Triptolide Inhibits Tumor Promoter-induced uPAR Expression *via* Blocking NF-κB Signaling in Human Gastric AGS Cells

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Abstract. *The overexpression of urokinase-type plasminogen activator receptor (uPAR) is closely related to tumor cell invasion. Therefore, strategies for down-regulating uPAR expression may be of clinical utility. This study examined the effects of triptolide, which is a diterpenoid triepoxide extracted from the Chinese herb Tripterygium wilfordii Hook F., on the induction of uPAR in human gastric cancer AGS cells. Triptolide inhibited the phorbol 12-myristate 13-acetate (PMA)-induced uPAR mRNA and protein expression in a dose-dependent manner, and reduced the uPAR transcriptional activity. The stability of the uPAR transcripts was not altered by the triptolide treatment. The signals involved in uPAR induction by PMA were investigated to determine the mechanisms for the triptolide-mediated regulation of uPAR. The inhibitors of extracellular signal-regulated kinases 1 and 2 (Erk-1/2, PD98059), c-Jun N-terminal kinases (JNK, SP600125) and nuclear factor-kappa B (NF-κB, Bay11-7082) inhibited the PMA-induced expression of uPAR, which suggests that PMA induces uPAR through multiple signals. Triptolide suppressed the PMA-induced activation of NF-κB but not Erk-1/2 and JNK. The inhibitory effect of triptolide on the activation of NF-κB was confirmed by an electrophoretic mobility shift assay and NF-κB dependent transcription studies. AGS cells treated with PMA showed a remarkably enhanced invasiveness, which was partially abrogated by triptolide and uPAR-neutralizing antibodies. This suggests that triptolide may exert at least part of its anti-invasive effect in gastric cancer by controlling the expression of uPAR through the suppression of NF-κB activation.*

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Although the incidence of gastric cancer has decreased over the last few decades, it is still the most common digestive tract cancer with a poor prognosis. Approximately 80% of gastric carcinomas in Western countries are of an advanced stage at presentation (1). Radiation therapy or chemotherapy does not significantly affect the length or quality of patient's life due to local invasion and metastasis of advanced gastric cancer. The development of effective anti-invasive strategies for gastric cancer is expected to help improve the treatment outcomes.

Extracts from the herb *Tripterygium wilfordii* Hook F. have been used in traditional Chinese medicine for more than two centuries to treat a variety of autoimmune and inflammatory diseases including rheumatoid arthritis. Triptolide (Figure 1) has been identified as one of the major components responsible for the immunosuppressive and anti-inflammatory effects of this herb (2). Recent studies have reported that triptolide also inhibited the proliferation of cancer cells *in vitro*, and reduced the growth of some tumors *in vivo* (3-5). In addition, triptolide inhibited experimental metastasis in a nude mouse model (4). However, the precise mechanisms by which triptolide inhibits tumor growth and metastasis are unknown.

Cancer invasion and metastasis are multifactorial processes that require the coordinated action of cell-secreted proteolytic enzymes and their inhibitors (6). Urokinase-type plasminogen activator (uPA), its inhibitors, and uPA receptor (uPAR) form a complex proteolytic system that has been implicated in cancer invasion and metastasis. uPA is a serine protease that can convert plasminogen to active plasmin (7). Furthermore, the uPA-uPAR binding interaction independently affected cell motility, integrin function, and gene expression (8). In a number of carcinomas, uPAR expression has been shown to be essential for the invasive and metastatic phenotype (9). The overexpression of uPAR increased the ability of cells to penetrate the barrier of the reconstituted basement membrane and, conversely, a blockade of the uPAR through the expression of a catalytically inactive enzyme or an antisense uPAR cDNA

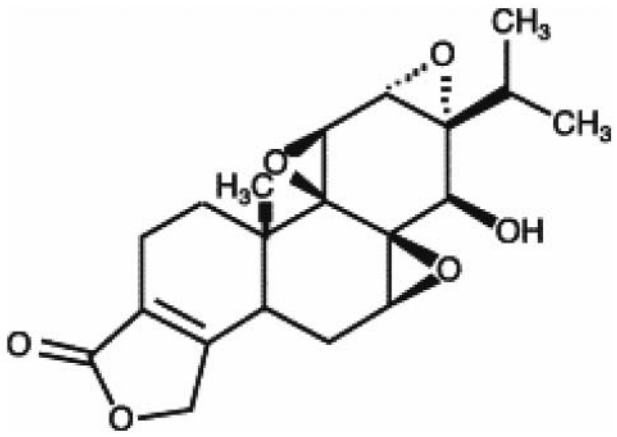

Figure 1. *Chemical structure of triptolide.*

remarkably decreased the invasiveness of cancer cells (10). In gastric carcinomas, increased levels of uPA and uPAR have been shown to be essential for maintaining the invasive and metastatic phenotypes, and have been considered prognostically significant (11).

This study examined the effects and underlying mechanisms of triptolide on the tumor promoter-induced cell invasiveness and uPAR expression in gastric cancer AGS cells.

Materials and Methods

Cell culture and culture conditions. Human gastric carcinoma AGS cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured at 37˚C in a 5% CO_2 atmosphere with RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin. The effects of triptolide on the phorbol 12-mysistate 13-acetate (PMA)-stimulated expression of uPAR were examined by harvesting the cells at different intervals and measuring the level of uPAR mRNA by Northern blot analysis.

In order to examine the role of specific signaling pathways in uPAR inhibition by PMA stimulation, the AGS cells were pretreated with 50 μM PD98059 (a specific inhibitor of MEK-1, an upstream effector of Erk-1/2; New England Biolabs Inc., Beverly, MA, USA), 20 μM SP600125 (a c-jun *N*-terminal kinase (JNK) inhibitor; Calbiochem, San Diego, CA, USA), 20 μM SB203580 (a specific P38 MAPK inhibitor, Calbiochem), 300 nM wortmannin (a PI-3 kinase/Akt inhibitor, Calbiochem) and 10 and 20 μM BAY 11-7082 (a nuclear factor-kappa B (NF-κB) inhibitor, Calbiochem) for 1 h before exposing the cells to PMA. The level of uPAR mRNA was then measured by Northern blot analysis.

Northern blot analysis. The total RNA extraction and Northern blot hybridization were performed using the methodologies described elsewhere (12). Each cDNA probe was radiolabeled with [α-^{32}P]deoxyribonucleoside triphosphate using the random-priming technique with the Rediprime labeling system (Amersham Corp., Arlington Heights, IL, USA). The probed nylon membranes were exposed to radiographic film (Life Technologies Inc., Grand Island, NY, USA).

MTT assay. The cells were grown to confluence in RPMI-1640 medium containing 10% FBS, harvested by trypsinization, and plated at 5,000 cells/well in a 96-well plate. The cells were growth arrested for 24 h by replacing the medium with fresh medium containing 0.5% FBS and 50 μg/ml of penicillin/streptomycin. Low serum levels were maintained during growth arrest in order to prevent the slow apoptosis that accompanies complete serum deprivation in cell lines. After 24 h, 0-100 nM triptolide was added to the medium containing 1% FBS, and the cells were incubated for 6 h. The cell viability was determined using an MTT assay in which the MTT was converted to formazan granules in the presence of molecular oxygen. After incubation, 10 μl of 5 mg/ml MTT were added to each well of the 96-well plates and incubated at 37˚C for 2 h. The formazan granules obtained were dissolved in 100% DMSO, and the absorbance at 562 nm was detected using a 96-well ELISA reader (Biotek Inc., Winooski, VT, USA).

Measurement of uPAR promoter assay. The transcriptional regulation of uPAR was examined by transient transfection with an uPAR promoter-luciferase reporter construct (pGL3-uPAR) (13). The plasmid pGL3/uPAR-promoter was kindly provided by Dr. Y. Wang (Australian National University, Canberra, Australia). The AGS cells ($5x10^5$) were seeded and grown until they reached 60-70% confluence, and pRLTK (an internal control plasmid containing the *Herpes simplex* thymidine kinase promoter linked to the constitutively active Renilla luciferase reporter gene) and pGL3-uPAR were cotransfected into the cells using FuGENE (Boehringer-Mannhein, Indianapolis, IN, USA) according to the manufacturer's protocol. pRLTK was transfected as a negative control. The cells were incubated in the transfection medium for 20 h. The effect of triptolide on PMA-induced uPAR was examined by treating the cells with 0-100 nM triptolide for 1 h before incubating them with 100 nM PMA for 4 h. Similary, the effects of the MAPK signaling pathway inhibitors on the uPAR promoter activity were determined by pretreating the cells with the inhibitors for 1 h before adding 100 nM PMA. After incubation, the cells were lysed and the luciferase activity was measured using a luminometer according to the manufacturer's protocol.

Transient transfection of NF-κB reporter. The NF-κB reporter construct was purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA). Once the cells had reached 80-90% confluence, they were washed with RPMI-1640 and incubated with RPMI-1640 in the absence of serum and antibiotics for 5 h. The cells were then transfected with the pGL3 vector containing 1 μg of the NF-κB reporter using FuGENE^6 (Boehringer-Mannheim) for 24 h. The roles of the specific signaling pathways in NF-κB inhibition by triptolide were determined by treating the transfected cells with different doses of triptolide for 1 h before incubating them with PMA for 4 h. After incubation, the cells were lysed and the luciferase activity was measured using a luminometer.

Western blot analysis. Protein extraction and Western blot analysis were performed using the methodologies described elsewhere (14). The primary antibody preparations used in this study were 1:1000 dilutions of rabbit policlonal anti-phosphospecific extracellular signal-regulated kinase (Erk-1/2) and anti-phospho-specific JNK (New England Biolabs Inc., Beverly, MA, USA) and the anti-uPAR antibody (American Diagnostica Inc., Hauppauge,

NY, USA). The secondary antibody was horseradish peroxidase-labeled anti-rabbit immunoglobulins from donkeys (Amersham Corp., Piscataway, NJ, USA) used at a 1:3000 dilution. The protein bands were visualized using a commercially available chemiluminescence kit (Amersham Corp.). The total protein levels were assayed by washing the blotted membrane with a stripping solution (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 62.5 mM Tris-HCl (pH 6.7)) for 30 min at 50˚C, and reprobing the membrane with rabbit polyclonal anti-actin antibodies diluted at 1:1000.

Extraction of nuclear proteins. Eighty to ninety percent confluent AGS cells were incubated overnight in a medium containing 1% FBS and treated with 100 nM PMA for various periods. The effect of triptolide on PMA-induced NF-κB-DNA complex formation was determined by pretreating the cells with 0-100 nM triptolide for 1 h before exposing them to PMA. The cells were then resuspended in 500 ml cold buffer A (50 mM Tris (pH 7.4), 150 mM NaCl, 0.2 mM EDTA, 3% (v/v) glycerol, and 1.5 mM $MgCl_2$). After allowing the cells to swell for 5 min on ice, they were lysed with 500 μl of buffer B [identical to buffer A, except containing 0.05% Nonidet P-40 (Sigma, St Louis, MO, USA)]. The homogenate was gently layered onto an equal volume cushion of buffer C (100 mM Tris (pH 7.4), 25% (v/v) glycerol, and 1.5 mM $MgCl_2$) and centrifuged for 5 min at 200 xg. The white nuclear pellet was resuspended in 75 μl of a cold high-salt lysis buffer (20 mM Hepes (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). This suspension was agitated for 30 min at 4˚C and then microcentrifuged for 15 min at 4˚C. The resulting supernatant was stored in aliquots at –80˚C. The protein was quantified spectrophotometrically using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

Electrophoretic mobility shift assay (EMSA). The EMSA was carried out using a gel shift assay system (Promega, Madison, WI, USA). Briefly, the oligonucleotides containing the consensus sequences for NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG-3') were end-labeled with [α-^{32}P] adenosine triphosphate (3000 ci/mmol; Amersham Pharmacia Biotech, Buckinghamshire, UK) using T4 polynucleotide kinase. The resulting oligonucleotides were then purified in Microspin G-25 columns (Sigma) and used as probes for EMSA. The nuclear extract proteins (6 μg) were preincubated with the binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 4% (v/v) glycerol, and 0.05 mg/ml poly (deoxyinosine-deoxycytosine)) for 5 min and then incubated with the labeled probe for 15 min at 37˚C. Each sample was electrophoresed in a 5% nondenaturing polyacrylamide gel in 0.5 x Tris borate-EDTA buffer at 150 V for 4 h. The gel was dried and subjected to autoradiography. In competition studies, a 50-fold excess of the unlabeled oligonucleotide was included in the reaction mixture along with the radiolabeled probe.

Matrigel invasion assay. The cell invasion assay was performed using BIOCOAT matrigel invasion chambers (Becton-Dickinson, Bedford, MA, USA) according to the manufacturer's protocol. The cells were grown in 6-well plates and treated with triptolide for 1 h, and then detached using 1 ml TrypLE (Invitorgen, Carlsbad, CA, USA). Briefly, 10^5 cells in 300 μl were added to each chamber with 100 nM PMA and allowed to invade the matrigel for 24 h. The non-invading cells in the upper surfaces of the membrane were removed from the chamber, and the invading cells in the lower surface of the membrane were stained using the Quick-Diff stain kit (Becton-Dickinson). After two washes with water, the chambers were allowed to air dry. The number of invading cells was counted using phase-contrast microscopy.

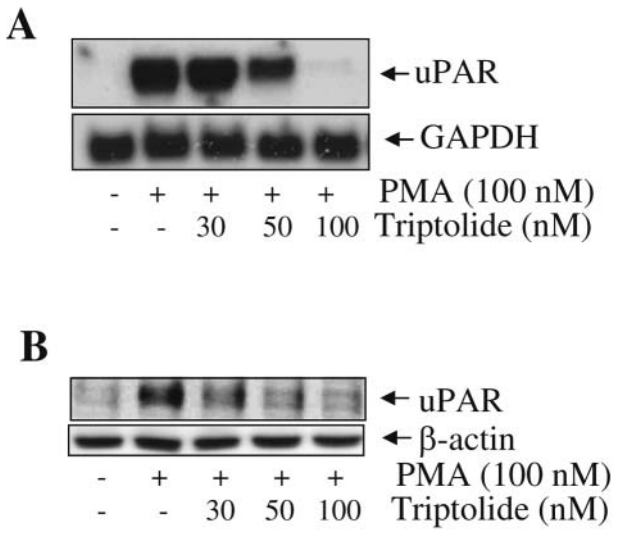

Figure 2. *Effect of triptolide on the PMA-induced expression of uPAR in AGS cells. The AGS cells were treated with 0-100 nM triptolide for 1 h, and then incubated with 100 nM phorbol 12- myristate 13-acetate (PMA) for 4 h. Northern and Western blot analyses were performed to determine the level of uPAR mRNA (A) and protein (B) expression, respectively. Glyceraldehyde-phosphate dehydrogenase (GAPDH) mRNA was used as a control.*

Result

Effect of triptolide on PMA-induced uPAR mRNA and protein expression. Triptolide inhibited the PMA-induced expression of uPAR mRNA in a dose-dependent manner (Figure 2A). As shown by Western blot analysis, the PMA-induced uPAR protein level was also decreased by the triptolide treatment (Figure 2B). Triptolide did not affect the cell viability at the concentrations used in this study.

Effects of triptolide on uPAR promoter activity and mRNA stability. The AGS cells transfected with pGL3-uPAR showed approximately 4-fold increases in the promoter activity after the 100 nM PMA treatments. However, as shown in Figure 3A, the AGS cells treated with triptolide before adding PMA showed a decrease in promoter activity in a dose-dependent manner. In order to determine if triptolide could alter the stability of uPAR mRNA, the AGS cells were cultured with 1 μg/ml actinomycin D for 0-8 h in the presence or absence of 50 nM triptolide, and the uPAR mRNA levels were determined. As shown in Figure 3B, triptolide did not affect the mRNA stability of the uPAR gene.

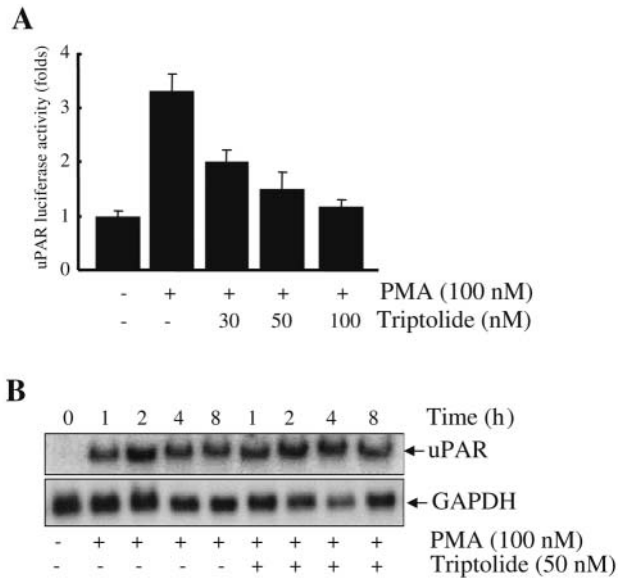

Figure 3. *Effect of triptolide on the uPAR transcription activity and mRNA stability in gastric AGS cells. (A) The cells transiently transfected with the uPAR-Luc plasmid were pretreated with the indicated concentrations of triptolide for 1 h before treating them with 100 nM PMA for 4 h. The cells were lysed and the luciferase activity was measured. The data represent the mean±S.D. of at least three independent experiments. (B) After the AGS cells had been cultured with 100 nM PMA for 4h, 1 mg/ml of actinomycin D was added to the cells in the presence or absence of triptolide (50 nM). The total RNA was extracted from the cells at the indicated times after adding actinomycin D, and the uPAR mRNA level was determined by Northern blotting.*

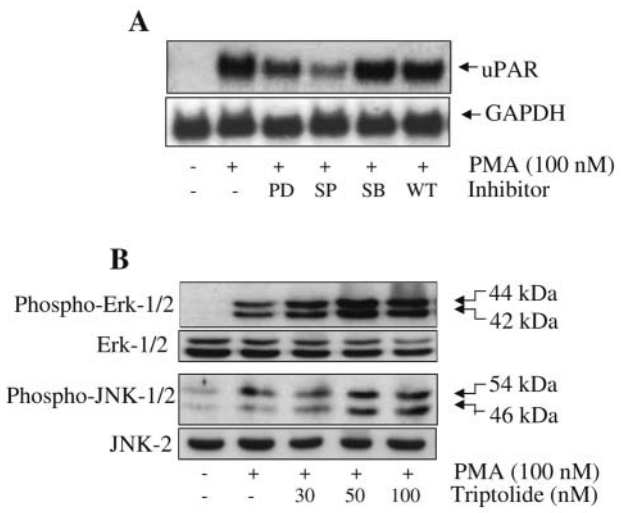

Figure 4. *Suppression of uPAR expression by the MAPK inhibitors and the effect of triptolide on the Erk-1/2 and JNK activation. (A) AGS cells were pretreated with 50 μM PD98059 (PD, MEK-1 inhibitor), 20 μM SP600125 (SP, JNK inhibitor), 20 μM SB203580 (SB, P38 inhibitor) and 300 nM wortmannin (WT, PI-3 kinase/Akt inhibitor) for 1 h and incubated with 100 nM PMA for 4 h. After incubation, the level of uPAR mRNA expression was determined by Northern blot analysis. (B) AGS cells were pretreated with 0-100nM triptolide for 1 h and treated with 100 nM PMA for 15 min. After incubation, the phosphorylated Erk-1/2 and JNK were determined by Western blot analysis.*

Effects of triptolide on the MAPKs signaling pathways in the uPAR expression. As shown in Figure 4A, PD98059 and SP600125 noticeably reduced the expression of uPAR mRNA, whereas wortmannin and SB203580 had only a negligible effect. These results suggested that both the Erk-1/2 and JNK pathways were involved in the PMA-induced expression of uPAR in the gastric cancer AGS cells. Since inhibitors of Erk-1/2 and JNK inhibited the PMA-induced expression of uPAR, the effects of triptolide on the activation of Erk-1/2 and JNK were examined. As shown in Figure 4B, triptolide did not decrease the level of Erk-1/2 and JNK phosphorylation induced by PMA. This suggests that triptolide inhibited the PMA-induced uPAR expression through an Erk-1/2- and JNK-independent mechanism.

Effects of triptolide on the NF-κB signaling pathways in the uPAR expression. As shown in Figure 5A, Bay 11-7082 (a specific NF-κB inhibitor) effectively blocked the PMA-induced expression of uPAR mRNA in a dose-dependent manner. When the transfected cells were pretreated with Bay 11-7082 before the PMA treatment, the induction of uPAR promoter activity by PMA was remarkably inhibited (data not shown). The NF-κB activities of the cells treated with triptolide before the PMA treatment were examined to determine if NF-κB is an important target for triptolide in AGS cells. As shown in Figure 5B, the PMA treatment caused a remarkable increase in the amount of NF-κB that could form a complex with the radiolabeled oligonucleotide probe in EMSA. However, the binding of NF-κB-DNA in the cells pretreated with triptolide decreased in a dose-dependent manner. Consistent with the EMSA result, the PMA treatment increased the NF-κB-dependent transcriptional activity, while the triptolide treatment decreased the PMA-induced NF-κB-dependent transcriptional activity in a dose-dependent manner (Figure 5C).

Effect of triptolide on PMA-induced AGS cell invasiveness. As shown in Figure 6A, the PMA treatment increased the AGS cell invasiveness in a modified Boyden invasion chamber up to approximately 200%, and this stimulatory effect was significantly abolished by the anti-uPAR antibody. In contrast, the Matrigel invasiveness was not lost after incubating the cells with nonspecific IgG. As shown in Figure 6B, the cells pretreated with triptolide lost the Matrigel invasiveness induced by PMA in a dose-dependent manner.

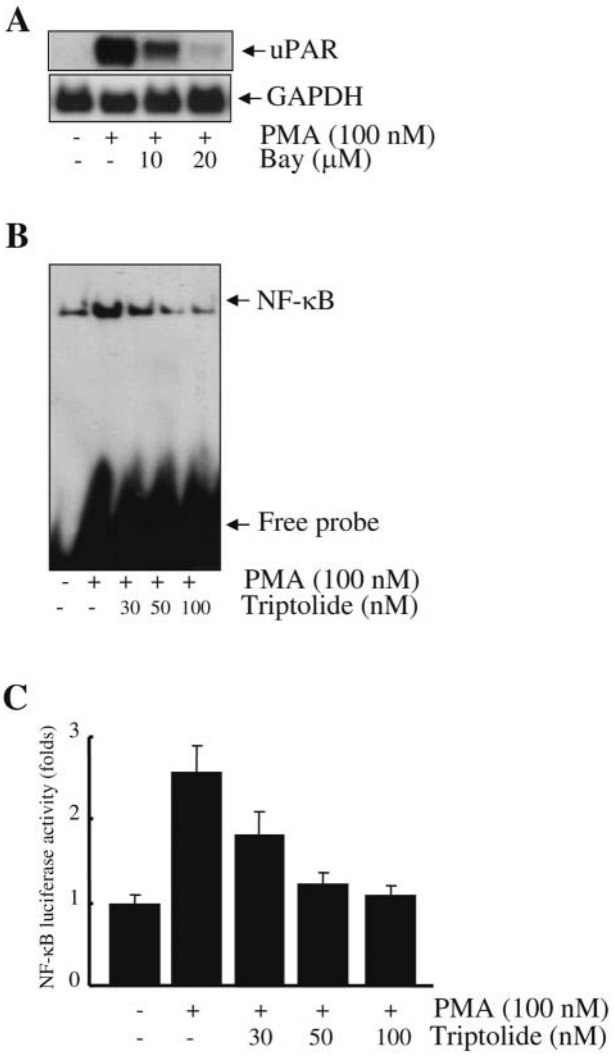

Figure 5. *Inhibition of uPAR expression by NF-κB inhibitor and the effect of triptolide on the NF-κB activation. (A) AGS cells were pretreated with 0-20 μM Bay11-7082 (NF-κB inhibitor) for 1 h, and incubated with 100 nM PMA for 4 h. After incubation, the level of uPAR mRNA in the cell lysates was determined by Northern blot analysis. (B) AGS cells were pretreated with the indicated concentrations of triptolide for 1 h before incubation with 100 nM PMA for 4 h. After incubation, the nuclear proteins were extracted from the cells and the level of NF-κB-DNA binding was determined by EMSA. (C) The cells were transiently transfected with a NF-κB-Luc plasmid, treated with the indicated concentrations of triptolide for 1 h and then with 100 nM PMA for 4 h. The cells were lysed and the luciferase activity was measured. The data represent the mean±SD of at least three independent experiments.*

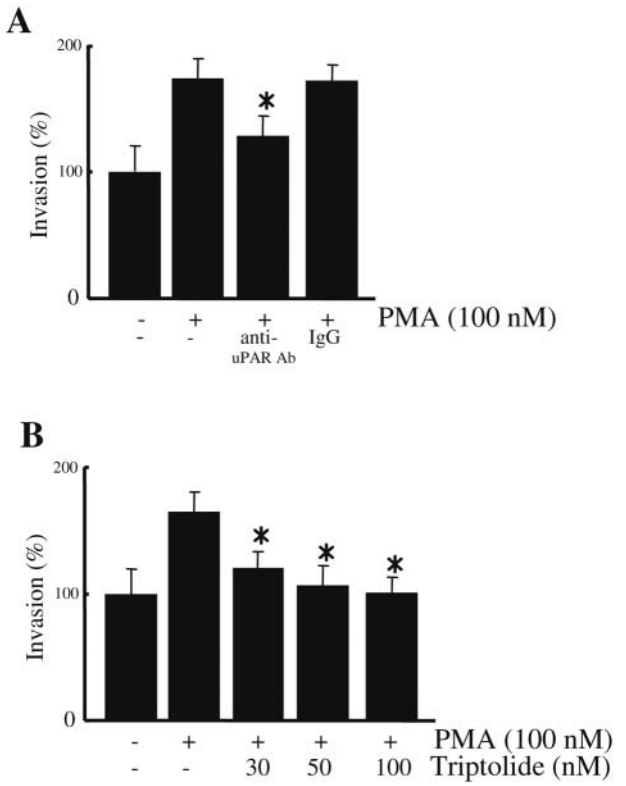

Figure 6. *Effect of triptolide on the PMA-induced AGS cell invasiveness. (A) AGS cells (10^5) were incubated in Biocoat Matrigel invasion chambers for 24 h with 100 nM PMA in the presence or absence of 50 μg/ml anti-PAR antibodies (anti-PAR) and nonspecific IgG (IgG). (B) AGS cells (10^5) pretreated with the indicated concentration of triptolide were incubated in Biocoat Matrigel invasion chambers for 24 h with 100 nM PMA. After incubation, the invading cells on the undersurface of the chambers were counted using a phase-contrast optical microscopy after staining using the Diff-Quick stain kit. The data represent the mean±S.D. from triplicate measurements. *p<0.05 versus PMA alone.*

Discussion

Localized gastric cancer can be cured by surgery. However, the high mortality in advanced gastric cancer is associated with the propensity of the tumor to metastasize. The interest in triptolide as a cancer chemopreventive agent in humans has intensified for several reasons. First, triptolide appears to be active against a broad spectrum of tumors resulting in the 50-90% inhibition of primary tumors derived from the breast, bladder, stomach and melanomas (15). Second, triptolide blocked the formation of metastases in experimental mouse models injected with B16 melanoma cells. Therefore, triptolide can inhibit the growth of both primary and secondary tumors. Third, triptolide enhances chemotherapy. It increased doxorubicin-mediated apoptosis by blocking p21-mediated growth arrest (16). Fourth, triptolide has a high level of antitumor activity. When tested against tumor cells growing in tissue culture, triptolide was more effective on a molar basis than other chemotherapeutic agents, such as taxol, adriamycin, mitomycin, and cisplatin. Similarly, in the mouse model system, triptolide was more potent in inhibiting the growth of tumor xenografts than adriamycin, mitomycin, or cisplatin (17).

This study has shown that triptolide inhibited the expression of uPAR mRNA and protein, possibly through the suppression of NF-κB activation, which resulted in the inhibition of cell invasiveness. Through a transient transfection study with the uPAR promoter reporter constructs, it was found that triptolide inhibited the uPAR promoter activity. However, the half-life of uPAR mRNA was not altered by the triptolide treatment. Therefore, uPAR expression might be regulated at the transcriptional level, but not at the post-transcriptional level.

Several studies have reported that MAPKs play important roles in regulating the uPAR gene, even though the profiles of MAPK activation appear to vary in a cell type-dependent manner. Recent studies have also demonstrated that the PMA-dependent stimulation of the uPAR gene expression requires JNK1-dependent and -independent signaling modules (18). This study found that PD98059 (a specific MEK-1 inhibitor, and an upstream effector of Erk-1/2), and SP600125 (a c-jun-*N*-terminal kinase inhibitor) significantly decreased the expression of uPAR mRNA, whereas SB203580 (a specific P38 MAPK inhibitor), and wortmannin (a PI-3 kinase/Akt inhibitor) had only a negligible effect. PMA also induced the activation of Erk-1/2 and JNK in the AGS cells. However, triptolide did not reduce the level of Erk-1/2 and JNK phosphorylation induced by PMA. This suggests that triptolide inhibited the PMA-induced expression of uPAR through Erk-1/2- and JNK-independent mechanisms. Several studies have reported that triptolide can regulate the MAPK and PI-3 kinase activities in various cell systems. Miyata *et al.* reported that triptolide induced antitumor proliferation through the activation of JNK by decreasing the PI-3 kinase level in human cells (19). Kim *et al.* also reported that triptolide inhibited the expression of murine-inducible nitric oxide synthase by down-regulating the lipopolysaccharide-induced activity of NF-κB and JNK (20). These differences in the regulation of MAPKs and PI-3 kinase by triptolide could be attributed to the different experimental systems employed.

The following observations in this study suggested that triptolide blocks the PMA-induced expression of uPAR through the suppression of NF-κB in the AGS cells. Firstly a specific NF-κB inhibitor (Bay 11-7082) significantly blocked the PMA-induced uPAR mRNA expression and promoter activity. Secondly the PMA treatment caused a remarkable increase in the amount of NF-κB that could form a complex with the radiolabeled oligonucleotide probe in EMSA. However, the level of NF-κB-DNA binding in the cells pretreated with triptolide decreased in a dose-dependent manner. Thirdly the triptolide treatment decreased the PMA-induced NF-κB-dependent transcriptional activity. The precise mechanism by which triptolide inhibited the activation of NF-κB is unknown. One explanation is that triptolide could suppress the production of reactive oxygen species (ROS) by PMA. ROS are known to activate NF-κB through the phosphorylation of I-kB (21). Therefore, triptolide might prevent the ROS induction of NF-κB activation in uPAR gene expression.

This study found that triptolide could block uPAR expression and cell invasiveness in human gastric cancer AGS cells, which suggests that the down-regulation of uPAR by triptolide is involved in decreasing cell invasiveness. However, further studies will be needed to clarify these mechanisms. Understanding the molecular mechanisms by which triptolide inhibits NF-κB activation and uPAR expression will serve as a basis for designing even more effective anti-invasion drugs.

Acknowledgements

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