 μmol/L of γ-tocotrienol, respectively. The median effective concentrations (EC_{50}) of γ-tocotrienol for inhibition of SGC-7901 cell viability were 65.68±5.21, 46.57±3.73 and 28.70±6.92 μmol/L at 24, 48 and 72 h, respectively.

3.2. γ-Tocotrienol inhibited SGC-7901 cell migration

An *in vitro* "scratch-wound healing" assay was performed to determine whether γ-tocotrienol affects SGC-7901 cell migration. Representative experiments are illustrated in Fig. 2. The number of migrated cells was reduced by 3.8±10.8% in SGC-7901 cells treated with 15 μmol/L γ-tocotrienol, by 19.8±4.8% with 30 μmol/L, by 41.8 ±10.2% with 45 μmol/L and by 60.9±20.53% with 60 μmol/L for 24 h. The migration of SGC-7901 cell was significantly decreased at doses of 30, 45 and 60 μmol/L γ-tocotrienol in a dose-dependent manner ($P<.05$).

3.3. Effect of γ-tocotrienol on cell chemotaxis motion for FN in SGC-7901 cells

The effect of γ-tocotrienol on the chemotaxis motion for FN in SGC-7901 cells was determined by the trans-well cell culture chambers coated with 10 μg FN on the lower surface. The number of migration cells was 42.5±3.1, 50.4±3.9, 55.0±4.4, 60.5±4.2 and 62.0±3.3 after treatment with 0, 15, 30, 45 and 60 μmol/L γ-tocotrienol for 48 h, respectively (Fig. 3). An increasing trend of migration capability was observed in the γ-tocotrienol treatments when compared to the control group.

3.4. γ-Tocotrienol inhibited SGC-7901 cell invasion into matrigel

In vitro assay of invasion was employed to determine whether γ-tocotrienol affected SGC-7901 cell invasion into matrigel. The number of invasion cells was 80.1±4.6, 74.9±3.1, 55.4±2.8, 39.6±3.3 and 23.2±2.9 at doses of 0, 15, 30, 45 and 60 μmol/L γ-tocotrienol for 48 h, respectively (Fig. 4). γ-Tocotrienol significantly inhibited the viability of matrigel invasion at doses of 30, 45 and 60 μmol/L in comparison with what the control group exhibited ($P<.01$). A dose response was observed.

3.5. γ-Tocotrienol decreased SGC-7901 cells to attach matrigel, FN and LN

To determine whether γ-tocotrienol inhibited SGC-7901 attachment to matrigel, FN and LN, cell attachment assay was performed. Results showed that the adhesive capabilities of matrigel, FN or LN were significantly decreased at doses of 15, 30, 45 and 60 μmol/L γ-tocotrienol when compared to the control after treatment for 48 h ($P<.01$) (Fig. 5).

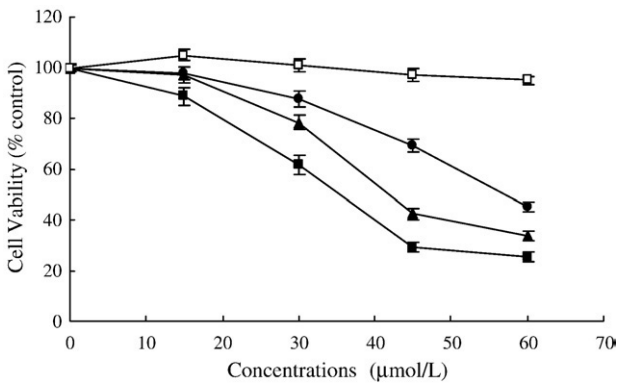

Fig. 1. The effect of γ-tocotrienol on the viability of SGC-7901 cells. Effect of γ-tocotrienol on the viability of SGC-7901 cells was determined by MTT assay. SGC-7901 cells were seeded in 24-well plates (1×10^5 cells/well) and treated with different concentrations of γ-tocotrienol separately for 24 (●), 48 (▲) and 72 h (■) (□, ethanol control for 72 h). Each concentration was repeated in four wells ($n=4$). The cell viability values were expressed relative to the negative control group. The result exhibited above is the mean of at least three independent experiments.

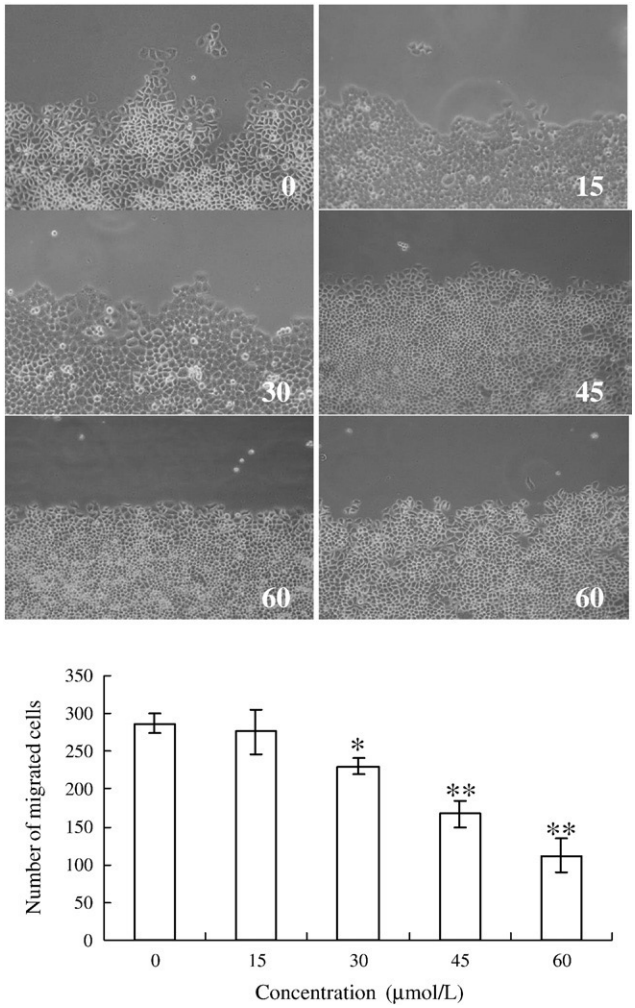

Fig. 2. The effect of γ-tocotrienol on the migration of SGC-7901 cells. Migration of SGC-7901 cells was measured by scratch-wound healing assay. SGC-7901 cells were treated with 0, 15, 30, 45 or 60 μmol/L γ-tocotrienol for 24 h. A wound track (approximately 5 mm in size) was scored in each dish with a plastic scraper, after incubation for 48 h with serum-free medium. Data represent the mean±S.D. *P<.05, **P<.01, compared to the control group; the experiments were repeated three times (n=3).

3.6. γ-Tocotrienol inhibited cell gelatinase activities of SGC-7901 cells

The type IV collagenase activity (72 kDa for MMP-2 and 92 kDa for MMP-9) in supernatants from SGC-7901 was treated with different concentrations of γ-tocotrienol for 48 h by zymography assay. γ-Tocotrienol significantly decreased the MMP-2 activity in SGC-7901 cells treated with 30, 45 and 60 μmol/L when compared to the control group (P<.05 or P<.01) (Fig. 6). The activities of MMP-9 also showed a significant decrease at doses of 45 and 60 μmol/L in comparison with the control group (P<.05) (Fig. 6). Both dose responses were observed.

3.7. Reverse transcription-PCR

The expression of measured genes in each sample was standardized to β-actin. All samples were analyzed in quadruplicate. The expression of mRNA levels of MMP-2 and MMP-9 in SGC-7901 cells treated with γ-tocotrienol for 48 h is shown in Fig. 7. The results illustrated that the expression of MMP-2 and MMP-9 decreases in a dose-dependent manner with the increasing concentrations of γ-tocotrienol. γ-Tocotrienol significantly decreased the expression of MMP-2 at doses of 30, 45 and 60 μmol/L and the expression of MMP-9 at doses of 45 and 60 μmol/L in SGC-7901 cells when compared to the control group in a dose-dependent manner (P<.01).

The mRNA expression levels of the tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2 in SGC-7901 cells treated with different concentrations of γ-tocotrienol for 48 h are shown in Fig. 8. The expression of TIMP-1 and TIMP-2 increased in a dose-dependent manner with the increasing concentrations of γ-tocotrienol. There was a significant increase in the expression of TIMP-1 and TIMP-2 in SGC-7901 cells treated with 30, 45 and 60 μmol/L γ-tocotrienol in comparison with the control group (P<.05 or P<.01). A dose response was observed.

The mRNA expression of nm23-H1 in SGC-7901 cells treated with γ-tocotrienol for 48 h is presented in Fig. 9. γ-Tocotrienol also showed a significantly increased expression of nm23-H1 in SGC-7901 cells in a dose-dependent manner (P<.01).

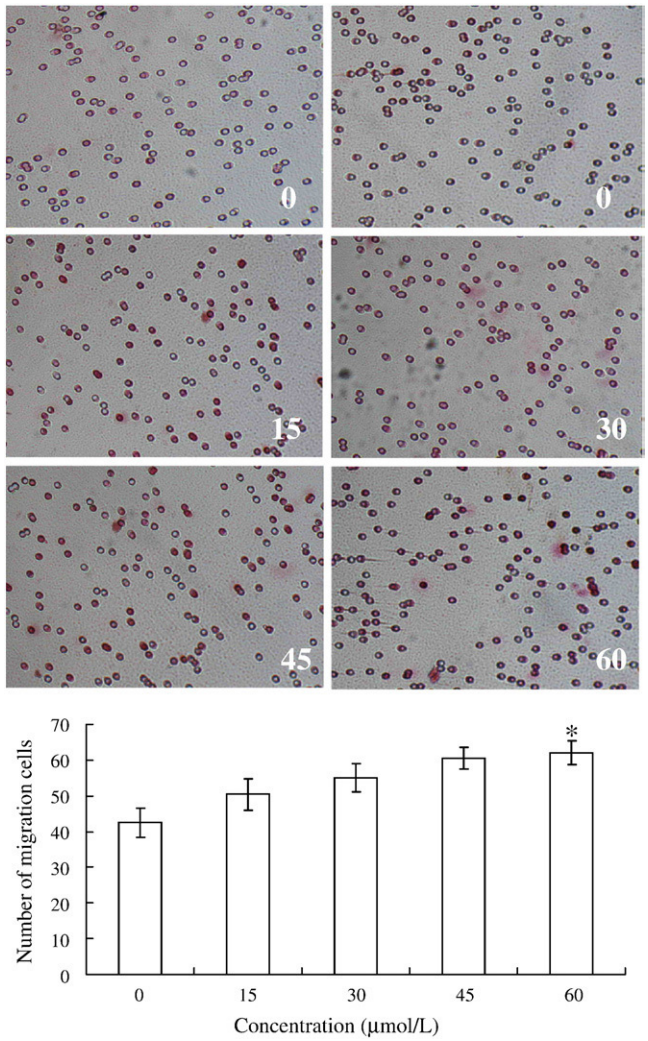

Fig. 3. The effect of γ-tocotrienol on cell chemotaxis motion of SGC-7901 cells. The trans-well cell culture chambers coated with 10 μg FN on the lower surface were used for calculation of cell chemotaxis motion. 0.1% BSA RPMI-1640 medium was placed in the lower chamber. SGC-7901 cells (5×10^4 vital cells per well) treated with 0, 15, 30, 45 or 60 μmol/L γ-tocotrienol for 48 h were placed in the upper chamber. After 4 h of incubation, cotton swabs were used to remove the cells on the upper surface of the filter. Cells on the lower side were stained with hematoxylin and eosin. The number of cells from at least 10 fields of view was randomly counted using a microscope (×200). The experiment was setup in independent triplicates (n=3). *P<.05, compared to the control group.

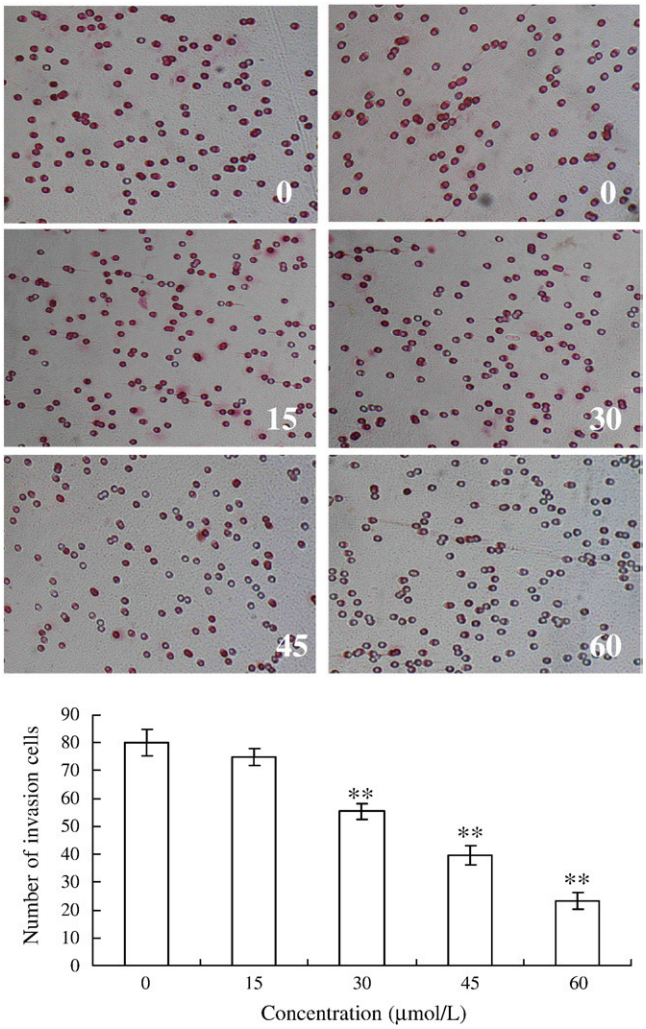

Fig. 4. The effect of γ-tocotrienol on matrigel invasion of SGC-7901 cells. The transwell microporous membrane was coated with 5 μg matrigel on the upper surface and 5 μg FN on the other surface to simulate the basal membrane, at 4°C overnight before use. 0.1% BSA RPMI-1640 medium was placed in the lower chamber. SGC-7901 cells (5×10^4 vital cells per well) treated with different concentrations of γ-tocotrienol (0, 15, 30, 45 and 60 μmol/L) for 48 h were placed in the upper chamber. After 4 h of incubation, cotton swabs were used to remove the cells on the upper surface of the filter. Cells on the lower side were stained with hematoxylin and eosin. The number of cells at least 10 fields of view was randomly counted using a microscope (× 200). The experiment was setup in independent triplicates ($n=3$). $^{**}P<.01$, compared to the control group.

4. Discussion

The intake of some fruits and vegetables has been suggested to be inversely associated with the risk of gastric cancer [3,4,30]. Several case-control studies have shown strong inverse associations between gastric cancer risk and higher consumption of some of these nutrients [31–35]. Results from prospective dietary studies are conflicting and show either a strong protection [5,36] or no clear inverse associations between the dietary intake of carotenoids and gastric cancer risk [37,38]. Tocotrienols have been shown to possess biological activity besides antioxidant activity, such as anticarcinogenic properties, which are evidenced mainly by studies from *in vivo* and *in vitro* trials. Recently, many results show that tocotrienol or palm oil consumption, which contains tocotrienol fractions, has led to the induction of apoptosis in many different kinds of malignant carcinoma cells. It is also attested by our research that γ-tocotrienol can exert a significant

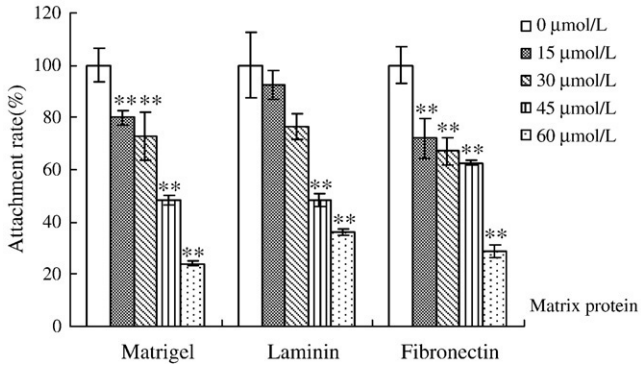

Fig. 5. The effect of γ-tocotrienol on the attachment of SGC-7901 cells. Matrigel, FN or LN (2 μg/well) was coated onto the surface of 96-well plates. Two percent BSA (20 μl/well) was incubated at 37°C for 1 h. SGC-7901 cells treated with different concentrations of γ-tocotrienol for 48 h were added to the wells (5.0×10^5 vital cells/ml, 50 μl/well) and incubated at 37°C for 1 h. Adherent cells were estimated by MTT. The experiment was setup in independent triplicates ($n=3$). $^{**}P<.01$, compared to the control group.

inhibitory effect on SGC-7901 cells in a dose-dependent manner. In our previous study, γ-tocotrienol induced human gastric adenocarcinoma SGC-7901 cell apoptosis through mitochondria-dependent apoptosis pathway and was associated with down-regulation of the Raf-ERK signaling pathway [24,25]. A study from eight adult healthy male volunteers showed that the maximum concentrations of γ-tocotrienol were 5.20±1.67 and 1.41±0.70 μmol/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 γ-tocotrienol). Tocotrienols have been shown to possess biological activity besides antioxidant activity, such as anticarcinogenic properties, which are evidenced mainly by studies from *in vivo* and *in vitro* trials [39]. The anticarcinoma effects studied in different cancer cell lines demonstrated that tocotrienols repressed cell proliferation and induced apoptosis. However, the activities of tocotrienol for the

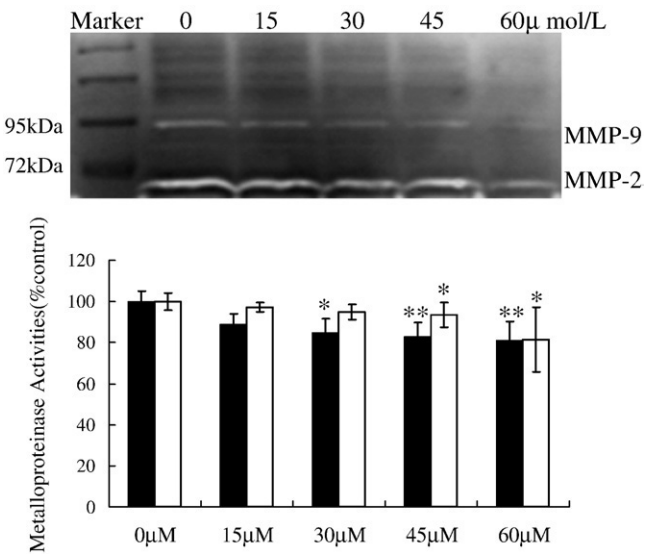

Fig. 6. The effect of γ-tocotrienol on cell gelatinase activities of SGC-7901 cells. Zymography was used to determine the effect of γ-tocotrienol on MMP-2 (■, 72 kDa) and MMP-9 (□, 92 kDa) activity after SGC-7901 cells were treated with various concentrations of γ-tocotrienol for 48 h. The supernatant was collected for the zymography assay. More details can be found in the Materials and methods section. The experiment was setup in independent triplicates ($n=3$). $^{*}P<.05$, $^{**}P<.01$, compared to the control group.

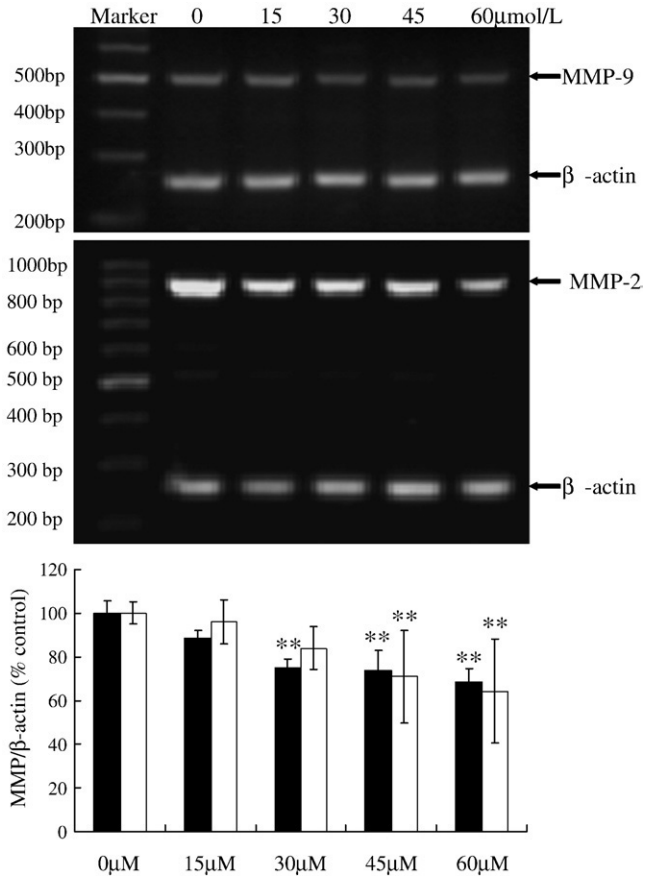

Fig. 7. The effect of γ-tocotrienol on the mRNA expression of MMP-2 and MMP-9 in SGC-7901 cells. Total RNA was isolated from SGC-7901 cells treated with various concentrations of γ-tocotrienol for 48 h. PCR primer sequences are shown in Table 2. The mRNA expression of MMP-2 (■) and MMP-9 (□) in SGC-7901 cells decreased with the increasing concentrations (0, 15, 30, 45 and 60 μmol/L) of γ-tocotrienol after incubation for 48 h. The experiment was setup in four independent replicates (n=4). $^{**}P$ <.01, compared to the control group.

invasion and metastasis in malignant carcinoma are not entirely understood. The effect of preventing invasion and metastasis on human gastric adenocarcinoma SGC-7901 cells by γ-tocotrienol was determined in this article.

Metastasis, the spread of cells from primary neoplasms to distant sites, as well as their succeeding growth in the new location, proves to be the most damaging aspect of cancer. As far as traditional theory is concerned, metastasis is treated as a series of sequential and interrelated steps, including dissociation of malignant cells in the primary tumor, local invasion, angiogenesis and intravasation of invading cells into the vasculature or lymphatic systems, survival in these channels, extravasations and proliferation at a distant site [40,41]. Metastatic spread of cancer is responsible for 90% of human cancer-related deaths and, as a result, is still one of the greatest impediments to cancer curing [42]. Understanding the mechanisms of the possible inhibitory effect of γ-tocotrienol on invasion and metastasis of gastric adenocarcinoma cells could provide us with a way to solve this problem, which proves to be meaningful given that gastric cancer is one of the most common malignancies in China as well as in other areas of the world.

Cancer aggressivity hinges mostly on tumor invasiveness, for which cell migration is a determinant manifestation. It is for this very reason that we center our primary attention on the effect of γ-tocotrienol on cell migration. γ-Tocotrienol contributes prominently to reduction in migration of cells that have been treated with the wound closure process.

During the metastatic cascade, tumor cells interact with various host cells as well as extracellular matrices and basement membrane components including LN, FN and type I collagen through certain adhesion molecules. Such adhesive interactions may lead to the enhancement of capabilities of survival, arrest or invasiveness of tumor cells and are one of the most important events in the metastatic process [43,44]. It is also revealed by previous research that prevention of tumor cell adhesion and migration is closely intertwined with inhibition of tumor cell invasion into the basement membrane [45,46]. Laminin, FN and type IV collagen are the principal components of extracellular matrix (ECM). We used the *in vitro* assays of ECM, FN and LN which can simulate the *in vivo* adhesive process. It shows that γ-tocotrienol could statistically significantly reduce the extent of cell adhesion to matrigel, FN or LN, compared to the control group. This result reveals that γ-tocotrienol can inhibit the invasion of SGC-7901 cells by reducing cell adherence to the basement membrane.

The prognosis of patients with cancer is influenced by tumor growth, local invasion and metastasis, and these processes are determined, in part, by the interaction of tumor cells with the ECM [47]. Most malignant cells fail to deposit ECM components such as FN, and, as a consequence, mobility of tumor cells is not limited by adhesion to the molecules within their microenvironment. Therefore, in this article, we studied the ability of cells to interact with and subsequently respond to ECM components. The response of chemotaxis to FN was shown to reflect the activation status of the FN

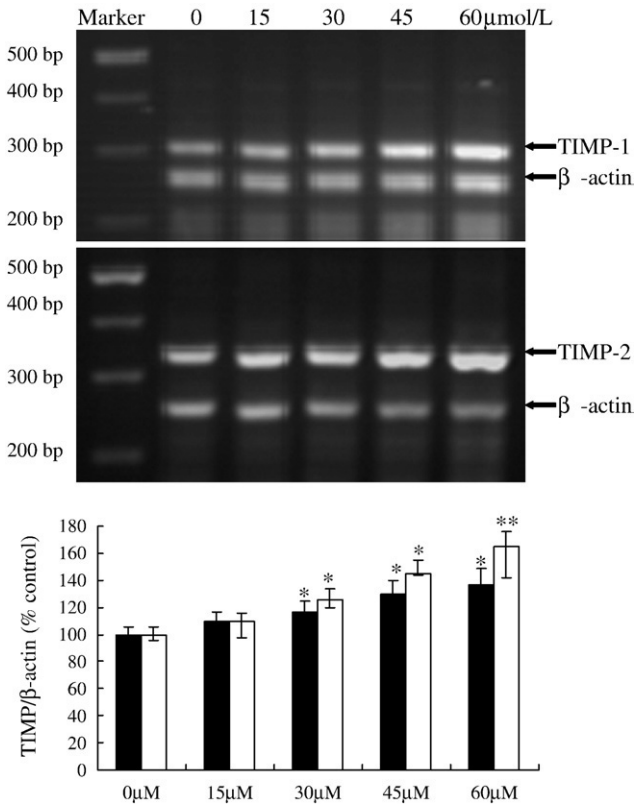

Fig. 8. The effect of γ-tocotrienol on the mRNA expression of TIMP-1 and TIMP-2 in SGC-7901 cells. Total RNA was isolated from SGC-7901 cells treated with various concentrations of γ-tocotrienol (0, 15, 30, 45 and 60 μmol/L) for 48 h. PCR primer sequences are shown in Table 2. The expression of TIMP-1 (■) and TIMP-2 (□) mRNA level in SGC-7901 cells increased with increasing concentrations (0, 15, 30, 45 and 60 μmol/L) of γ-tocotrienol after incubation for 48 h. The experiment was setup in four independent replicates (n=4). $^{*}P$<.05, $^{**}P$<.01, compared to the control group.

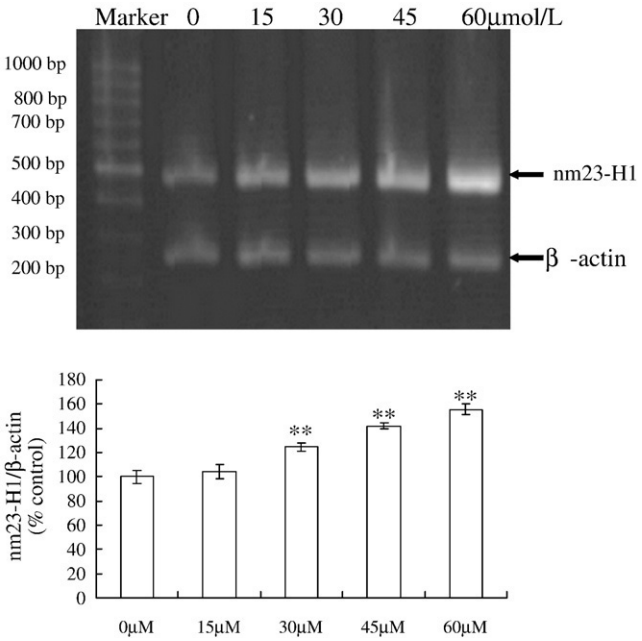

Fig. 9. The effect of γ-tocotrienol on the mRNA expression of nm23-H1 in SGC-7901 cells. Total RNA was isolated from SGC-7901 cells treated with various concentrations of γ-tocotrienol (0, 15, 30, 45 and 60 μmol/L) for 48 h. PCR primer sequences are shown in Table 1. The expression of nm23-H1 in SGC-7901 cells significantly increased in the γ-tocotrienol treatments after incubation for 48 h. The experiment was setup in four independent replicates (n=4). $^{**}P$<.01, compared to the control group.

receptors and to correlate inversely with their *in vivo* growth potential. Trans-wells were used for researching the cells responding chemotactically or failing to respond to FN. In the present study, SGC-7901 cells treated by γ-tocotrienol showed a higher potential to attach, but a lower chemotactic response, to FN when compared with the control group. The results showed that the weakly metastatic cell line showed a higher adhesiveness to FN and had more deposition of FN on the cell surface compared with the highly metastatic cell line [27]. The changes in matrigel invasive capability of SGC-7901 cells induced by γ-tocotrienol were determined. γ-Tocotrienol showed a tendency of attenuation in matrigel invasive ability in SGC-7901 cells by the trans-well microporous membrane coated with matrigel and FN to simulate the ECM (Fig. 4).

Matrix metalloproteinases are some of the most important components in the metastatic process, given their capacity to degrade ECM proteins [48]. MMPs are a family of zinc endopeptidases, which consists of at least 20 different members [49,50], and they play a pivotal role in various cellular metabolic processes. Among the previously reported human MMPs, MMP-2 (72 kDa) and MMP-9 (92kDa) are considered to be particularly ideal targets for anticancer drugs because both enzymes degrade gelatins — major components of the basement membrane. The expression of both enzymes is correlated with an aggressive, advanced invasive or metastatic tumor phenotype [51–53]. As γ-tocotrienol has shown to remarkably reduce matrigel invasion ability, zymographic assay was used to investigate the activities of MMP-2 and MMP-9 in SGC-7901 cells. The results showed that γ-tocotrienol inhibited the activation and reduced the mRNA level of MMP-2 in a dose-dependent manner. γ-Tocotrienol could also decrease the mRNA expression of MMP-9, but failed in the activation of MMP-9. This phenomenon requires further investigation.

Multiple reports from a variety of model systems have revealed that overexpression of TIMPs, especially TIMP-1 and TIMP-2, can inhibit tumor growth, invasion and metastasis; nevertheless, contradictory findings have also been published [54,55]. In a previous study, the expressions of these inhibitors are not found to be relevant to the regulation of MMP-2 and MMP-9 expressions [56]. In our study, the effect of γ-tocotrienol on TIMP-1 and TIMP-2 expression was determined by RT-PCR. γ-Tocotrienol could increase the expression of TIMP-1 and TIMP-2 in SGC-7901 cells. Thus, γ-tocotrienol may decrease the MMP-2 and MMP-9 expression, and increase the TIMP-1 and TIMP-2 expression to reduce the metastatic capabilities of SGC-7901 cells.

In addition, nm23-H1 is one of the main anti-oncogenes. A great number of experiments indicate that inactivation of these genes, that is, genetic instability, resulted in metastasis [57–59]. Leone et al. [60] found that nm23-H1 had a function on the prevention of tumor metastasis by inhibiting the ability of cancer cells to clone. In this study, γ-tocotrienol also increased the expression of nm23-H1 in SGC-7901 cells.

In conclusion, the present study indicates that γ-tocotrienol inhibited the invasion and migration ability of SGC-7901 cells in cell migration, cell attachment and matrigel invasion. These findings showed that γ-tocotrienol may reduce the matrigel invasion capabilities through down-regulation of the MMP-2 and MMP-9 expression, and up-regulation of the TIMP-1 and TIMP-2 expression in SGC-7901 cells. However, we found that γ-tocotrienol did not differ in the chemotactic response to FN in SGC-7901 cells. This needs to be studied further.

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