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Inhibitive effect of triptolide on invasiveness of human fibrosarcoma cells by downregulating matrix metalloproteinase-9 expression

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ABSTRACT

Objective: To explore the molecular mechanisms of antitumor properties of triptolide, a bioactive component isolated from the Chinese herb *Tripterygium wolfordii* Hook F. **Methods:** Human fibrosarcoma HT-1080 cells were treated with different doses of triptolide for 72 h. Then the expression and activity of matrix metalloproteinase (MMP)-2 and -9 were measured and the invasiveness of triptolide-treated HT-1080 cells was compared with that of anti-MMP-9-treated HT-1080 cells. **Results:** 18 nmol/L triptolide inhibited the gene expression and activity of MMP-9, but not those of MMP-2, in HT-1080 cells. In addition, both 18 nmol/L triptolide and 3 μg/mL anti-MMP-9 significantly reduced the invasive potential of HT-1080 cells, by about 50% and 35%, respectively, compared with the control. Whereas there was no significant difference between the effect of 18 nmol/L triptolide and that of anti-MMP-9 on invasive potential of HT-1080 cells. **Conclusions:** These data suggest that triptolide inhibits tumor cell invasion partly by reducing MMP-9 gene expression and activity.

1. Introduction

Matrix metalloproteinases (MMPs) are a large family of over 25 zinc-dependent proteolytic enzymes, which are capable of degrading virtually every component of the extracellular matrix (ECM), and have been implicated in a variety of key normal and pathologic processes, including wound healing, organogenesis, inflammation, auto-immunity and carcinogenesis. Traditionally, MMPs have been subdivided into collagenases, gelatinases, stromelysins, and membrane-type MMPs, according to their substrate specificity, primary structure, and cellular location[1]. Studies over the past 20 years have revealed that MMPs play a complex and key role in tumor growth, progression, metastasis and angiogenesis[2]. Of MMP family, MMP-2 and MMP-9 are mainly involved in the metastasis process.

Triptolide (PG490), a diterpene triepoxide, has been identified as a crucial active component in extracts of the Chinese herb *Tripterygium wilfordii* Hook F. (TWHF), and exerts potent anti-inflammatory, anti-cancer, and anti-fertility activities *in vivo* and *in vitro*[3,4]. Most studies indicated that the antitumor activity of triptolide may attributes to inhibition of proliferation and induction of apoptosis of tumor cells[5-7]. Meanwhile, previous studies also showed triptolide as a potent inhibitor of MMPs in some cell types. In 2003, Lu *et al* demonstrated that triptolide inhibited the IL-1 β -inducing expression of MMP-1,-2,-3, and -9 in corneal fibroblasts at both mRNA and protein levels[8]. It is consistent with the previous observation that triptolide directly suppressed the production of MMP-1 and MMP-3, and simultaneously up-regulated the production of tissue inhibitor of metalloproteinase (TIMP)-1 and -2 induced by IL-1 α in human synovial fibroblasts[9]. Triptolide also down-regulated the synthesis of MMP-3 and MMP-13 in chondrocytes partly by interfering with the DNA binding activities of transcription factor AP-1 and NF- κ B[10]. These studies raise the question that whether triptolide could down-regulate the expression and production of MMPs in tumor microenvironment and thus inhibit the invasion and metastasis of tumors.

In the study, we investigated whether triptolide modulated the expression and activities of MMP-2 and MMP-9 in human fibrosarcoma HT-1080 cells, and compared its effect on invasiveness of the cells with that of anti-MMP-9 *in vitro*.

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2. Materials and methods

2.1. Cell culture and drug treatment

Human fibrosarcoma HT-1080 cells were purchased from the China Center for Type Culture Collection (Wuhan, P. R. China) and cultured in MEM containing non- essential amino acids (Invitrogen, CA), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin. Cells were maintained in a 5% CO_2 humidified incubator at 37 ℃. Triptolide (PG490, molecular weight 360, purity ⩾98%) was obtained from Sigma (USA), and prepared as previously described[11]. Triptolide was dissolved in dimethyl sulfoxide (DMSO) and stock solution (1 mg/mL) and was stored at -20 ℃. It was freshly diluted to the indicated concentration with MEM before use and directly added to cell cultures. The DMSO concentration in all experiments never exceeded 0.001% (V/V).

2.2. MTT assay

The effect of triptolide on cell viability and proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, HT-1080 cells were seeded in a 96-well plate at a density of 2×10^4 cells per well and incubated overnight. The cells were then treated with various concentrations of triptolide (0, 5, 10, 15, 20, 25, or 30 nmol/L) for 72 h, with changes of reagents every 24 h (MEM supplemented with just 1% FBS). Next, 20 μL of MTT (Sigma, USA) solution (5 mg/mL in PBS) was added to each well and the cells were incubated for additional 4 h at 37 ℃. MTT solution in medium was aspirated off and 100 μL of dimethyl sulfoxide (DMSO) was added to each well before absorbance at 492 nm was measured.

2.3. RNA extraction and reverse transcription PCR

Total RNA was extracted from 2.8×10^6 cells using TRIzol reagent (Invitrogen, CA) according to the manufacturer's instructions, and quantitated by spectrophotometry. The integrity and quantity of RNA were verified by ethidium bromide staining of the 28S and 18S ribosomal RNA bands. First strand cDNA was prepared from 3 μg of total RNA in 20 μL of reaction volume using a RevertAid™ First Strand cDNA Synthesis Kit (Ferments, Lithuania). After reverse transcription, MMP-9 (sense: 5′-CAACATCACCTATTGGATCC-3′, and antisense: 5′-TGGGTGTAGAGTCTCTCGCT-3′), MMP-2 (sense: 5′-GGCCCTGTCACTCCTGAGAT-3′, and antisense: 5′-GGCATCCAGGTTTCGGGGA-3′), and β-actin (sense: 5′-CGCGAGAAGATGACCCAGAT-3′, and antisense: 5′-GCACTGTGTTGGCGTACAGG-3′) were amplified in a TGradient Thermocycler (Whatman Biometra, Germany) as follows: 94 ℃ for 4 min, and then 94 ℃ for 30 sec, 55 ℃ for 50 sec, and 72 ℃ for 60 sec for 30 cycles, and finally, 72 ℃ for 10 min.

2.4. Gelatin zymography

To measure the activity of MMP-2 and MMP-9, cell-culture supernatants (serum-free) were collected and assayed by gelatin zymography, using 7.5% SDS-PAGE gels containing gelatin (1% w/v; Sigma, MO, USA). After electrophoresis, the gels were washed twice for 30 min each in 2.5% Triton X-100 to remove SDS and then incubated overnight at 37 ℃ in Tris-HCl (pH 7.6), 10 mM $CaCl_2$, 50 mM NaCl, and 0.02% Brij-35. After incubation, the gels were stained with 0.25% Coomassie brilliant blue R-250 and destained in a solution of 40% methanol and 10% acetic acid.

2.5. Matrigel invasion assay

HT-1080 cell invasion was assayed using a 6.4 mm BD Falcon™ cell Culture Insert with an 8.0 μm pore polyethylene terephthalate (PET) membrane (BD Biosciences, Durham, NC, USA). Prior to each experiment, the PET filters were coated with Growth Factor Reduced BD Matrigel™ Matrix (BD Biosciences, Durham, NC, USA). After treatment with the indicated concentrations of triptolide or anti-MMP-9 for 72 h, 1×10^5 cells, in 0.2 mL of serum-free medium, were plated onto the upper compartment of the chamber, and the lower chamber was filled with 500 μL of MEM supplemented with 5% FBS. After incubation (without triptolide/anti-MMP-9) for another 36 h, the non-invaded cells on the upper surface of the membrane were removed with a cotton swab, and the invaded cells on the lower surface were stained with 0.1% crystal violet for 20 min and destained with 33% acetic acid. The eluate was collected, and absorbance was measured at 492 nm using a multiwell spectrophotometer (Bio-Tek Instruments, Italy).

2.6. Statistical analysis

All experiments were performed in triplicate. Data were expressed as means±SD and were analyzed using a Student`s *t*-test, with *P* values less than 0.05 as statistically significant. Statistical analyses were performed using SPSS software.

3. Results

3.1. Cytotoxic effect of triptolide on HT-1080 cells

As shown in Figure 1, triptolide IC_{50} for HT-1080 cells was about 25 nmol/L after 72 h of treatment. Thus, doses of triptolide in following experiments were 6, 12, and 18 nmol/L to exclude toxicity.

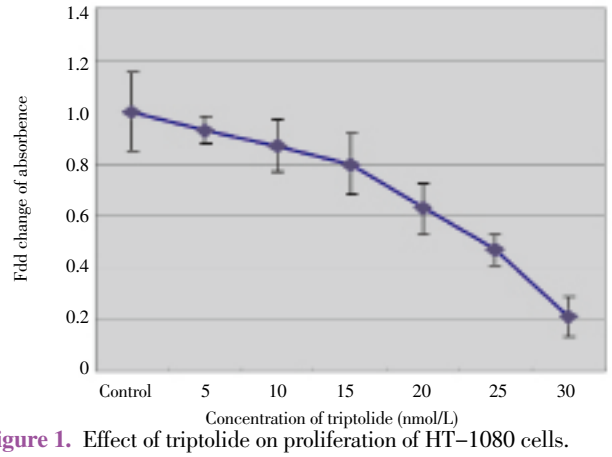

Figure 1. Effect of triptolide on proliferation of HT-1080 cells.

3.2. Effects of triptolide on the mRNA expression and activity of MMP-2 and MMP-9

Figure 2 showed that triptolide significantly inhibited the gene expression of MMP-9 at a concentration of 18 nmol/L, compared with the control (0.36±0.04 versus 0.59±0.11, P <0.05). However, triptolide at indicated concentration had no inhibitive effect on the mRNA expression of MMP-2 in HT-1080 cells.

Results also showed that 18 nmol/L triptolide reduced MMP-9 activity almost completely but had little effect on MMP-2 activity (Figure 3).

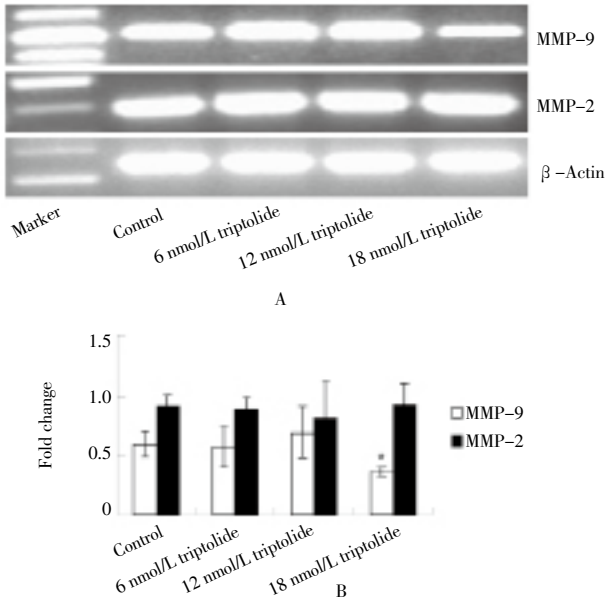

Figure 2. Effect of triptolide on the mRNA expression of MMP-2 and MMP-9 in HT-1080 cells.
(A) RT-PCR result of MMP-2 and MMP-9 expression. Marker = 100 bp ladder; (B) Fold change of MMP-2 and MMP-9 expression, * P < 0.05 compared with control.

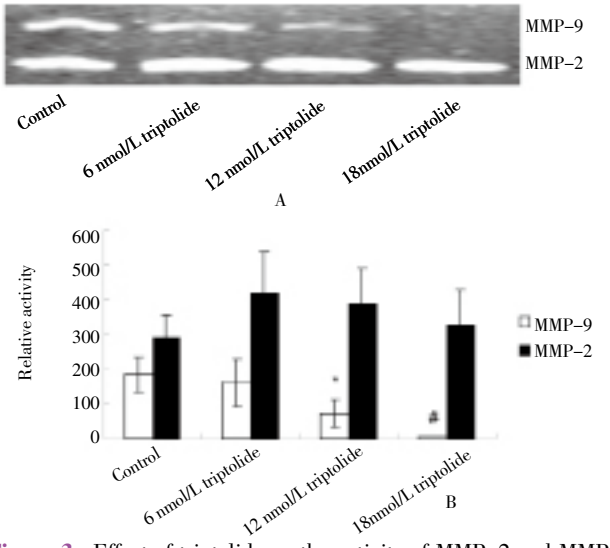

Figure 3. Effect of triptolide on the activity of MMP-2 and MMP-9 in HT-1080 cells.
(A) Content of gelatinases in serum-free supernatants; (B) Relative activity of MMP-2 and MMP-9, *P<0.05 and # P<0.01 compared with control.

3.3. Effect of triptolide on HT-1080 cell invasion

The modified Matrigel invasion assay showed that compared with the control, incubation with 18 nmol/L triptolide or 3 μg/mL anti-MMP-9 for 72 h significantly reduced the number of HT-1080 cells which invaded through the Matrigel coating, by about 50% and 35%, respectively,. However, no significant difference was found between the invasiveness of 18 nmol/L triptolide treated HT-1080 cells and that of anti-MMP-9 treated cells (Figure 4),

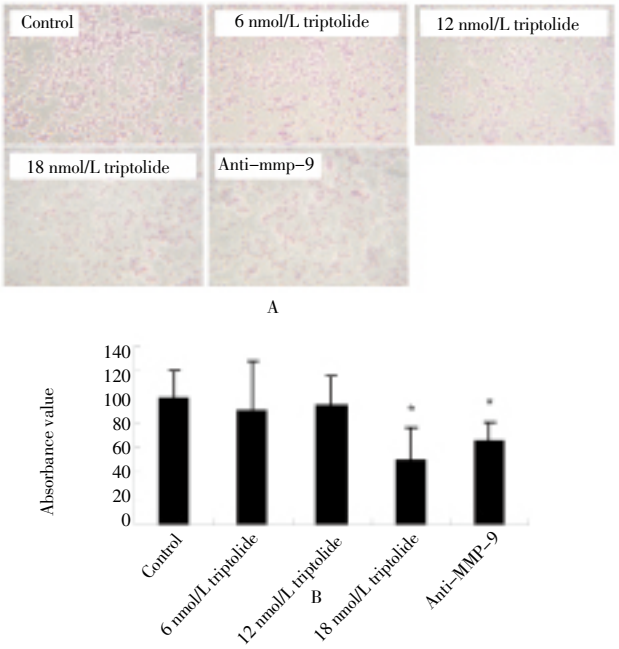

Figure 4. Effect of triptolide on invasiveness of HT-1080 cells *in vitro*. (A) Electroscopic result of HT-1080 cells; (B) Absorbance value, * P < 0.05 compared with control.

4. Discussion

Most cancer patients do not die from local complications of their primary tumor growth, but rather from the development and spread of the tumor. Therefore, metastasis is one of hallmarks of malignant tumor and a major cause of death among cancer patients. Several reports have indicated that triptolide can reduce the growth and metastasis of tumors *in vivo* and *in vitro*, via inhibition of heat shock protein 70 (HSP70), CXC chemokine receptor 4 (CXCR4), or urokinase-type plasminogen activator receptor (uPAR)[5,12,13]. In the present study, we demonstrated that triptolide effectively inhibited the invasion of highly matastatic human fibrosarcoma HT-1080 cells to extracellular matrix (ECM) at a nontoxic concentration, implying the potent antimetastatic activity of triptolide.

Neoplasm metastasis involves a series of complex processes that spread tumor cells from the primary site to a distant location. MMP-9, also known as gelatinase B, has been involved in many steps of tumor metastasis, including tumor growth, invasion, migration, host immune escape, extravasation and angiogenesis[2,14-16]. In this study, we showed that 18 nmol/L triptolide inhibited almost completely the gene expression and activity of MMP-9 in HT-1080 cells. The data suggest that the anti-metastatic activity of triptolide might be, at least partly, due to inhibition of

MMP-9.

Furthermore, our experiments also showed that the invasion ability of 18nmol/L triptolide treated HT-1080 cells was much lower than that of anti-MMP-9 treated cells, though no significant difference was detected between each other. These results indicate that triptolide may inhibit HT-1080 cell metastasis through mechanisms other than downregulating MMP-9 expression.

On the other hand, culture of HT-1080 cells treated with 18nmol/L triptolide also had a high number of invading cells, which might be due to the long incubating time and the high invasive potential of HT-1080 cells.

Although MMP-9 shares fairly broad substrate specificity and structural features with MMP-2, both enzymes differ considerably in terms of transcriptional regulation. The 5′ flanking sequence of the MMP-9 gene harbors TATA boxes and several consensus motifs for AP-1, NF-κB, Sp-1, and Ets transcription factors, and transcription of the gene requires synergistic cooperation of AP-1 with either NF-κB or Sp-1[17, 18]. In contrast, the promoter of MMP-2 contains no AP-1 binding site nor TATA box, and the expression of this gene is mainly determined by Sp-1, which binds to a proximal GC box [17]. These could be presumed to be one of the reasons for the different effect of triptolide on the gene expression of MMP-9, compared with MMP-2.

Our previous study demonstrated that triptolide up-regulated the DNA methylation level of MMP-9 gene in HT-1080 cells[19]. Furthermore, Chicoine et al found an inverse correlation between level of methylation of the MMP-9 promoter and the level of MMP-9 expression in a series of lymphoma cell lines[20]. Given the importance of DNA methylation pattern in gene expression, further studies are required to investigate whether triptolide inhibits MMP-9 expression by elevated DNA methylation level of the gene in a variety of cells.

The over-expression of MMPs in the tumor microenvironment depends not only on the cancer cells, but also on the neighboring stromal cells, which are induced by the cancer cells in a paracrime manner. To determine the effect of triptolide exert on MMPs expression in other cell types, further studies are required also.

In conclusion, we demonstrated that triptolide inhibited the invasiveness of human fibrosarcoma HT-1080 cells *in vitro*, partly by directly reducing MMP-9 gene expression and activity. Our results cast new light on the molecular mechanisms underlying the anti-tumor effects of triptolide.

Conflict of interest statement

We declare that we have no conflict of interest.

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