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Inhibitory effect of emodin on tumor invasion through suppression of activator protein-1 and nuclear factor-κB

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Abstract

3-Methyl-1,6,8-trihydroxyanthraquinone (emodin) is an active component from the rhizome of *Rheum palmatum*, a widely used traditional Chinese herb. In this study, we found that emodin significantly inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced in vitro invasion of human cancer cells including HSC5 and MDA-MB-231 cells. Matrix metalloproteinases (MMPs) are known to be associated with cancer invasion. Zymographic analysis showed that emodin suppressed TPA-induced MMP-9 activity in a concentration-dependent manner. We further demonstrated that emodin reduced the transcriptional activity of activator protein-1 (AP-1) and nuclear factor kappaB (NF-κB), two important nuclear transcription factors involved in MMP-9 expression. Emodin suppressed the phosphorylation of two mitogen-activated protein kinases, extracellular signal-regulated protein kinase and c-Jun N-terminal kinase, but not p38 kinase, leading to reduced c-Jun phosphorylation and AP-1 DNA-binding. Moreover, emodin inhibited TPA-induced degradation of inhibitor of kappaBα, nuclear translocation of p65, and NF-κB DNA-binding activity. Taken together, these results suggest that emodin inhibits the invasiveness of human cancer cells by suppressing MMP-9 expression through inhibiting AP-1 and NF-κB signaling pathways.

Keywords: Matrix metalloproteinase; Invasion; NF-κB; AP-1; Chemoprevention; Emodin

1. Introduction

Tumor metastasis is the leading cause of deaths in cancer patients [1]. The process of metastasis consists of a complex cascade of interdependent steps, such as intravasation by tumor cells, circulation in lymph and blood vessels, arrest at distant vascular bed, extravasation, proliferation of cells as a secondary colony, and induction of angiogenesis. Cell invasion, the process of translocation of neoplastic cells across extracellular matrix barriers, is one of essential biological events required for tumor metastasis [2].

Expression of MMPs has been associated with tumor invasion and metastasis due to their ability to proteolytically degrade extracellular matrix components such as collagen, fibronectin, and laminin [3]. MMPs are a large family of proteases sharing a diverse range of substrates and currently divided into several subgroups: gelatinases, collagenases, stromelysins, stromelysin-like MMPs, matrilysins, membrane-type MMPs, and other MMPs [4]. Particularly the gelatinases, MMP-2 and MMP-9, are known to be essential for cancer cell invasion as they can degrade type IV, V, and VII collagens, gelatin, and some other components of extracellular matrix [4]. MMP-2 is constitutively present in tissues and mainly regulated at a post-transcriptional level by interacting with tissue inhibitor of metalloproteinase-2 [5]. In contrast, the expression of MMP-9 is largely controlled at the transcriptional level and can be stimulated by growth factors, cytokines, UV, and TPA [6].

3-Methyl-1,6,8-trihydroxyanthraquinone (emodin) is an anthraquinone derivative from the rhizome of *Rheum*

Abbreviations: MMP, matrix metalloproteinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; AP-1, activator protein-1; NF-κB, nuclear factor kappaB; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electropheresis; DTT, dithiothreitol; MAPK, mitogen-activated protein kinases; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; EMSA, electrophoretic mobility shift assay; IκBα, inhibitor of kappaBα; S.D., standard deviation

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palmatum, a herb widely used in traditional Chinese medicine as a laxative [7]. It has been reported that emodin possesses a number of biological activities such as vasorelaxative [8], immunosuppressive [9], hepatoprotective [10], and anti-tumor activity [7,11]. Emodin has been shown to be a tyrosine kinase inhibitor that can restrict the activity of $p56^{lck}$ [12], *HER*-2/*neu* [13], and *ras*-oncogene [14], which are involved in the signaling pathways of cell proliferation, transformation and differentiation. Recent studies suggest that emodin could also induce apoptosis in several kinds of cancer cells [15–17]. However, so far there is little evidence showing the possible effects of emodin on tumor invasion and metastasis. Here we investigated the inhibitory effect of emodin on cell invasion using a skin squamous cancer cell line HSC5 and a breast cancer cell line MDA-MB-231. Our findings showed that emodin significantly inhibited the invasiveness of TPA-stimulated cancer cells. We further demonstrated that emodin effectively suppressed AP-1 and NF-κB signaling pathways, leading to decreased MMP-9 expression and activity. These results suggest that emodin inhibits the invasiveness of human cancer cells by suppressing MMP-9 expression through inhibiting both AP-1 and NF-κB activity.

2. Materials and methods

2.1. Chemicals and reagents

3-Methyl-1,6,8-trihydroxyanthraquinone, TPA, parthenolide, PD98059, SB203580, and antibody against α-tubulin were purchased from Sigma. SP600125 was from Calbiochem. Antibodies against c-Jun, JNK, ERK, p38, phospho-c-Jun, phospho-JNK, phospho-ERK, phospho-p38 and Histone H1 were obtained from Cell Signaling. Antibodies against MMP-9, IκBα, p65, and Sp-1 were provided by Santa Cruz. pAP-1-Luc, pNF-κB-Luc and β-galactosidase plasmid were from Clonetech.

2.2. Cell culture

HSC5, derived from human skin squamous cell carcinoma, was kindly provided by Dr. S. Kondo (Yamagata University, Yamagata, Japan). MDA-MB-231 (human breast cancer cell line) was purchased from ATCC. Both cell lines were cultured in monolayers at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 U/ml penicillin.

2.3. Cell invasion assay

Invasion assay was conducted as described previously [18] with modifications using BD Matrigel® invasion chamber. Cells (2×10^5/ml) were inoculated into the culture inserts and stimulated with 80 nM TPA after 2 h pretreatment of different concentrations of emodin. After 12 h incubation, the lower surfaces of the membrane were fixed with 100% methanol and stained with Giemsa solution. Cells that had invaded to the lower surface of the membranes were counted in high-power fields under a microscope.

2.4. Gelatin zymography

Gelatin zymography was performed essentially as reported earlier with modifications [19]. Cells were seeded onto six-well plates in DMEM with 10% FBS and were cultured to 80% confluence. Cells were then washed and maintained in serum-free medium for 24 h prior to designated treatments with TPA and emodin as indicated in the text. The conditioned media were collected after 12 h and standardized with cell number (5×10^5 cells), mixed with nonreducing sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 25% (v/v) glycerol, 0.01% bromophenol blue) and subjected to electrophoresis on a 10% SDS-PAGE gel containing 0.1% (w/v) gelatin. The resulting gels were washed in 10 mM Tris (pH 8.0) containing 2.5% (v/v) Triton X-100, and then incubated for 16 h in developing buffer (50 mM Tris–HCl, pH 7.5, 0.2 M NaCl, 10 mM $CaCl_2$, and 1 μM $ZnCl_2$) at 37 °C. Gels were subsequently stained using 0.5% Coomassie blue R-250 in 5% (v/v) methanol and 10% (v/v) acetic acid for 1 h and destained in 10% (v/v) methanol, 5% (v/v) acetic acid until the bands were seen.

2.5. Western blot

Cells were starved in serum-free medium for 24 h, and then treated as designated. Cell pellets were lysed in lysis buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% (v/v) glycerol) with protease inhibitor cocktail. After a brief sonication lysates were clarified by centrifugation at 20,000 × *g* for 15 min at 4 °C to remove cell debris, and the protein content was measured using Bio-Rad Dc protein assay kit. Aliquots of the lysates were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with primary antibody followed by second antibody and visualized using Super-Signal® West Dura Kit, according to the manufacturer's protocol. Densitometric measurements of the bands in western blot analysis were performed using digitalized scientific software program Kodak 1D 3.5 purchased from Kodak.

For detection of MMP-9 protein level in the medium, conditioned medium was standardized with cell number and incubated for 2 h with gelatin-agarose beads to achieve 10-fold concentrations of the gelatinases. After recovered by centrifugation, beads were washed and resuspended in sample buffer, and heated for 5 min at 100 °C before being subjected to 10% SDS-PAGE gel.

2.6. Preparation of cytosolic and nuclear extract and EMSA

Assays were performed as described previously with modification [20]. In brief, cells were harvested and washed twice with cold phosphate buffer saline (PBS) buffer. The pellet was resuspended in cytosolic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsufonyl fluoride (PMSF), 0.3% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin) on ice for 15 min and the supernatants were collected as cytoplasmic extracts after centrifugation with 2000 × *g* for 10 min. The pellets were resuspended with nuclear protein extraction buffer (20 mM HEPES, pH 7.9, 0.45 M NaCl, 25% (v/v) glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin) and incubated on ice for 30 min. The nuclear proteins were collected by centrifugation with 20,000 × *g* at 4 °C for 15 min. Equal amounts of nuclear extract were mixed with 2 μg of poly(dI-dC), 2 μg of bovine serum albumin, and 100,000 cpm of a ^{32}P-labeled oligonucleotide on ice in 5 × binding buffer (100 mM HEPES/KOH, pH 7.9, 1 mM DTT, 300 mM KCl, and 20% (v/v) glycerol) at room temperature for 30 min. The DNA/protein complex was electrophoresed on a non-denaturing 5% polyacrylamide gel. The gel was dried after electrophoresis and exposed to an X-ray film.

2.7. Luciferase assay

HSC5 cells were subcultured in 24-well plates in triplicate 24 h before transfection. pAP-1-Luc or pNF-κB-Luc was co-transfected with β-galactosidase reporter plasmid using Lipofectamine™ 2000 in antibiotics-free medium. After 18 h incubation, the cells were treated as designated. Cells were then lysed and reporter gene activity was determined with the Promega luciferase assay system using LUMI-ONE luminometer. β-Galactosidase activity was measured using Promega β-galactosidase enzyme assay system and used for normalization of transfection efficiency.

2.8. Statistical analysis

Data are presented as means ± S.D., and analyzed by Student's *t*-test.

3. Results

3.1. Suppressive effect of emodin on TPA-induced invasion of HSC5 and MDA-MB-231 cells

To determine whether emodin could inhibit the invasive property of HSC5 cells, we used transwell plates to measure the cell invasion property following TPA stimulation. As shown in Fig. 1A and B, emodin inhibited the invasiveness of TPA-treated HSC5 cells through Matrigel® coated membrane in a concentration-dependent manner. To rule out the possibility that such inhibition is due to its cytotoxicity, the cell viability of emodin-treated cells was determined using MTT assay. The result showed that emodin had no significant effect on cell viability at concentration ranged from 10 to 40 μM within 14 h treatment (data not shown), suggesting that emodin inhibits cell invasion without obvious cellular cytotoxicity. The inhibitory effect of emodin was also conducted on MDA-MB-231, a breast cancer cell line with high invasive capacity. Similar dose–response inhibitory effect was observed in this cell line (Fig. 1C), indicating that this inhibitory effect was not unique to HSC5 cells.

3.2. Inhibition of TPA-induced MMP-9 activity and expression by emodin

MMP-9 and MMP-2 are the key enzymes in the degradation of type IV collagen, which acts as the "backbone" of cellular basement membrane [3]. Gelatin zymography was performed to examine the activities of MMP-9 and MMP-2 using conditioned medium, which was collected and measured after 12 h TPA stimulation with or without emodin pretreatment. Both MMP-2 and MMP-9 were detected in the conditioned media of HSC5 cells (Fig. 2A), while TPA significantly increased the MMP-9 activity. Emodin pretreatment inhibited MMP-9 activity in a concentration-dependent manner. At 40 μM, emodin reduced MMP-9 activity to the basal level. In contrast, the activity of MMP-2 was not significantly affected by emodin, nor by TPA.

In order to determine whether the down-regulation of MMP-9 activity was due to decreased expression of MMP-9, we next determined the level of MMP-9 protein in the conditioned medium secreted by HSC5 cells. The result showed that emodin inhibited the TPA-induced expression of MMP-9 in a concentration-dependent manner (Fig. 2B). At 40 μM, emodin suppressed the MMP-9 expression to the basal level. Similar inhibitory effect was also observed on MDA-MB-231 cells (data not shown).

3.3. Suppressive effect of emodin on AP-1 signaling pathways

Transcriptional regulation plays an important role in the expression of MMPs, and AP-1 is a major transcription factor that regulates MMP-9 expression [6]. We measured the transcriptional activity of AP-1 by transient transfection with an AP-1 luciferase construct. As shown in Fig. 3A, TPA significantly stimulated the transcriptional activity of AP-1 and emodin efficiently inhibited the TPA-induced AP-1 luciferase activity in HSC5 cells. These data suggest that AP-1 is likely to be involved in the inhibitory effect of emodin on TPA-induced MMP-9 expression.

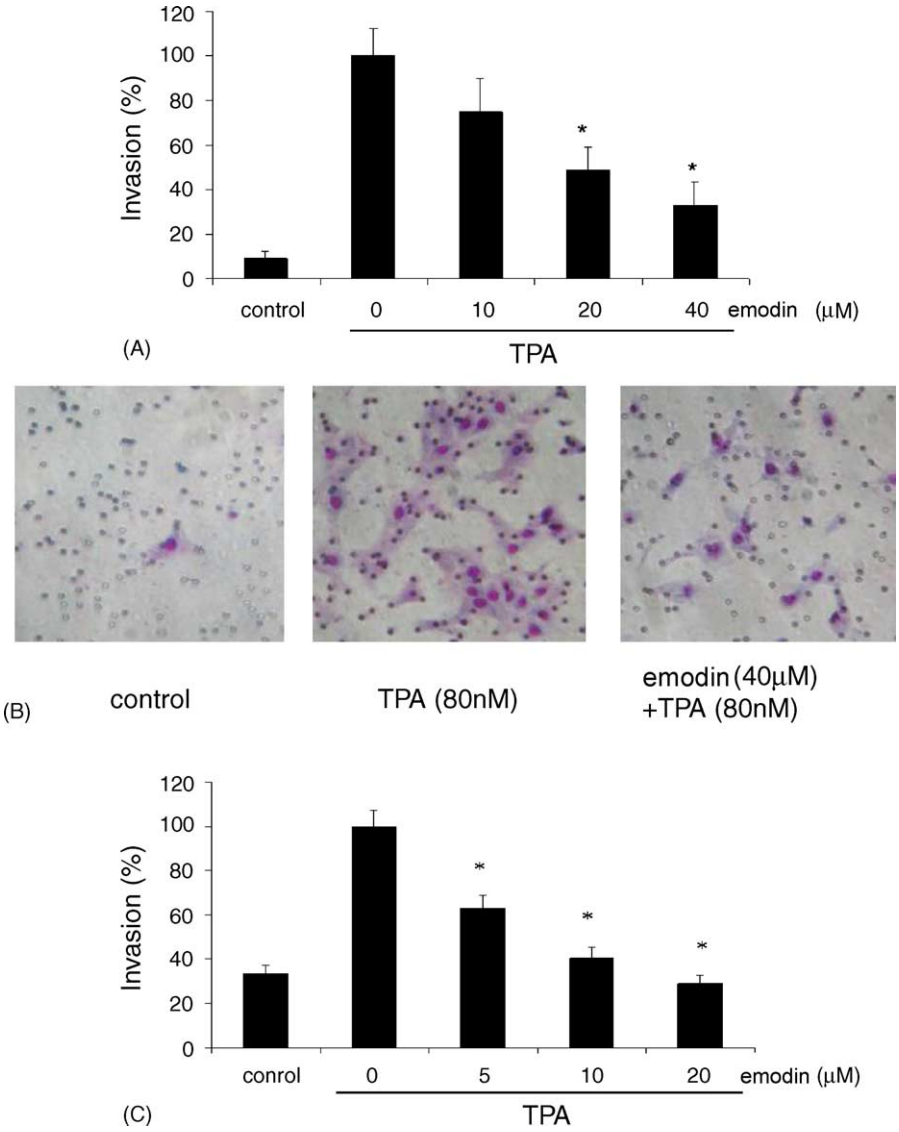

Fig. 1. Inhibition of TPA-induced cell invasion by emodin. (A) Inhibitory effect of emodin on invasiveness of HSC5 cells. Cells (2×10^5/ml) were seeded onto Matrigel® coated membranes of transwell plates and then pretreated with 0–40 μM emodin for 2 h, followed by incubation with 80 nM TPA for additional 12 h. The lower surfaces of the membranes from the transwell units were fixed with 100% methanol and stained with Giemsa solution. Cells that had invaded to the lower surface of the membranes were counted under a light microscope. (B) Photographs of HSC5 cells after invasion through Matrigel. (C) Inhibitory effect of emodin on invasiveness of MDA-MB-231 cells. Cell invasion assay was conducted the same as in A, except for 0–20 μM emodin pretreatment. Data are presented as means ± S.D. All experiments were done in triplicate. ($^*P < 0.05$ compared to the TPA group and analyzed by Student's *t*-test).

To understand the possible mechanisms involved, we further examined the effect of emodin on the DNA-binding activity of AP-1 using EMSA. TPA significantly increased DNA-binding activity of AP-1, and emodin inhibited the binding activity in a concentration-dependent pattern (Fig. 3B), being consistent with the effects of emodin on the transcriptional activity of AP-1 (Fig. 3A). No obvious change of AP-1 DNA-binding activity was observed in the cells treated with emodin alone.

c-Jun is one of the main components of transcription factor AP-1, and also, serves as the target for ERK, JNK and p38 kinase [21,22]. It is well known that c-Jun phosphorylation is essential for the binding and transcriptional activation at TPA-responsive element, which is bound by AP-1 [21,22]. Therefore, we next determined whether c-Jun phosphorylation could be inhibited by emodin. The result indicates that TPA markedly enhanced c-Jun phosphorylation, and emodin significantly impaired c-Jun phosphorylation in a concentration-dependent manner, without affecting the c-Jun protein level (Fig. 3C).

3.4. Inhibitory effect of emodin on the phosphorylation of JNK and ERK, but not p38

The MAPK pathways can influence AP-1 transactivation by increasing the level of AP-1 components or altering the phosphorylation of their subunits such as c-Jun [21,22]. To determine which class of MAPK is involved in

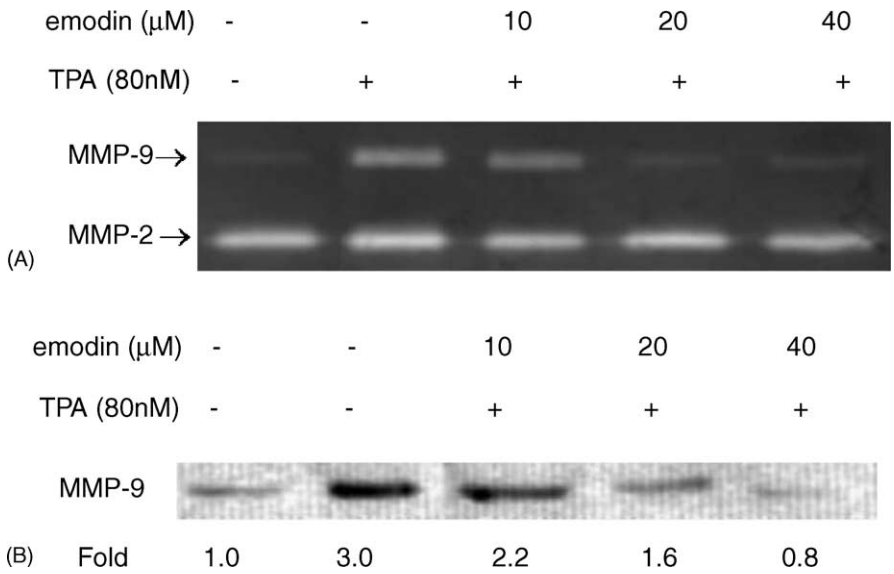

Fig. 2. Effect of emodin on TPA-induced activity and expression of MMP-9 and MMP-2. (A) Concentration-dependent decrease of TPA-induced MMP-9 activity after emodin treatment. HSC5 cells were incubated with 80 nM TPA for 12 h after 2 h pretreatment of 0–40 μM emodin. Conditioned media were harvested and cells were counted. Aliquots of conditioned medium normalized for differences of cell number were subjected to gelatin zymography. (B) Western blot analysis of TPA-induced MMP-9 expression in 10 × concentrated conditioned medium. Cell treatment, conditioned medium collection and normalization are the same as Fig. 2A. Western blot was conducted as described in Section 2.

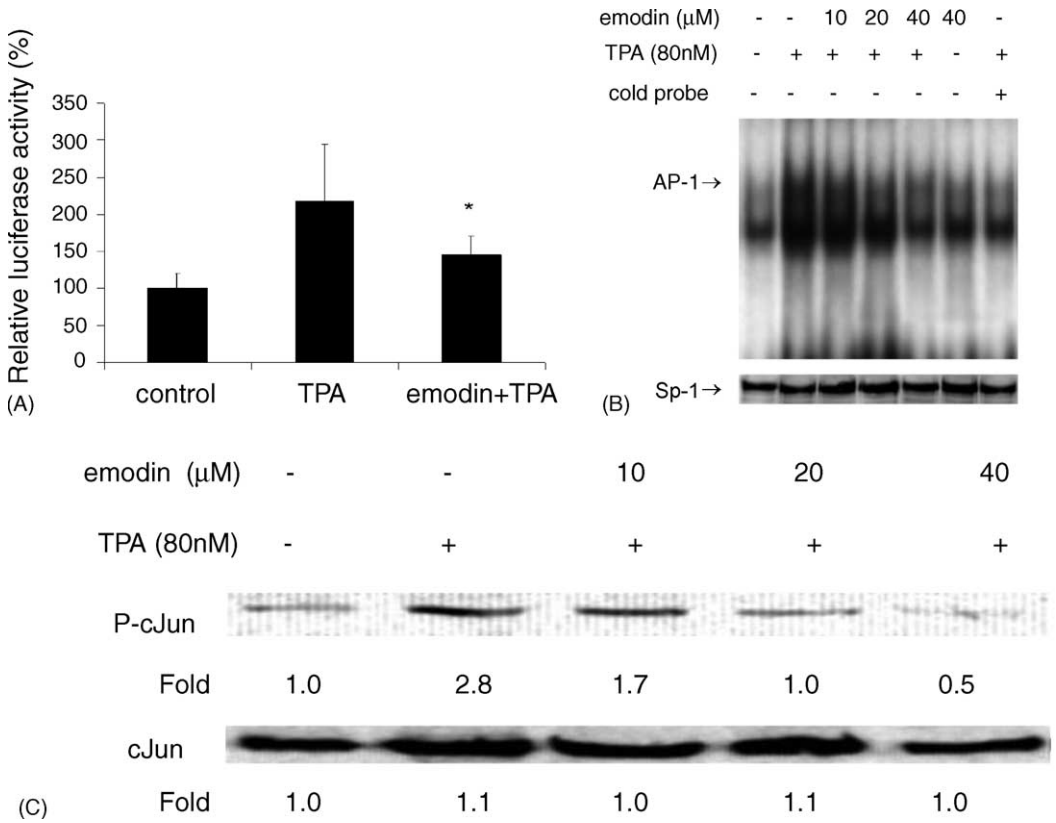

Fig. 3. Inhibitory effect of emodin on AP-1 signaling pathways. (A) Inhibitory effect of emodin on TPA-induced transcriptional activity of AP-1. pAP-1-Luc was transfected into HSC5 cells together with β-gal as normalization of transfection efficiency. Cell treatment and luciferase activity measurement is the same as described in Fig. 3A. Data are presented as means ± S.D. of three independent experiments and expressed as percentage. (* $P < 0.05$ compared to the TPA group and analyzed by Student's *t*-test). (B) Concentration-dependent inhibition by emodin on TPA-induced AP-1 binding activity. Cells were pretreated with 0–40 μM emodin for 2 h, followed by 2 h TPA stimulation. The nuclear extract was subjected to EMSA as described in Section 2. Cold probe, unlabeled oligonucleotides for AP-1. Sp-1 is served as a loading control measured by Western blot. (C) Inhibitory effect of emodin on TPA-induced c-Jun phosphorylation. Cells were pretreated with 0–40 μM emodin for 2 h. The cells were subsequently stimulated with 80 nM TPA for another 2 h. Total cell lysate protein of 40 μg was subjected on 10% SDS-PAGE. Western blot was done as described in Section 2.

emodin-mediated inhibition of AP-1 transactivation, we examined the effect of emodin on the phosphorylation and activation of ERK, JNK, and p38 kinase. Our preliminary data showed that phosphorylation of MAPK occurred at 1–3 h after TPA treatment in HSC5 cells (data not shown). We thus investigated the effect of emodin on JNK, ERK, and p38 phosphorylation in cells stimulated by 80 nM TPA for 2 h. PD98059 (a specific ERK inhibitor), SP600125 (a specific JNK inhibitor) and SB203580 (a specific p38 inhibitor) were used as respective positive controls. As shown in Fig. 4, emodin inhibited TPA-induced ERK and JNK phosphorylation in a concentration-dependent manner, while having no evident effect on phosphorylation of p38. Total protein level of ERK, JNK, and p38 did not change with TPA and/or emodin treatment. These results suggest that inhibition of ERK and JNK phosphorylation

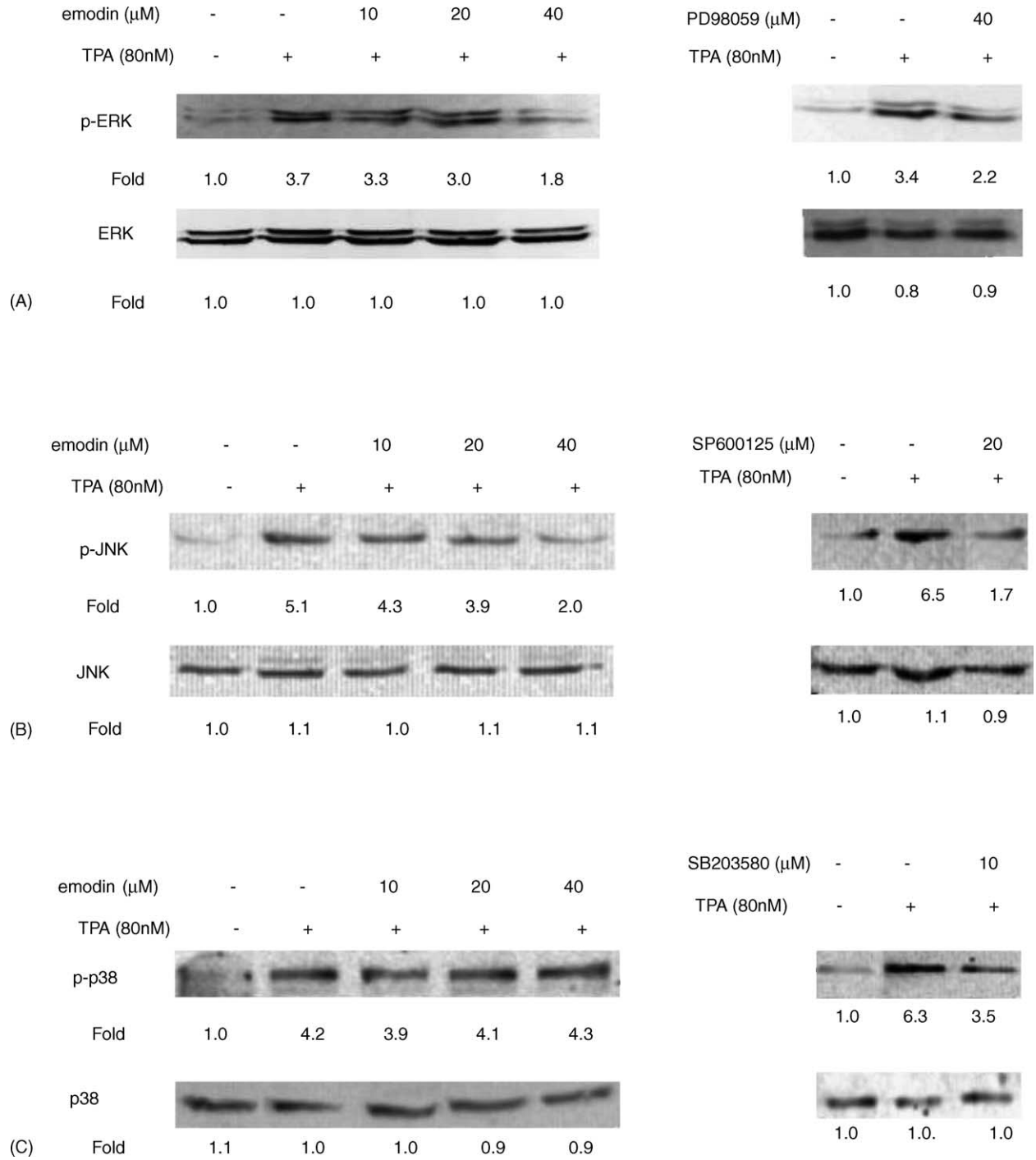

Fig. 4. Effect of emodin, ERK inhibitor, JNK inhibitor, and p38 inhibitor on TPA-induced ERK, JNK and p38 phosphorylation. HSC5 cells were pretreated with 0–40 μM emodin, 40 μM PD98059, 20 μM SP600125, or 10 μM SB203580 for 2 h. Cells were then stimulated with 80 nM TPA for another 2 h. Western blot of whole cell lysates was performed as described in Section 2. (A) Change of ERK phosphorylation. (B) Change of JNK phosphorylation. (C) Change of p38 phosphorylation.

by emodin is one of the underlying mechanisms involved in its inhibitory effect on AP-1 transactivation and the down-regulation of MMP-9.

3.5. Inhibitory effect of emodin on NF-κB signaling pathway

NF-κB is another major transcription factor that regulates MMP-9 expression [6]. Here we first tested the transcriptional activity of NF-κB by using a NF-κB luciferase reporter plasmid. The result in Fig. 5A showed that TPA significantly stimulated the NF-κB transactivation; whereas, emodin significantly inhibited TPA-induced luciferase activity. These data, together with the results from Fig. 2, suggests the possible involvement of NF-κB in the inhibitory effect of emodin on TPA-induced MMP-9.

To further determine the molecular mechanism involved in emodin-mediated suppression on NF-κB transactivation, we examined the effect of emodin on the DNA-binding activity of NF-κB. EMSA result revealed that TPA caused significant increase of NF-κB DNA-binding, and emodin inhibited NF-κB DNA-binding in a concentration-dependent fashion, being consistent with the effect of emodin on the transcriptional activity of NF-κB (Fig. 5A). No obvious change of NF-κB DNA-binding activity was observed in the cells treated with emodin alone.

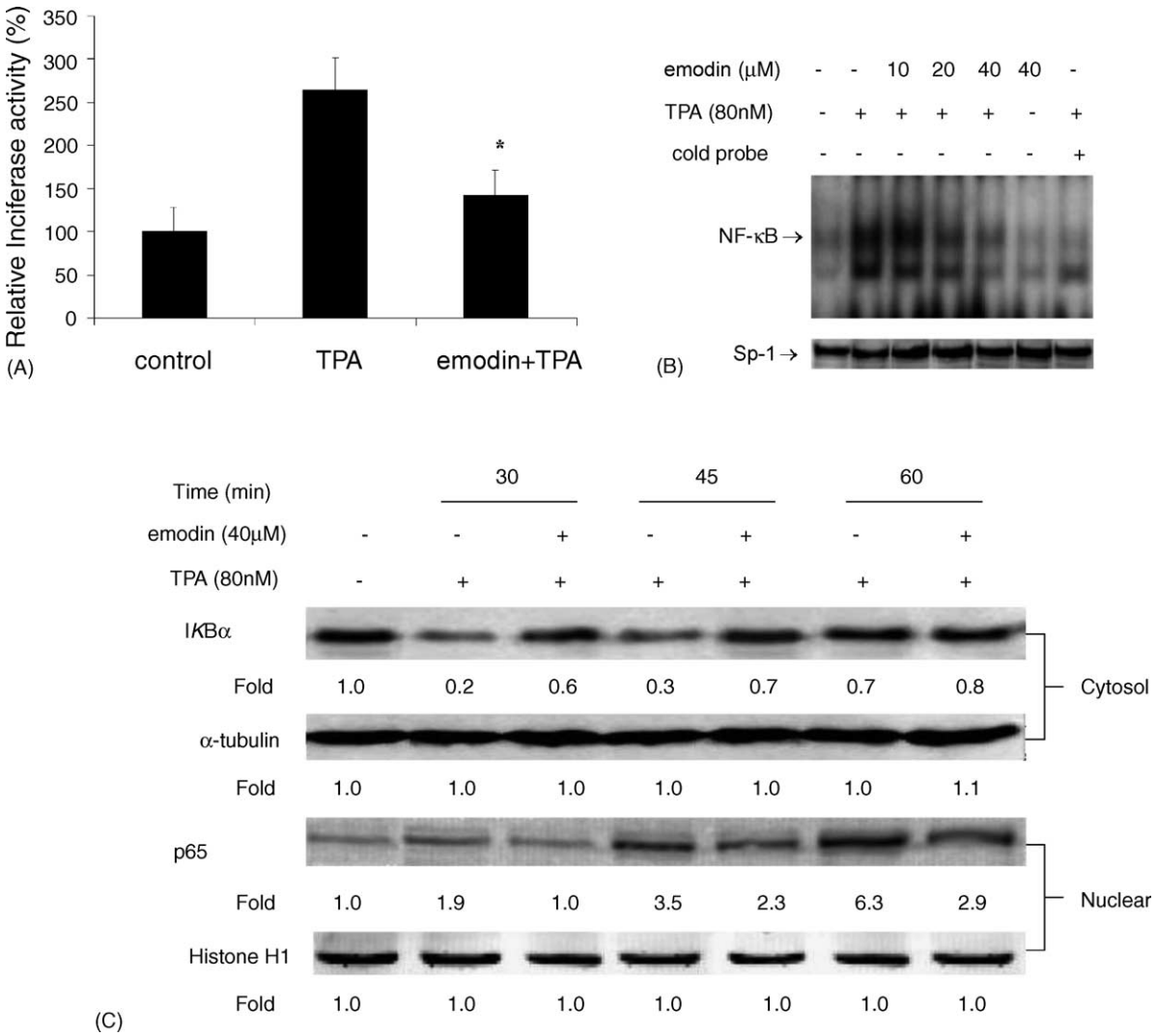

Fig. 5. Inhibitory effect of emodin on NF-κB signaling pathway. (A) Inhibitory effect of emodin on TPA-induced transcriptional activity of NF-κB. pNF-κB-Luc was transfected into HSC5 cells together with β-gal as normalization of transfection efficiency. After 2 h pretreatment with or without 40 μM emodin, the cells were cultured with 80 nM TPA for additional 6 h. Luciferase activity was measured as described in Section 2. Data are presented as means ± S.D. of three independent experiments and expressed as percentage. ($^{*}P < 0.05$ compared to the TPA group and analyzed by Student's *t*-test). (B) Concentration-dependent inhibition by emodin on TPA-induced NF-κB binding activity. Cells were pretreated with or without 0–40 μM emodin for 2 h, followed by TPA stimulation for 1 h. The nuclear extract was subjected to EMSA as described in Section 2. Cold probe, unlabeled oligonucleotides for NF-κB. Sp-1 is served as a loading control measured by Western blot. (C) Inhibitory effect of emodin on IκBα degradation and p65 nuclear translocation. Cells were pretreated with 40 μM emodin for 2 h and then stimulated with 80 nM TPA up to 1 h. Cytosolic and nuclear proteins were prepared and analyzed by Western blot. α-Tubulin and histone H1 are served as cytosolic and nuclear loading control, respectively.

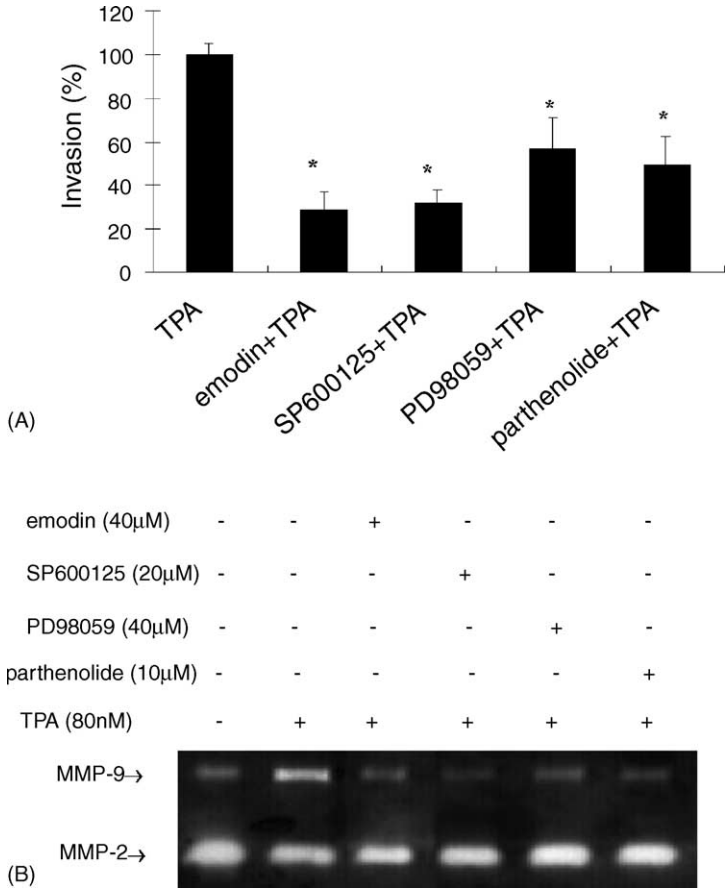

Fig. 6. Inhibitory effect of emodin, SP600125, PD98059, and parthenolide on TPA-induced cell invasion (A) and MMP-9 production (B). HSC5 cells were stimulated for 12 h with 80 nM TPA after 2 h pretreatment of 40 μM emodin, 20 μM SP600125, 40 μM PD98059, or 10 μM parthenolide respectively. Invasion assay and gelatin zymography were done as described in Section 2. Invasion data are presented as means ± S.D. of three independent experiments and expressed as percentage. ($^*P < 0.05$ compared to the TPA group and analyzed by Student's *t*-test).

It is well known that TPA activates NF-κB via IκBα phosphorylation and degradation, followed by p65 nuclear translocation [23,24]. We thus studied the effect of emodin on the degradation of IκBα and nuclear translocation of p65 in TPA-stimulated cells. As shown in Fig. 5C, TPA treatment caused significant decrease of IκBα at 30 min and 45 min. This reduction was associated with an increasing translocation of p65 into the nucleus. Pretreatment of the cells with 40 μM emodin for 2 h significantly inhibited both IκBα degradation and p65 nuclear translocation. Treatment of cells with emodin alone had no effect on IκBα level (data not shown). The reduced IκBα degradation and p65 nuclear translocation are thus found to be consistent with the reduced NF-κB DNA-binding capacity (Fig. 5B) and transcriptional activity (Fig. 5A).

3.6. Inhibition of TPA-induced MMP-9 activity and cell invasion by ERK Inhibitor, JNK Inhibitor and NF-κB Inhibitor

To further elucidate the involvement of ERK, JNK, and NF-κB in emodin's effect against cancer cell invasion, we investigated the effects of their specific inhibitors on cell invasion and MMP-9 production to mimic the actions of emodin in HSC5 cells. The cells were incubated with TPA for 12 h after 2 h pretreatment of emodin, PD98059, SP600125, or parthenolide (a known NF-κB inhibitor) [25], respectively. Cell invasion assay and gelatin zymography data showed that these compounds inhibited invasion and MMP-9 activity of HSC5 cells, similar to the effect of emodin (Fig. 6B). Therefore, it is believed that ERK, JNK, and NF-κB are involved in the up-regulation of MMP-9 and cell invasion in TPA-treated HSC5 cells and they serve as the molecular targets for emodin in its anti-invasiveness effect.

4. Discussion

Emodin, an active component from the rhizome of *R. palmatum*, has been reported to exhibit anti-tumor effects in various cancer cells [11]. However, currently little is known about its effect against tumor invasion and metastasis. In this study, we investigated emodin's effect against cancer cell invasion and the mechanisms involved. We found that nontoxic levels of emodin could efficiently

suppress the invasiveness of TPA-treated cancer cells (Fig. 1) by decreasing the activity and production of MMP-9 through inhibiting AP-1 and NF-κB signaling pathways.

MMPs play important roles in the degradation of extracellular matrix to allow tumor invasion and metastasis [3]. Among various human MMPs reported previously, MMP-9 can digest type IV collagen, the ''backbone'' of basement membrane, and is regarded as a metastatic marker in transformed cell lines [3]. Moreover, there is substantial evidence on the inhibition of MMP-9 and suppression of invasiveness and metastasis of cancer cells using various chemopreventive or chemotherapeutic agents such as genistein, aspirin, and penta-*O*-galloyl-β-D-glucose [26–29]. In the present study, we showed that emodin inhibited TPA-induced MMP-9 expression and activity in a dose-dependent fashion (Fig. 2). The inhibitory effect of emodin on MMP-9 expression provides convincing explanation for its suppression of invasiveness of HSC5 cells observed in the cell invasion assay (Fig. 1). Similar suppressive effect of emodin was also observed in MDA-MB-231 breast cancer cells, suggesting that emodin-mediated decrease of MMP-9 expression and cell invasiveness is also effective in other types of cancer cells.

The expression of MMP-9 is largely controlled at the transcriptional level [6]. In the promoter region of MMP-9 gene there are two AP-1 binding sites and one NF-κB binding site [30]. Mutations in AP-1 and NF-κB binding sites can reduce or even abolish the induction of MMP-9 by TPA in rat mesangial cells [31] . In this study we found that emodin markedly suppressed the TPA-induced AP-1 DNA-binding (Fig. 3B) and transcriptional activity (Fig. 3A). Besides, emodin inhibited TPA-induced phosphorylation of c-Jun in a concentration-dependent manner (Fig. 3C). Since the activity of AP-1 can be modulated through the phosphorylation of c-Jun [32], a major component of AP-1, it is thus believed that emodin's inhibitory effect on AP-1 DNA-binding activity and transcriptional activation could be through suppressing the phosphorylation of c-Jun.

It is well established that c-Jun phosphorylation is mediated by MAPK pathways [22]. Generally there are three classes of MAPKs: ERK, JNK, and p38 MAPKs. Activation of MAPK occurs through phosphorylation of specific threonine and tyrosine, and the components of AP-1, such as c-Jun, can be phosphorylated after the translocation of activated MAPKs to the nucleus [33]. Our data showed that emodin inhibited the phosphorylation of JNK and ERK, but not p38 (Fig. 4). Since c-Jun is the phosphorylation target of both JNK and ERK [21], it is likely that emodin inhibits the phosphorylation of c-Jun through suppressing JNK and ERK activation. It has been shown previously, that activation of JNK and/or ERK can induce cancer invasion through c-Jun and AP-1 signaling pathway [34,35]. Some chemopreventive agents, such as aspirin and penta-*O*-galloyl-β-D-glucose, can inhibit cancer cell invasion and AP-1 transactivation through suppressing JNK and/or ERK activation [26,27]. In this study, SP600125, a specific JNK inhibitor and PD98059, a specific ERK inhibitor, both effectively inhibited TPA-induced phosphorylation of ERK and JNK (Fig. 4), MMP-9 expression and cell invasion (Fig. 6), thus confirming that the inhibition of ERK and JNK cascades is critical in diminishing TPA-induced cell invasion. Therefore, it is believed that emodin may suppress TPA-induced MMP-9 expression and cancer cell invasion through JNK and ERK pathways.

At present, it is still not clear how emodin affects MAPK signaling pathways. Several studies have demonstrated that TPA can activate the GTP-binding protein ras that plays an important role in regulating the MMP-9 expression [36–38]. Not only the classical Raf/$MEK_{1/2}$/$ERK_{1/2}$ mitogenic cascade but also the p38 and JNK signaling cascades can be activated by TPA in a ras-dependent fashion [39,40]. Since emodin has been shown to display highly selective activity against *ras*-oncogene [41], it is possible that this suppression leads to inhibition of TPA-induced $ERK_{1/2}$ and JNK phosphorylation, followed by inhibition of MMP-9 expression and reduced invasiveness in TPA-stimulated cells.

On the other hand, NF-κB is another nuclear transcription factor that is known to be important in the regulation of MMP-9 expression [30]. NF-κB, one of the main molecular targets of chemopreventive phytochemicals[42], is a transcription factor involved in multiple cellular processes, including cytokine gene expression, cellular adhesion, apoptosis, and metastasis [43]. In unstimulated cells, NF-κB is generally sequestered in the cytoplasm by the IκB proteins. When stimulated, IκB is phosphorylated and degraded. NF-κB, released from the inhibitors, translocates from cytoplasm into the nucleus, binds to the promoter region of genes containing its specific sites and activates gene transcription [43]. In this study, we demonstrated that emodin suppressed TPA-mediated IκBα degradation and p65 nuclear translocation (Fig. 5C), thus inhibiting TPA-induced NF-κB DNA-binding (Fig. 5B) and transcriptional activation (Fig. 5A). It has been reported earlier that emodin inhibited TNF-induced NF-κB activation in human vascular endothelial cells [44]. Therefore, it appears that emodin is capable of acting against NF-κB activation in various cell types and with different stimuli. Our results also showed that nontoxic level of parthenolide, a specific inhibitor of NF-κB signaling pathway, inhibited cell invasion and MMP-9 expression (Fig. 6), suggesting that NF-κB pathway is involved in the invasion and metastasis of TPA-stimulated cancer cells; and NF-κB serves as one of molecular targets for emodin in its effect against cancer invasion.

Taken together, here we provide clear evidence that emodin inhibits TPA-induced cell invasiveness and MMP-9 expression in human cancer cells through suppressing both AP-1 and NF-κB signaling pathways. Such findings suggest that emodin could be of therapeutic value in preventing invasion or metastasis of human cancer.

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