

Triptolide inhibits ovarian cancer cell invasion by repression of matrix metalloproteinase 7 and 19 and upregulation of E-cadherin

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Abbreviations: ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition

Abstract

Triptolide, a compound extracted from the traditional Chinese medicine preparation of *Tripterygium wilfordii* Hook F., has been reported to have anti-inflammatory and anti-cancer activities. However, its effect on ovarian cancer invasion is unknown. We observed that MMP7 and MMP19 expression increased in ovarian cancer tissue. Triptolide treatment inhibited the migration and invasion of ovarian cancer cells SKOV3 and A2780 at the concentration of 15 nM. We also observed that triptolide suppressed MMP7 and MMP19 promoter activity in a dose-dependent manner,

down-regulating the expressions of these promoters on mRNA and protein level. Moreover, triptolide enhanced E-cadherin expression in ovarian cancer cells. *In vivo*, triptolide inhibited tumor formation and metastasis in nude mice, and suppressed MMP7 and MMP19 expression; it also enhanced E-cadherin expression in tumor in a dose-dependent manner. Over expression of MMP7 and MMP19, or suppression of E-cadherin expression partially abolished the inhibitory effect of triptolide on invasion of ovarian cancer cells. To summarize, triptolide significantly inhibited the migration and invasion of ovarian cancer cells by suppression of MMP7 and MMP19 and up-regulation of E-cadherin expression. This study shows that triptolide is a good candidate for the treatment of ovarian cancer and reduction of metastasis.

Keywords: cadherins; matrix metalloproteinase 19; matrix metalloproteinase 7; ovarian neoplasms; triptolide

Introduction

Triptolide is a compound originally extracted from the traditional Chinese medicinal preparation of *Tripterygium wilfordii* Hook F. With its broad-spectrum anticancer activity, triptolide has a considerable potential as chemotherapeutic agent. It inhibits colon cancer, breast cancer, renal cell carcinoma, and cervical carcinogenesis process (Wang *et al.*, 2009; Kim *et al.*, 2010; Li *et al.*, 2011; Tan *et al.*, 2011). Recently, it has been found to be effective in the treatment of ovarian cancer (Zhou *et al.*, 2010); it has been shown that triptolide increases the proportion of cells in the S-phase of the cell cycle and induces apoptosis. Numerous biological molecules inhibited by triptolide have been viewed as its possible targets. Triptolide changes the expression of cell cycle regulators, apoptosis-related factors and cell proliferation markers; for example, it up-regulates LRAP, CDH4, and SFRP1 and down-regulates the expression of cystatin, TNNT 1, and

L1-CAM (Westfall *et al.*, 2008; Li *et al.*, 2010). However, the effects of triptolide on ovarian cancer cell invasion have not been investigated.

Its high invasion and metastatic potential make ovarian cancer a particularly life-threatening disease and affect the efficacy of chemotherapy treatment. Invasion and migration of cancer cells are complicated processes in which many types of molecules are involved. Rho GTPases, such as Rac (Wang *et al.*, 2009), RhoA (Jackson *et al.*, 2011), and Cdc42 (Du *et al.*, 2009) play crucial roles in cytoskeleton reorganization, adhesion and motility. Degradation of extracellular matrix (ECM) is an important step in cancer invasion into neighboring organs and initiation of metastasis. Matrix metalloproteinases (MMPs) have been implicated in cell motility and invasion by the virtue of their ability to degrade ECM components. Induced expression or overexpression of MMPs is observed in the pathogenesis of diverse diseases, such as periodontitis, rheumatoid arthritis, and skin wound healing (Vu and Werb, 2000; Sorsa *et al.*, 2004; Kanbe *et al.*, 2011). Moreover, MMPs have been found to play a crucial role in the development of many cancers. MMPs promote the epithelial-to-mesenchymal transition (EMT) associated with malignant behavior by cleaving the cell-adhesion molecule E-cadherin and liberating TGF- β . They speed up tumorigenesis by inducing angiogenesis (Bergers *et al.*, 2000), and induce tumor cells invasion and metastasis process by the degradation of surrounding ECM.

MMP-7 is involved in the progression of many cancers, for example, gastric adenocarcinoma, colon cancer, hepatocellular carcinoma, pancreatic cancer, breast cancer, cervical and ovarian cancer. Pertinent to this study, MMP7 deletion in *Apc^{Min/+}* mice has been shown to reduce strongly the intestinal tumor burden (Wilson *et al.*, 1997). Wang *et al.* have shown that MMP-7 is overexpressed in epithelial ovarian cancer, and recombinant MMP-7 promotes invasion *in vitro* (Wang *et al.*, 2005). These observations suggest that MMP-7 is a viable target for the treatment of ovarian cancer.

MMP19 is widely expressed in normal human tissues. However, it is associated with ovulation and angiogenic processes and deregulated in diverse pathological conditions such as rheumatoid arthritis and cancer, suggesting its importance in cancer development (Pendas *et al.*, 2004; Chan *et al.*, 2011). MMP19-null mice show a decreased susceptibility to skin tumors induced by chemical carcinogens. Moreover, MMP19 can be used as a marker for tumor invasiveness in patients with oropharyngeal squamous cell carcinoma. MMP19 is highly expressed in astroglial tumors, and its expression might facilitate the invasion of glioma cells through

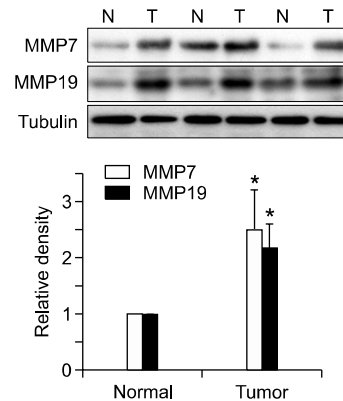


Figure 1. MMP7 and MMP19 expression increased in human ovarian cancer tissue. Equal amounts of protein lysates from ovarian cancer tissue (lanes T) and adjacent normal tissue (lanes N) from three different patients were analyzed by Western blotting. Tubulin was used as an internal control. Density of bands was quantified by Total lab software.

brain extracellular matrix (Lettau *et al.*, 2010).

In this study, on the basis of the observation that MMP7 and MMP19 is overexpressed in ovarian tumor tissue, we treated ovarian cancer cells SKOV3 and A2780 with triptolide. This treatment inhibited the migration and invasion of ovarian cancer cells, decreased the expression of MMP7 and MMP19 through inhibition of their promoters, and induced E-cadherin expression.

Results

MMP7 and MMP19 expression increased in ovarian cancer tissue

To investigate the involvement of MMP7 and MMP19 in ovarian cancer, we examined MMP7 and MMP19 expression in ovarian cancer tissue and in adjacent normal tissue using Western blotting. MMP7 and MMP19 expression was elevated significantly in 46% and 38% of analyzed carcinoma tissue samples, respectively, in comparison with adjacent normal tissue controls, indicating crucial roles for these proteins in ovarian cancer development. Representative photographs are shown in Figure 1.

Triptolide inhibited ovarian cancer cells proliferation and migration

First, we evaluated the effect of triptolide on ovarian cancer cell proliferation. Figure 2A shows that triptolide inhibited ovarian cancer cell proliferation in a dose-dependent manner in both SKOV3 and A2780 cells. This effect is first clearly noticeable after 72 h incubation with 50 nM triptolide. To study the effect of triptolide on ovarian cancer cell motility,

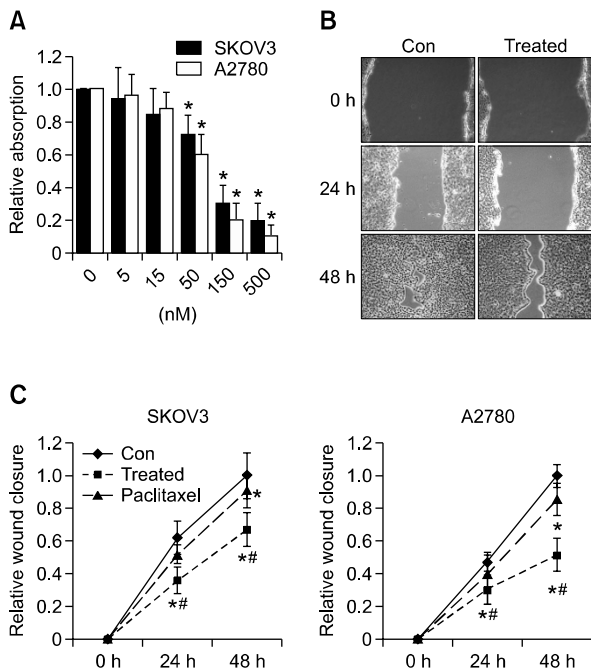


Figure 2. Effects of triptolide on the migratory abilities of ovarian cancer cells. (A) SKOV3 and A2780 cells were exposed to triptolide at the concentration from 5 to 500nM, survival of cells were detected by MTT assay. (B) After incubation with 15 nM triptolide or Paclitaxel for 24 h and 48 h, the migratory abilities of SKOV3 and A2780 cells were evaluated by the wound healing assay. Photographs shown here are representative images for SKOV3 cells. (C) Relative wound closure was calculated by comparing wound width to the wound width for the control cells. * $P < 0.01$ vs. untreated cells, # $P < 0.01$ vs. Paclitaxel.

we used scratch wound healing assay, measuring the extent of cell migration into the scratched area. Control cells were confluent at 48 h after scratching. To examine the effect of triptolide on cell migration, we selected the concentration of triptolide that did not affect cells' proliferation, and the cells were incubated with 15 nM triptolide (the concentration not affecting cell proliferation). Cell migration was significantly inhibited, by 23% for SKOV3 cells and 48% for A2780 cells, 48 h after wounding (Figures 2B and 2C). Paclitaxel is a chemotherapy agent used frequently to treat ovarian cancer patients; we used it as a positive control to evaluate the anti-cancer efficacy of triptolide. We observed that although Paclitaxel inhibited ovarian cancer cell migration, the Paclitaxel-treated cells migrated significantly faster than the cells treated with 15 nM triptolide.

Triptolide inhibited ovarian cancer cell invasion

We next investigated the effect of triptolide on invasive properties of ovarian cancer cells. Matrigel transmembrane invasion assay was used; the cells

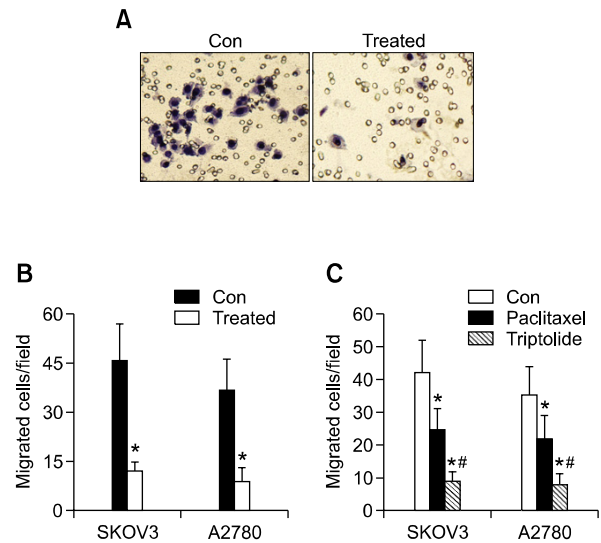


Figure 3. Effect of triptolide on the invasive abilities of ovarian cancer cells. SKOV3 and A2780 cells were treated with triptolide or Paclitaxel at 15nM. (A) Representative photos of SKOV3 cells that migrated through 8 μ m pore transwell membranes with matrigel. (B, C) Invasiveness of SKOV3 and A2780 cells was evaluated by counting cells that migrated through 8 μ m pore transwell membranes with matrigel. * $P < 0.01$ vs. untreated cells, # $P < 0.01$ vs. Paclitaxel.

that migrated across a membrane toward the source of serum attractant were stained with crystal violet. Representative images of staining for migrated SKOV3 cells are shown in Figure 3A. We found that 15 nM triptolide markedly blocked the invasive capacity of SKOV3 and A2780 cells (Figure 3B). Although Paclitaxel inhibited ovarian cancer cell invasion, the inhibitive effect of triptolide was significantly greater than observed for the cells incubated with 15 nM Paclitaxel for 48 h (Figure 3C).

Triptolide inhibited MMP7 and MMP19 expression in ovarian cancer cells

ECM degradation mediated by MMPs such as MMP7 and MMP19 is an essential step in tumor invasion and metastasis. Since MMP7 and MMP19 are overexpressed in ovarian cancer tissue, we investigated the effect of triptolide on MMP7 and MMP19 expression in ovarian cancer cells. SKOV3 and A2780 cells were exposed to 0, 5 or 15 nM triptolide for 24 h. The results of Western blotting showed that the levels of MMP7 and MMP19 expression were reduced in both cell lines (Figure 4). Similarly, RT-PCR showed that MMP7 and MMP19 expression decreased on the mRNA level. Furthermore, we employed luciferase reporter assay to evaluate MMP7 and MMP19 promoter activities. Figure 5 shows that in SKOV3 and A2780 cells, *MMP7* and *MMP19* promoter activity were

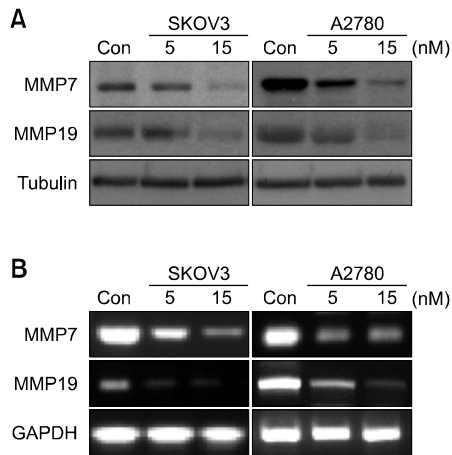


Figure 4. Effect of triptolide on MMP7 and MMP19 expression. (A) SKOV3 and A2780 cells were incubated with triptolide at 0, 5 and 15 nM concentration for 24 h. MMP7 and MMP19 expression at protein level were evaluated by Western blot. Tubulin was used as an internal control. (B) SKOV3 and A2780 cells were incubated with triptolide at 0, 5 and 15 nM for 24 h. MMP7 and MMP19 expression at mRNA level were evaluated by RT-PCR. GAPDH was used as an internal control.

substantially inhibited in a dose-dependent manner when the cells were incubated with triptolide (Figure 5). Knocking down MMP7 and MMP19 by siRNA significantly reduced cells migration (Figure 5B). We next overexpressed MMP7 and MMP19 in SKOV3 and A2780 cells by transfecting the cells with HA-tagged MMP7 and MMP19 vector (Figure 5B). We found that overexpression of MMP7 or MMP19 partially alleviates the inhibitory effect of triptolide on invasion of ovarian cancer cells.

Triptolide up-regulated E-cadherin expression in ovarian cancer cells

Detachment from the neighboring cells is a crucial step for cancer cell metastasis. E-cadherin is a key molecule in the control of cell-cell adhesion; in cancer cells, this molecule is either partially or entirely missing from the membranes. We tested the effect of 0, 5 or 15 nM triptolide on E-cadherin expression. Western blotting results showed that E-cadherin expression increased in a dose-dependent manner after triptolide treatment for 24 h (Figure 6A). Using SKOV3 and A2780 cells transfected with shRNA against E-cadherin, we found that the resulting suppression of E-cadherin expression partially abolished the inhibitory effect of triptolide on invasion of ovarian cancer cells (Figure 6B).

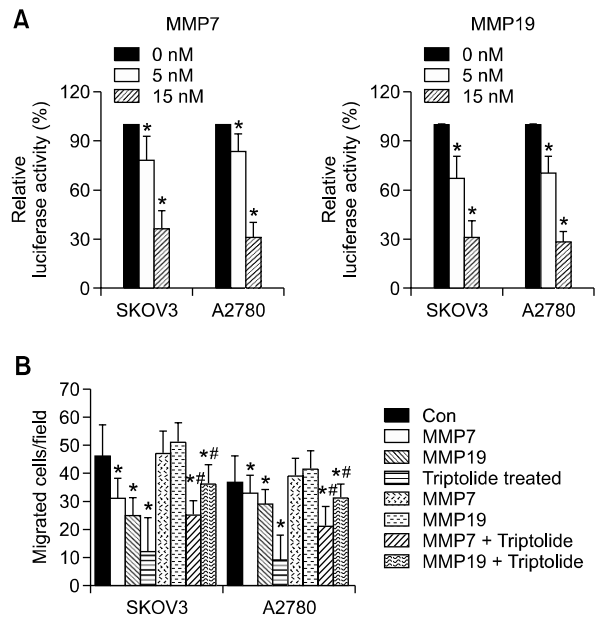


Figure 5. Effect of triptolide on MMP7 and MMP19 promoter activity. (A) MMP7 and MMP19 promoter activities were tested by luciferase assay after treatment with 0, 5 and 15 nM triptolide for 24 h. * $P < 0.01$ vs. untreated cells. (B) MMP7 and MMP19 were knocked down by siRNA (labeled as MMP7 KD or MMP19 KD) or overexpressed (labeled as MMP7 or MMP19) in SKOV3 and A2780 cells. Invasiveness of SKOV3 and A2780 cells was evaluated by counting the cells that migrated through 8 μ m pore transwell membranes with matrigel. * $P < 0.01$ vs. control cells, # $P < 0.01$ vs. Triptolide-treated cells.

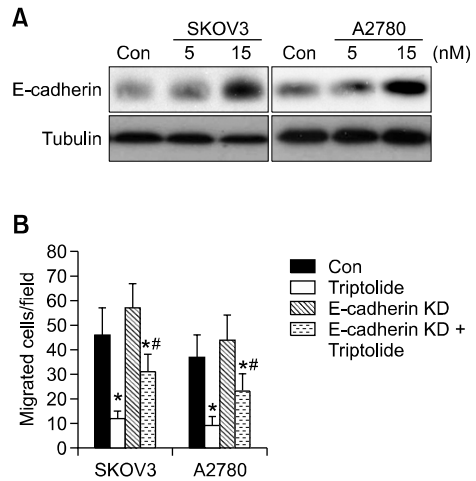


Figure 6. Effect of triptolide on E-cadherin expression. (A) SKOV3 and A2780 cells were incubated with triptolide at 0, 5 and 15 nM for 24 h. E-cadherin expression at protein level was evaluated by Western blotting. Tubulin was used as an internal control. (B) E-cadherin expression was suppressed (E-cadherin KD) in SKOV3 and A2780 cells by transfecting with shRNA against E-cadherin. Invasiveness of SKOV3 and A2780 cells was evaluated by counting the cells that migrated through 8 μ m pore transwell membranes with matrigel. * $P < 0.01$ vs. control cells, # $P < 0.01$ vs. Triptolide-treated cells.

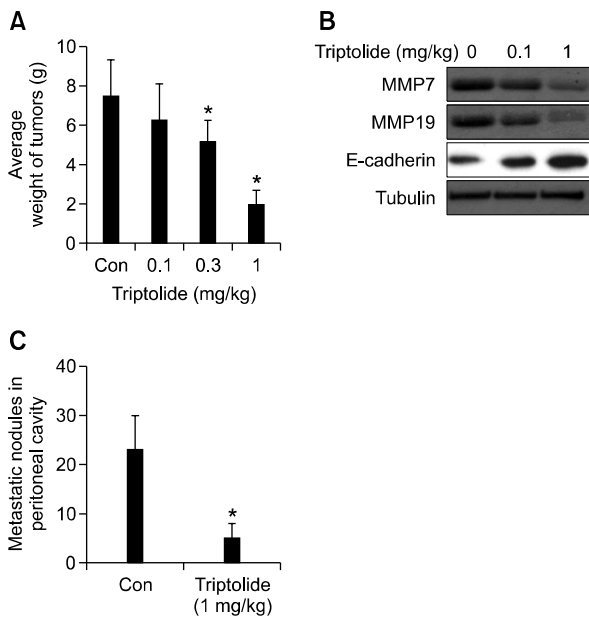


Figure 7. Triptolide inhibited tumor formation in nude mice. (A) SKOV3 cells were injected subcutaneously into nude mice. Triptolide at 0, 0.1, 0.3 or 1 mg/kg/day was orally administered daily for three weeks, and tumor xenografts were removed and weighed. * $P < 0.05$ comparing to untreated mice. (B) Protein lysates were prepared from tumors. MMP7, MMP19 and E-cadherin were detected by Western blot. (C) SKOV3 cells were injected (i.p.) into nude mice. Triptolide at 0 and 1 mg/kg/day was orally administered daily for five weeks, and metastasis nodules were examined and quantified. * $P < 0.05$ comparing to untreated mice.

Triptolide inhibited ovarian cancer tumors formation and metastasis *in vivo*, suppressed MMP9 and MMP17 expression, and enhanced the expression of E-cadherin

Next, we turned to the *in vivo* analysis of triptolide effect on ovarian cancer. When triptolide was administrated orally to mice with established tumor xenografts, it had a significant, dose-dependent inhibitory effect on the growth of tumors, beginning with the dosage of 0.1 to 1 mg/kg (Figure 7A). Furthermore, Western blots of protein extracts from the tumor xenografts showed that triptolide had the same effect *in vivo* as *in vitro*. The levels of MMP9 and MMP17 were lower, and E-cadherin levels increased in the tumors of mice treated with triptolide (Figure 7B).

35 days after nude mice were injected with SKOV3 cells (i.p.), disseminated and enlarged metastatic nodules were found throughout the peritoneal cavity in control mice. The number of metastatic nodules was significantly reduced (by ~80%) in mice treated with triptolide at the concentration of 1 mg/kg (Figure 7C). These results suggested that triptolide can efficiently inhibit the metastasis of SKOV-3 ovarian cancer cells.

Discussion

Ovarian cancer is one of the major causes of cancer deaths in women (Bomalaski, 1999). It has a high mortality due to its clinically occult dissemination and metastasis (Saad *et al.*, 2010). The spread of ovary cancer cells occurs by direct migration and invasion of adjacent organs, as well as throughout the peritoneal cavity by flow of peritoneal fluid (Naora and Montell, 2005; Gubbles *et al.*, 2010). Because of this unique and furtive mode of dissemination, efforts to develop chemotherapies for ovarian cancer have been largely unsuccessful (Dutta *et al.*, 2010; McKenzie *et al.*, 2011). Therefore, a better understanding of the cellular and molecular mechanisms that are responsible for ovarian cancer metastasis is needed.

Tumor invasion and metastasis are associated with a complex cascade of proteolytic events, including degradation of extracellular matrix (ECM) proteins and basement membrane (Coussens *et al.*, 2002). Matrix metalloproteinases (MMPs) are proteolytic enzymes that play an important role in cancer progression through enhancement of cell motility, invasion, and lymph node metastasis by degrading components of the basement membrane, thereby facilitating carcinoma cell intravasation and dissemination (Curran and Murray, 1999; Nagase and Woessner, 1999). MMP group of proteins expressed and secreted by carcinoma and stromal cells consists of 23 members including 17 secreted as soluble enzymes, and 6 membrane type-metalloproteinases (Radisky and Radisky, 2010). MMPs may also modulate carcinoma cells physiology *in vivo* through cleaving of growth factors, cell surface receptors, cell adhesion molecules, or chemokines/cytokines. For instance, as a consequence of cleaving of the pro-apoptotic factors, MMPs may be able to produce a more aggressive phenotype *via* generation of apoptosis-resistant cells (Gonzalez *et al.*, 2010). In the present study, we detected increased expression of MMP7 and MMP19 in ovarian cancer tissue in comparison with the adjacent normal tissue, suggesting a crucial role for those proteins in ovarian cancer progression.

Triptolide has attracted a lot of attention because of its potential anti-inflammatory and anti-cancer activities (Yang *et al.*, 2003; Corson and Crews, 2007). Our previous study has shown that it inhibits the proliferation of colorectal cancer cells *in vitro* and *in vivo* by interrupting the IL6R-JAK/STAT pathway (Wang *et al.*, 2009). In this study, we examined the effect of triptolide on ovarian cancer cells. The results showed that triptolide significantly inhibited migration and invasion of ovarian cancer cells SKOV3 and A2780 *in vitro*. Since ECM

degradation mediated by MMPs is an essential step in tumor invasion and metastasis, we hypothesized that triptolide might be able to alter the expression and activity MMPs such as MMP7 and MMP19. We observed that triptolide suppressed MMP7 and MMP19 expression at both mRNA and protein levels in SKOV3 and A2780 cells. Further investigation showed that *MMP7 and MMP19* promoter activities were substantially inhibited in a dose-dependent manner by treatment with triptolide. *In vivo*, triptolide significantly reduced the growth of primary SKOV3 xenografts in nude mice. MMP7 and MMP19 expression in tumor tissues dramatically decreased in mice treated with triptolide, confirming the results of *in vitro* analysis.

The separation of cancer cells from the surrounding cells is another important step in cancer dissemination and metastasis process (Baum *et al.*, 2011). Cells are held together by the interaction of cell-cell adhesion molecules. Cadherins, a family of intercellular adhesion protein molecules including E-cadherin, play a major part in mammalian cell adherence (Berx *et al.*, 2001). E-cadherin mediates intercellular adhesion through homophilic associations with the extracellular domains of E-cadherin of a neighboring cell. In cancer cells, E-cadherin is down-regulated, allowing cells to detach from each other and from the ECM (Cavallaro *et al.*, 2002). Some chemical compounds have been reported to retard cancer cell motility by increasing the expression of E-cadherin. For instance, Artemisinin enhances E-cadherin activity, resulting in greater cell-cell adhesion, and therefore reduces metastasis of hepatocellular carcinoma cells (Weifeng *et al.*, 2011). Here, we observed that triptolide markedly increased the expression of E-cadherin not only in SKOV3 and A2780 cells *in vitro*, but also in xenografts and metastasis of SKOV3 *in vivo*, in a dose-dependent manner. This upregulation of E-cadherin expression might at least partially decrease cell migration and invasion, due to the enhanced cell-cell adhesion.

In summary, we found that triptolide significantly inhibited migration and invasion in ovarian cancer cells. This was achieved by suppression of MMP7 and MMP19 expression. We also observed that triptolide up-regulated the E-cadherin expression, thereby improving cell-cell adhesion, resulting in retarded cell motility. This study suggests that triptolide is potentially a very useful compound for the development of chemotherapeutic treatments for ovarian cancer.

Methods

Tissue sample collections

A collection of tissue specimens, 20 normal and 20 ovarian tumor samples (5 serous borderline ovarian tumors, and 15 serous ovarian cystadenocarcinomas), were obtained from the Department of Gynaecology and Obstetrics of Tangdu Hospital, with Institutional Review Board approval.

Cell culture

Human ovarian cancer cell lines SKOV3 and A2780 were cultured on cell plates at 37°C, in 5% CO₂, in RPMI1640 (Sigma) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

Drug preparation

Triptolide was purchased from Sigma, and dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 500 nM. The solution was filtered through a 0.22 μm micropore filter, stored at -20°C, and diluted in cell culture medium.

MTT assay

SKOV3 and A2780 cells were plated in 96-well plates (5 × 10³/well). 24 h later, triptolide was added to a final concentration of 0, 1.5, 5, 15, 50, or 150 nM for 72 h. 20 μl of 5 mg/ml MTT (Sigma, St. Louis, MO) was added to each well for 4 h at 37°C. 150 μl of dimethylsulfoxide was added to dissolve the crystals. The results were obtained using an enzyme-linked immunosorbent assay reader (Bio-RAD, San Diego, CA); the measurement wavelength was 490 nm.

Wound healing assay

Wound healing assays were used to evaluate cell migration capability in 2D space. Confluent SKOV3 and A2780 cells were treated with triptolide or Paclitaxel (15 nM) for 48 h. Then a scratch wound in the monolayer was made by dragging a 1 ml pipette tip across the layer. Cells were cultured with RPMI1640 containing triptolide or Paclitaxel as described above, and the extent of wound closure was followed by microscopy at 24 h and 48 h. The experiments were repeated three times.

Invasion assay

Invasion assays were used to investigate cell migration capability in 3D gel. Briefly, SKOV3 and A2780 cells were treated with triptolide at the concentration of 15 nM for 24 h, and then 3 × 10⁴ cells were seeded into the upper chambers of 8 μm pore transwell. To examine the invasion through the matrigel barrier, 3 × 10⁴ cells in 100 μl of RPMI1640 and 15 nM triptolide or Paclitaxel were added to the upper chamber of each well. The cells were allowed to migrate for 20 h; migrated cells were fixed, stained, counted from six random fields and the results averaged. The experiment was repeated three times.

Western blotting

Protein extraction and immunoblot analysis were performed on cells or tumor tissue lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% v/v Triton X-100, 1 mM EDTA, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM Na₃VO₄). Lysates were centrifuged and supernatants collected. Cell lysate aliquots (20 µg) were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and incubated with primary antibody, either rabbit polyclonal anti-MMP7, anti-MMP19 (diluted 1:300; Abcam), anti-E-cadherin (diluted 1:1000; Cell Signaling), or tubulin (diluted 1:5000; Sigma Chemical Co.) overnight at 4°C. After repeated washing, membranes were incubated with horseradish-peroxidase-conjugated anti-mouse secondary antibody (1:2000; Santa Cruz Biotechnology). Bands were visualized using an enhanced chemiluminescence system (Amersham Pharmacia Biotech), quantified using TotalLab TL100 software (Nonlinear Dynamics, Newcastle upon Tyne, UK), and b-actin was used to normalize for different protein amounts. Each experiment was performed in triplicate.

RT-PCR

Total RNA was extracted from ovarian cancer cells using Trizol reagent (Invitrogen, Life Technologies, CA) following the manufacturer's protocol. cDNA was synthesized from 2 µg of total RNA using Superscript reverse transcriptase (Life Technologies, CA). Primer sequences used for MMP7 detection were as follows, sense: 5'-GGATGGTAGCAGTC TAGGGATTAAC-3'; and antisense: 5'-GGAATGTCCCAT ACCCAAAGAA-3'. For MMP19 detection, sense: 5'-TGCC CACAGAACCCAGTCC-3', antisense: 5'-GGTATTCCCAC CTGATGGGGTAG-3'. The housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) was used for normalization (sense: 5'-TGATGACATCAAGAAGGTG GTGAAG-3'; and antisense: 5'-TCCTTGGAGGCCATGTG GGCCAT-3'). The amplification conditions were 95°C for 1 min followed by 30 cycles of 94°C for 30 s, 59°C for 20 s, and 72°C for 45 s with a 5 min 72°C extension. PCR products were analyzed by 1.2% agarose gel electrophoresis with ethidium bromide for UV light transilluminator visualization.

Transfection of ovarian cancer cells

2×10^5 SKOV3 and A2780 cells were seeded out in 6-well plates. Mixture of 0.5 µg siRNA against MMP7 or MMP19 (Santa Cruz, sc-41554, 106229) with 5 µl transfection reagent (Santa Cruz, sc-29528) was added into cell culture medium. On the other hand, cells in each well were transfected with 2 µg HA-tagged MMP7 and MMP19 vectors, or shRNA against E-cadherin (Santa Cruz, sc-35242), using 6 µl Fugene 6 transfection reagent (Roche). Expression of MMP7, MMP19 and E-cadherin was detected by Western blotting.

Promoter activity

The promoter activities of MMP7 and MMP19 were analyzed using the dual luciferase assay (Promega, Madison, WI) using a *firefly luciferase* vector containing an 1898-bp

stretch of the 5'-flanking region (-1898/LUC) of the *MMP7* or *MMP19* gene. For transient transfection, SKOV3 and A2780 cells (5×10^4 cells/well) were transfected with a total amount of 0.1 µg DNA using Lipofectamine 2000 (Invitrogen) in serum-free RPMI1640 containing 0.03 mM CaCl₂. For each transfection 0.06 µg of -1898/LUC vector and 0.02 µg pRL of *renilla luciferase* vector (an internal control) were co-transfected with 0.005, 0.01, or 0.02 µg (5, 10, or 20% of plasmid DNA used for transfection) of either MMP7 or MMP19 vector. Transfected cells were lysed after 48 h of cultivation. Luminescence was measured using a single-sample luminometer (TD/20, Turner Design, CA) according to the manufacturer's manual (Promega). All assays were performed in quadruplicate in three separate experiments. The average of these quadruplicates was taken to obtain the relative *luciferase* activity, which is expressed in relative light units as defined by the ratio of *firefly* to *renilla luciferase* activity multiplied by the factor of 10⁴. Data are presented as average \pm SD and show the percentage of relative light units change against the control. The results were analyzed using Student's two-tailed paired *t*-test.

Tumor formation and metastasis of ovarian cancer *in vivo*

To establish xenografts of ovarian cancer cells in mice, SKOV3 cells were injected subcutaneously into the flanks of 5- to 6-week-old BABL/c nude mice, at 5×10^6 cells/site, using 8 mice per group. After growing for 30 days, the tumor xenografts reached approximately 100 mm³. Thereafter, triptolide at 0, 0.1, 0.3 or 1 mg/kg/day was orally administered on a daily basis. At the end of three weeks, mice were sacrificed, and tumor xenografts were removed and weighed.

To examine the effect of triptolide on ovarian cancer cell metastasis, 5×10^5 SKOV3 cells were injected intraperitoneally (i.p.) into mice. Animals were killed and the number and extent of overt metastases were then quantified, 35 days after cell injection.

Statistical analysis

Results from cell phenotype characterization were analyzed by Student's *t*-test, using Statistical SPSS software package (SPSS Inc, Chicago). Differences were considered statistically significant at $P < 0.05$.

Acknowledgements

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