

Silibinin inhibits TPA-induced cell migration and MMP-9 expression in thyroid and breast cancer cells

SOO-JIN OH*, SEUNG PIL JUNG*, JEONGHUN HAN, SANGMIN KIM,
JEE SOO KIM, SEOK JIN NAM, JEONG EON LEE and JUNG-HAN KIM

Department of Surgery, Samsung Medical Center, Sungkyunkwan University
School of Medicine, Gangnam-gu, Seoul 135-710, Republic of Korea

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Abstract. Matrix metalloproteinases (MMPs) play an important role in cancer metastasis, cell migration and invasion. Herein, we investigated the effects of silibinin on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced cell migration and MMP-9 expression in thyroid and breast cancer cells. Our results revealed that the levels of MMP-9 mRNA and protein expression were significantly increased by TPA but not MMP-2 in TPC-1 and MCF7 cells. To verify the regulatory mechanism of TPA-induced MMP-9 expression, we treated TPC-1 and MCF7 cells with the MEK1/2 inhibitor, UO126, and TPA-induced MMP-9 expression was significantly decreased. We also found that TPA-induced cell migration and MMP-9 expression was significantly decreased by silibinin. In addition, TPA-induced phosphorylation of MEK and ERK was also inhibited by silibinin. Taken together, we suggest that silibinin suppresses TPA-induced cell migration and MMP-9 expression through the MEK/ERK-dependent pathway in thyroid and breast cancer cells.

Introduction

Silibinin is the main component of the silymarin complex and is isolated from the seeds of *Silybum marianum*, also known as milk thistle (1). Silibinin has a wide range of pharmacologic effects including the induction of apoptosis, and the inhibition of cell proliferation, cell invasion and angiogenesis (2,3). Recently, Kim *et al* reported that silibinin triggers cell cycle arrest through the downregulation of cyclin B1 and cdc2 and

upregulation of p21 expression in triple-negative breast cancer cells (4). In addition, silibinin was found to effectively delay the development of spontaneous mammary tumors and decrease the tumor mass in Her2/neu transgenic mice (5).

Migration of cells is a dynamic process that occurs in tissue remodeling, inflammation and wound repair and is regulated by a variety of extracellular factors, including extracellular matrix (ECM) proteins (6,7). Tumor cell migration is associated with the early stages of metastasis (8). Metastasis is considered responsible for more than 90% of cancer-related deaths (9). During metastasis, cancer cells are involved in numerous interactions with various factors such as the ECM, growth factors, cytokines and basement membranes (8). Consequently, cancer cells acquire motility and local invasive capability. In addition, cancer cell-mediated tissue remodeling is observed to have a strong positive correlation with matrix metalloproteinase (MMP) levels (10).

MMPs are major critical molecules that assist tumor cells during metastasis (11). They play an important role in ECM degradation and cancer cell invasion. Overexpression of MMPs contributes to tumorigenesis and tumor progression through multiple pathways (12). MMP-9 is one of two gelatinases and is able to degrade type IV collagen, which is abundant in basement membranes (13). High serum levels of MMP-9 are associated with a higher tumor grade, poor overall survival and secondary metastasis in melanoma and breast cancer tissue (10,14).

In the present study, we investigated the relationship between silibinin and cell migration in thyroid and breast cancer cells. Here, our results revealed that 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced MMP-9 expression and cell migration were suppressed by silibinin in both thyroid and breast cancer cells.

Materials and methods

Reagents and cell cultures. Dulbecco's modified Eagle's medium (DMEM), antibiotics and 10% zymogram gel were purchased from Life Technologies (Rockville, MD, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Silibinin was purchased from Sigma (St. Louis, MO, USA). Mouse recombinant MMP-9 was purchased from R&D Systems (Minneapolis, MN, USA). TPA was purchased

Correspondence to: Professor Jung-Han Kim or Dr Sangmin Kim, Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, Republic of Korea
E-mail: jhkim15@skku.edu
E-mail: ksm3005@hotmail.com

*Contributed equally

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from Tocris (Ellisville, MO, USA). The secondary peroxidase-conjugated antibodies and ECL Prime reagents were purchased from Amersham (Buckinghamshire, UK).

Papillary thyroid cancer TPC-1 and breast cancer MCF7 cells were grown in a humidified atmosphere of 95% air and 5% CO₂ at 37°C in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Each cell line was maintained in culture medium supplemented without FBS for 24 h.

Drug treatment. Cells were maintained in culture medium without FBS for 24 h, and then the culture medium was replaced with fresh medium without FBS, and the cells were further incubated with the indicated concentrations of silibinin for 24 h. In the experiments involving silibinin, the cells were pretreated with 50 µM silibinin for 60 min prior to treatment with 20 nM TPA for 24 h.

Zymography. Zymography was performed on 10% polyacrylamide gels that had been cast in the presence of gelatin as previously described (15). Briefly, samples (100 µl) were resuspended in a loading buffer and run on a 10% SDS-PAGE gel containing 0.5 mg/ml gelatin without prior denaturation. After electrophoresis, the gels were washed to remove SDS and incubated for 30 min at room temperature (RT) in a renaturing buffer (50 mM Tris, 5 mM CaCl₂, 0.02% NaN₃ and 1% Triton X-100). Next, the gels were incubated for 48 h at 37°C in a developing buffer [50 mM Tris-HCl (pH 7.8), 5 mM CaCl₂, 0.15 M NaCl and 1% Triton X-100]. The gels were subsequently stained with Coomassie Brilliant Blue G-250, destained in 30% methanol, and flooded with 10% acetic acid to detect gelatinase secretion.

cDNA synthesis and real-time PCR. Total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Isolated RNA samples were then used for RT-PCR. Samples (total RNA, 1 µg) were reverse-transcribed into cDNA in 20-µl reaction volumes using a first-strand cDNA synthesis kit for RT-PCR, according to the manufacturer's instructions (MBI Fermentas, Hanover, MD, USA).

Gene expression was quantified by real-time PCR using the SensiMix SYBR kit (Bioline Ltd., London, UK) and 100 ng of cDNA per reaction. The sequences of the primer sets used for this analysis were: human MMP-9 (forward, 5'-CCC GGA CCA AGG ATA CAG-3'; reverse, 5'-GGC TTT CTC TCG GTA CTG-3'), MMP-2 (forward, 5'-GGC CTC TCC TGA CAT TGA CCT T-3'; reverse, 5'-GGC CTC GTA TAC CGC ATC AAT C-3'), and β-actin as an internal control (forward, 5'-AAA CTG GAA CGG TGA AGG TG-3'; reverse, 5'-CTC AAG TTG GGG GAC AAA AA-3'). An annealing temperature of 60°C was used for all of the primers. PCRs were performed in a standard 384-well plate format with an ABI 7900HT real-time PCR detection system. For data analysis, the raw threshold cycle (C_T) value was first normalized to the housekeeping gene for each sample to obtain ΔC_T. The normalized ΔC_T was then calibrated to control cell samples to obtain ΔΔC_T.

Cell viability. Total cell numbers following treatment with silibinin were evaluated by Quick Cell Proliferation Assay

Kit II (Biovision, Mountain View, CA, USA) according to the manufacturer's protocol. Briefly, TPC-1 human papillary thyroid cancer cells (5×10⁴/well) were grown in a 96-well plate in 100 µl/well of culture media in the absence or presence of the indicated concentrations of silibinin. After incubating the cells for 24 h, 10 µl WST reagent was added to each well. Viable cells were quantified photometrically at 480 nm.

Wound healing assay. TPC-1 thyroid cancer cells were seeded in 6-well plates and were cultured for 24 h. A monolayer of cells was scratched with a 200-µl pipette tip to create a wound, and then this was washed twice in PBS to remove any suspended cells. The cells were pretreated with silibinin (50 µM) for 60 min prior to treatment with TPA; the monolayer of cells was then treated with 20 nM TPA for 24 h in serum-free media. The cells migrating from the leading edge were photographed at 0 and 24 h using a CK40 inverted microscope (Olympus, Tokyo, Japan).

Statistical analysis. Statistical significance was determined using the Student's t-test. The results are presented as the means ± SEM. All p-values were two-tailed and significance was set at a p-value <0.05.

Results

Expression of MMP-9 and MMP-2 in thyroid and breast cancer cells following TPA treatment. The levels of MMP-9 and MMP-2 mRNA and protein expression in the TPC-1 and MCF7 cells were determined following treatment with TPA at the indicated concentrations for 24 h. We analyzed the levels of MMP-9 and MMP-2 mRNA (in cell lysates) and protein (in culture media) expression using real-time PCR and zymography, respectively. Our results revealed that the levels of MMP-9 protein expression were significantly increased in the TPC-1 thyroid (Fig. 1A) and MCF7 breast (Fig. 1C) cancer cells. However, MMP-2 protein expression was not altered following TPA treatment in the TPC-1 cells (Fig. 1A), although MMP-2 expression was not detected in the MCF7 cells following TPA treatment (Fig. 1C). In addition, the levels of MMP-9 mRNA were also increased following TPA treatment (Fig. 1B and D). MMP-9 mRNA expression was increased by 28.8±6.6- and 30.6±2.5-fold in the TPC-1 thyroid (Fig. 1B) and MCF7 (Fig. 1D) breast cancer cells, respectively, following treatment with 20 nM TPA when compared with the control level.

TPA-induced MMP-9 expression is inhibited by the MEK1/2 inhibitor, UO126. To verify the regulatory mechanism of TPA-induced MMP-9 expression, we examined the effect of the MEK1/2 inhibitor, UO126, on TPA-induced MMP-9 expression. After pretreatment of TPC-1 and MCF7 cells with 10 µM UO126 for 30 min, we treated the cells with 20 nM TPA for 24 h. The levels of TPA-induced MMP-9 protein expression were significantly decreased by UO126 in the TPC-1 thyroid (Fig. 2A) and MCF7 breast cancer cells (Fig. 2C). In addition, TPA-induced MMP-9 mRNA expression was also decreased following pretreatment with UO126 (Fig. 2B and D). MMP-9 mRNA expression was increased by 29.4±2.4- and 41.8±0.6-fold, respectively, in the TPC-1 thyroid (Fig. 2B) and

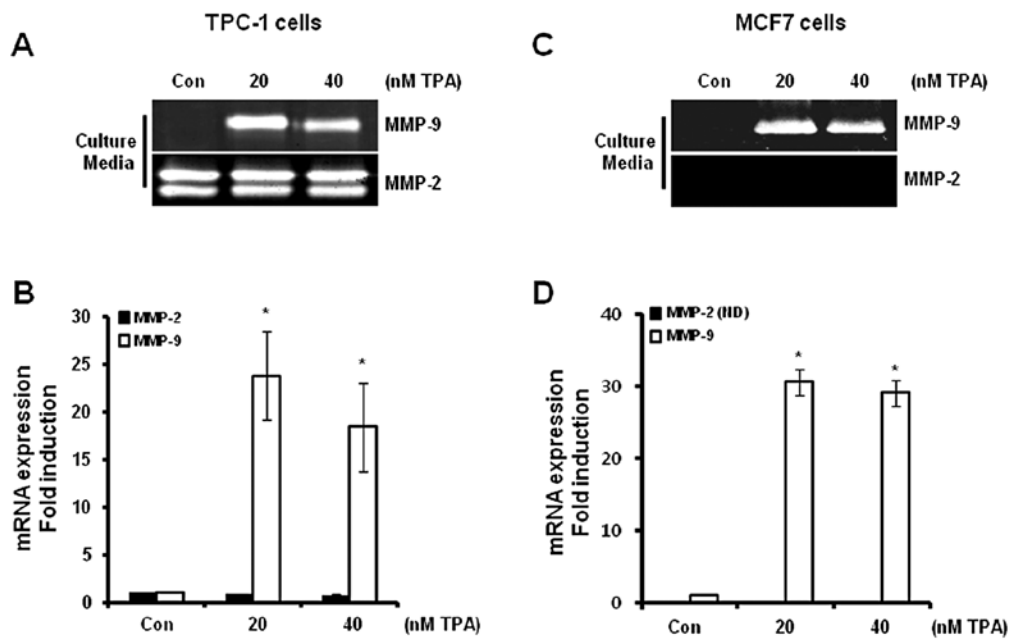


Figure 1. Expression of MMP-9 and MMP-2 in TPC-1 thyroid and MCF7 breast cancer cells following TPA treatment. After serum starvation for 24 h, TPC-1 (A and B) and MCF7 (C and D) cells were treated with the indicated concentrations of TPA for 24 h. The cell culture media and cell lysates were harvested for detection of MMP-9 and MMP-2 protein (A and C) and mRNA (B and D), respectively. Expression of MMP-9 and MMP-2 protein (cell culture media) and mRNA (cell lysates) was analyzed by zymography (A and C) and real-time PCR (B and D), respectively. The results are representative of three independent experiments. The values shown are the means \pm SEM. * $P < 0.05$ vs. control. Con, control.

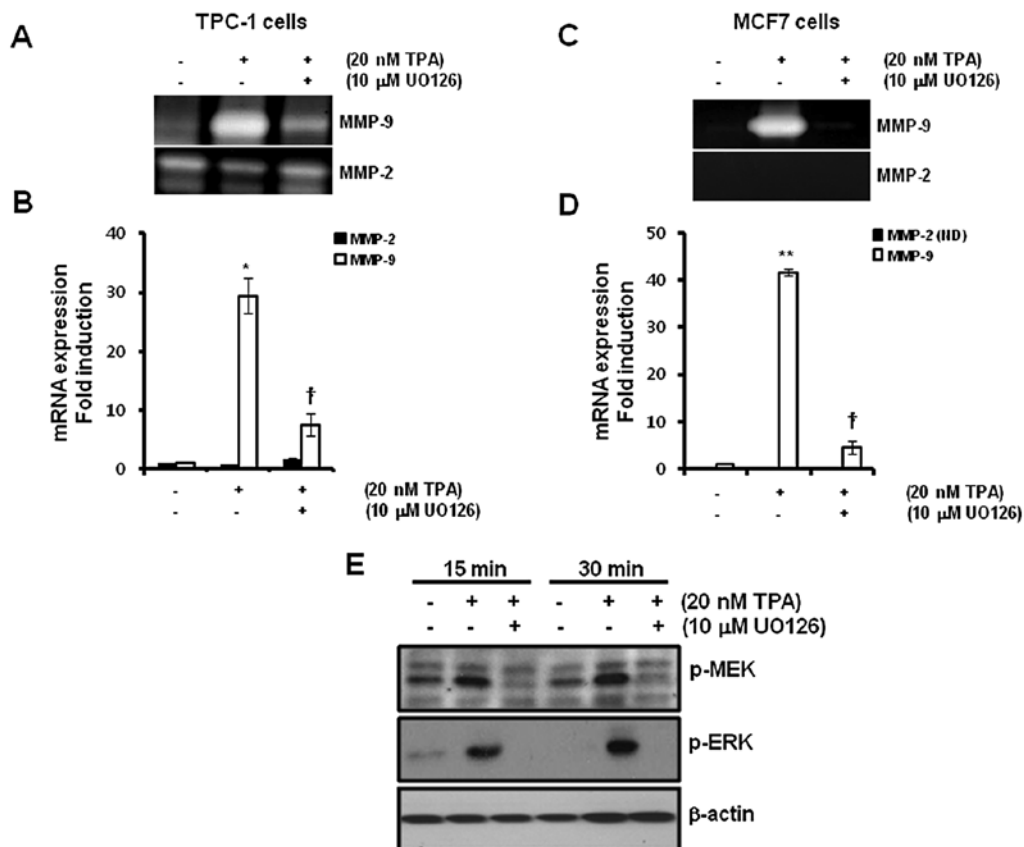


Figure 2. TPA-induced MMP-9 expression is suppressed by UO126 in TPC-1 thyroid and MCF7 breast cancer cells. After serum starvation for 24 h, TPC-1 (A and B) and MCF7 (C and D) cells were pretreated with UO126 for 30 min prior to further incubation with 20 nM TPA for 24 h. The cell culture media and cell lysates were harvested for detection of MMP-9 protein (A and C) and mRNA (B and D), respectively. Expression of MMP-9 and MMP-2 protein (cell culture media) and mRNA (cell lysates) was analyzed by zymography (A and C) and real-time PCR (B and D), respectively. (E) After serum starvation for 24 h, TPC-1 cells were pretreated with UO126 for 30 min prior to further incubation with 20 nM TPA for the indicated times. The phosphorylation of MEK and ERK was analyzed by western blotting in total cell lysates. The results are representative of three independent experiments. The values shown are the means \pm SEM. * $P < 0.05$ vs. control, † $P < 0.05$ vs. TPA-treated cells. Con, control.

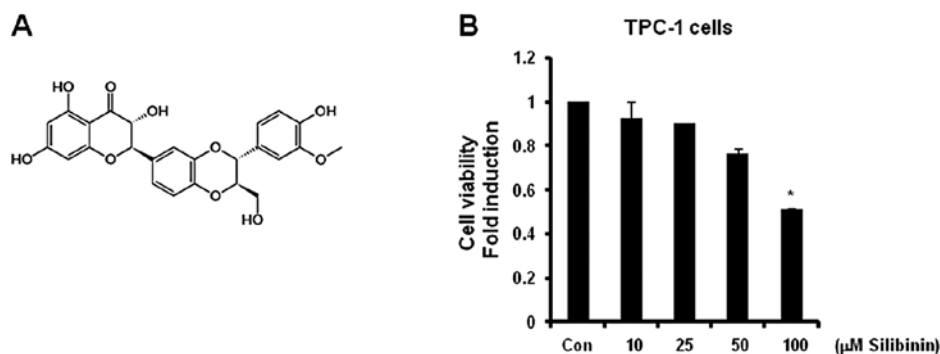


Figure 3. Effect of silibinin on cell viability. (A) The chemical structure of silibinin. (B) After serum starvation for 24 h, TPC-1 cells were treated with silibinin at the indicated concentrations for 24 h. These results are representative of three independent experiments. The values shown are the means \pm SEM. * $P < 0.05$ vs. control. Con, control.

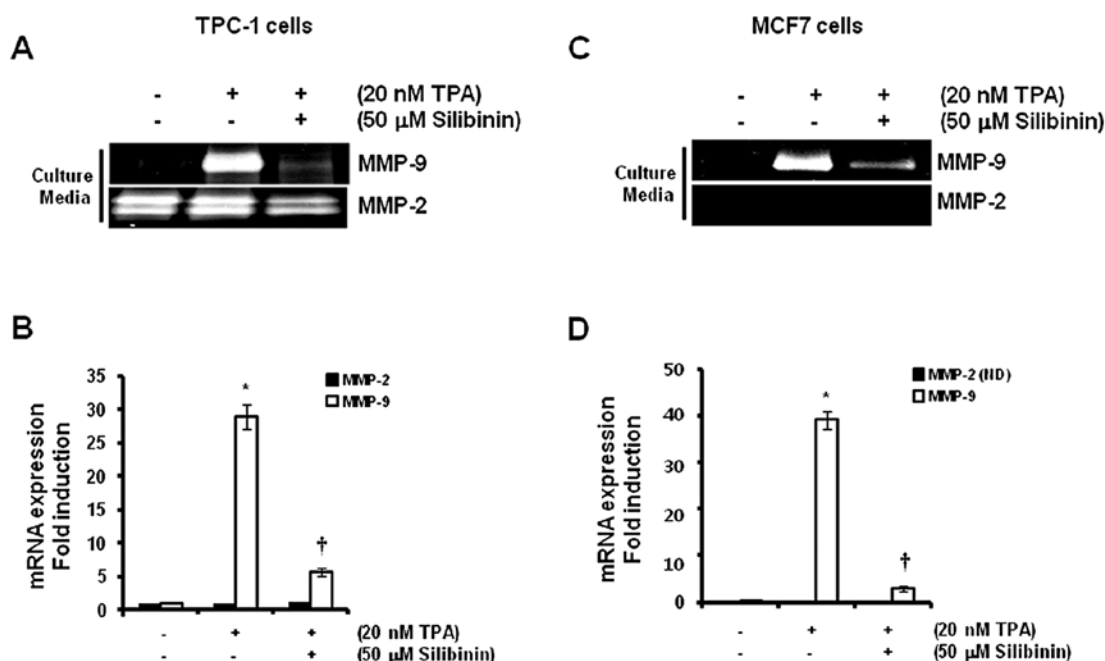


Figure 4. TPA-induced MMP-9 expression is decreased by silibinin. After serum starvation for 24 h, TPC-1 (A and B) and MCF7 (C and D) cells were pretreated with 50 μ M silibinin for 60 min prior to further incubation with 20 nM TPA for 24 h. The cell culture media and cell lysates were harvested for detection of MMP-9 and MMP-2 protein (A and C) and mRNA (B and D), respectively. Expression of MMP-9 protein (cell culture media) and mRNA (cell lysates) was analyzed by zymography (A and C) and real-time PCR (B and D), respectively. The results are representative of three independent experiments. The values shown are the means \pm SEM. * $P < 0.05$ vs. control, † $P < 0.05$ vs. TPA-treated cells. Con, control.

MCF7 (Fig. 2D) breast cancer cells, following treatment with 20 nM TPA when compared with control levels. In contrast, TPA-induced MMP-9 expression was significantly decreased by 7.5 \pm 1.9 and 4.5 \pm 1.3-fold in the TPC-1 thyroid (Fig. 2B) and MCF7 (Fig. 2D) breast cancer cells, respectively, following treatment with 10 μ M UO126 when compared with the control levels.

Next, we confirmed the effect of UO126 on TPA-induced phosphorylation of MEK and ERK in TPC-1 thyroid cancer cells. As expected, the phosphorylation of MEK and ERK was increased following TPA treatment (Fig. 2E). In contrast, TPA-induced phosphorylation of MEK and ERK was significantly decreased by UO126 (Fig. 2E).

TPA-induced MMP-9 expression is completely suppressed by silibinin. The chemical structure of silibinin is represented in

Fig. 3A. Our results revealed that the cell viability was significantly (50% of the control level) decreased following treatment with 100 μ M silibinin (Fig. 3B). Therefore, we treated cells with 50 μ M silibinin in the subsequent studies.

To investigate the effect of silibinin on TPA-induced MMP-9 expression, we pretreated cells with 50 μ M silibinin for 60 min prior to treatment with 20 nM TPA. We found that TPA-induced MMP-9 protein expression was significantly decreased by silibinin treatment (Fig. 4A and C). In addition, the levels of expression of MMP-9 mRNA increased significantly (29.0 \pm 2.6- and 39.1 \pm 2.5-fold) in the TPC-1 thyroid (Fig. 4B) and MCF7 breast (Fig. 4D) cancer cells, respectively, following treatment with a concentration of 20 nM TPA when compared to control levels. In contrast, TPA-induced MMP-9 mRNA expression was decreased to 5.7 \pm 0.6-fold in TPC-1 thyroid cancer cells by 50 μ M silibinin (Fig. 4B). MCF7 breast

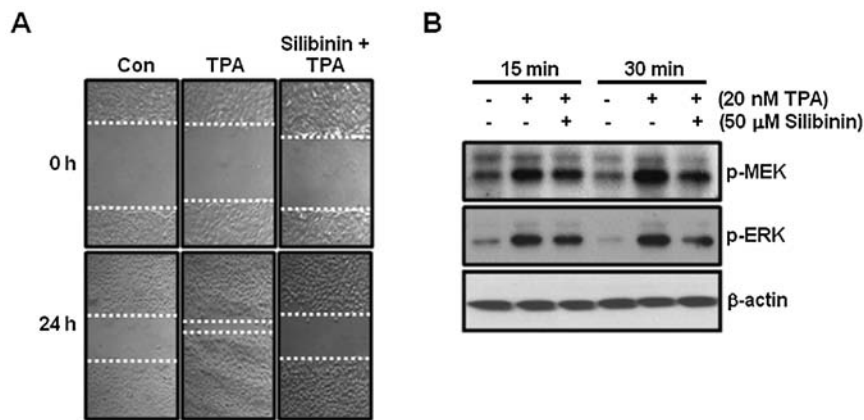


Figure 5. TPA-induced cell migration is suppressed by silibinin. (A) After serum starvation for 24 h, TPC-1 cells were pretreated with 50 μ M silibinin for 60 min prior to further incubation with 20 nM TPA for 24 h. The migration abilities of the cells were analyzed by a wound healing assay, as described in Materials and methods. (B) After serum starvation for 24 h, TPC-1 cells were pretreated with 50 μ M silibinin for 60 min prior to further incubation with 20 nM TPA for the indicated times. The phosphorylation of MEK and ERK was analyzed in total cell lysates by western blotting. The results are representative of three independent experiments. Con, control.

cancer cells also showed similar results (Fig. 4D) following treatment with 50 μ M silibinin when compared with the control level.

TPA-induced cell migration is completely suppressed by silibinin. In the next experiment, we examined the effect of silibinin on TPA-induced cell migration in TPC-1 thyroid cancer cells. As shown in Fig. 5A, TPA-induced cell migration was completely blocked by 50 μ M silibinin treatment.

Next, we investigated the effect of silibinin on TPA-induced phosphorylation of MEK and ERK in TPC-1 thyroid cancer cells. As expected, the phosphorylation of MEK and ERK was increased following TPA treatment (Fig. 5B). In contrast, TPA-induced phosphorylation of MEK and ERK was decreased by silibinin (Fig. 5B). Therefore, we demonstrated that silibinin suppressed TPA-induced cell migration as well as inhibited MMP-9 expression.

Discussion

Phorbol esters, such as TPA, are natural molecules that are recognized as potent tumor promoters and can potently trigger multiple cellular events such as protein kinase C (16,17). TPA was found to significantly enhance cell migration abilities of human cancer cells including hepatoma and breast cancer cells (18,19). In addition, TPA-induced migration and invasion of glioblastoma cells were prevented by blocking PKC α -dependent pathways (20). Consistent with these reports, we found that TPA increased cell migration in thyroid and breast cancer cells. Herein, we investigated the inhibitory effect of silibinin on TPA-induced cancer cell migration.

MMPs are positively associated with tumor progression including tumor differentiation, metastasis and poor prognosis (21,22). In addition, inhibition of MMPs decreases cell invasion while the activation of MMPs yields increased tumor cell invasion (23). MMP-9 is one of the gelatinases and is expressed in a large number of cell types, including epithelial and inflammatory cells (24). MMP-9 has been associated with the development and extent of metastases in lymph nodes (25). Although we did not present the data, TPA-induced cell

migration was significantly suppressed by a broad-spectrum MMP inhibitor, PD166793, in both TPC-1 and MCF7 cells. Therefore, we demonstrated that a broad-spectrum MMP inhibitor, PD166793, directly or indirectly affects TPA-induced cell migration through the regulation of MMP activity.

The region of the MMP-9 promoter contains *cis*-acting regulatory elements for transcription factors, including two AP-1 sites and an NF- κ B site (26,27). The DNA binding activity of a variety of transcription factors such as NF- κ B and AP-1 is regulated by ERK activity (26-28). Recently, we reported that TPA-induced transcriptional activity of AP-1 is mediated through the Raf/MEK/ERK-dependent pathway (3). In addition, the transcriptional expression of MMP-9 was found to be directly regulated by AP-1 DNA binding activity (28) and was completely suppressed by the MEK1/2 inhibitor, UO126 (3). In accordance with these studies, TPA-induced MMP-9 expression was significantly decreased by silibinin. TPA-induced phosphorylation of MEK and ERK was also suppressed.

Conclusively, we found that silibinin suppresses the TPA-induced phosphorylation of MEK and ERK in thyroid cancer cells. In addition, cell migration and MMP-9 expression were completely inhibited by silibinin. Therefore, silibinin may be a promising drug for the treatment of thyroid and breast cancer.

Acknowledgements

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