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Silibinin prevents TPA-induced MMP-9 expression and VEGF secretion by inactivation of the Raf/MEK/ERK pathway in MCF-7 human breast cancer cells

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Abstract

Matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF) expression are pivotal steps in cancer metastasis. Herein, we investigated the effect of silibinin, a major constituent (flavanolignan) of the fruits of *Silybum marianum*, on 12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced MMP-9 and VEGF expression in MCF-7 human breast cancer cells. The expression of MMP-9 and VEGF in response to TPA was increased, whereas TPA-induced MMP-9 and VEGF expression was decreased by silibinin. To investigate the regulatory mechanism of silibinin on TPA-induced MMP-9 and VEGF expression, we pretreated cells with various inhibitors, such as UO126 (MEK1/2 inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor). Interestingly, TPA-induced MMP-9 expression was significantly inhibited by UO126, but not by SP600125 and SB203580. In addition, we pretreated cells with 100 μM silibinin prior to TPA treatment. TPA-induced MEK and ERK phosphorylation was significantly decreased by silibinin in MCF7 cells. TPA-induced VEGF expression was also suppressed by UO126. On the other hand, we found that adenoviral constitutive active-MEK (Ad-CA-MEK) significantly increased MMP-9 and VEGF expression. Taken together, we suggest that the inhibition of TPA-induced MMP-9 and VEGF expression by silibinin is mediated by the suppression of the Raf/MEK/ERK pathway in MCF-7 breast cancer cells.

Keywords: Silibinin; *Silybum marianum*; 12-O-tetradecanoyl phorbol-13-acetate (TPA); Vascular endothelial growth factor (VEGF); Matrix metalloproteinase-9 (MMP-9); MCF-7 human breast cancer cells

Introduction

Silibinin is a major bioactive flavanolignan that has been isolated from milk thistle seeds, and has been used as a traditional medicine (Singh and Agarwal 2002). Silibinin was reported to be a chemopreventive agent against skin cancer in a murine model (Singh and Agarwal 2002), and known to trigger cell cycle arrest and apoptosis in lung cell carcinoma cells, such as SHP-77 and A-549 cells (Sharma et al. 2003). However, the role of silibinin has not been fully elucidated for breast cancer. Herein, we investigated the effect of silibinin on TPA-induced MMP-9 and VEGF expression in the MCF-7 breast cancer cell line.

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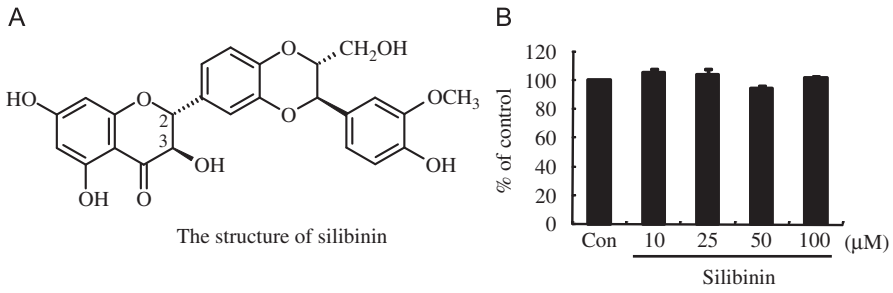

Fig. 1. The effect of silibinin on cell viability in MCF-7 cells. After serum starvation for 24 h, cells were treated with silibinin at the concentrations indicated for 24 h in fresh serum-free media. (A) The structure of silibinin. (B) Cell viabilities were analyzed by the MTT assay, as described in the Materials and Methods. These results are representative of three independent experiments. The values shown are the means ± SEM. Con—control.

The matrix metalloproteinases (MMPs) are a major group of enzymes that regulate cell-matrix composition (Deryugina and Quigley 2006). MMPs are zinc-binding endopeptidases that can degrade virtually all extracellular matrix (ECM) components (Overall and Dean 2006). Abnormally severe expression of MMPs contributes to various pathologic processes, including rheumatoid arthritis, osteoarthritis, angiogenesis, invasion, and metastasis in carcinoma (Chambers and Matrisian 1997; Egeblad and Werb 2002; Murphy and Docherty 1992). MMP-9 belongs to the gelatinase subgroup of the MMP family and the expression and activity of MMP-9 have been associated with different stages of carcinoma progression through breakdown of the basement membrane (Stetler-Stevenson et al. 1993; Zeng and Guillem 1996). Zucker et al. (1993) reported that MMP-9 is increased in the plasma of patients with colon and breast cancers and these inductions do not only regulate tumor invasiveness, but also tumor growth and angiogenesis (Sang 1998).

VEGF is a one of the most important pro-angiogenic growth factors (Leung et al. 1989) and suggested to be a potential therapeutic target in solid tumors, including breast cancer (Jensen et al. 1982). VEGF is highly expressed by malignant and non-malignant cells in response to extracellular stimuli, such as inflammation, hypoxia, and cytokines (Bouck et al. 1996; Ferrara 1999). VEGF-overexpressed breast cancer cells have both greater cell growth and greater metastatic potential (McLeskey et al. 1998), and the role of VEGF has been investigated both *in vitro* and *in vivo* with breast cancer models.

The aim of the current study was to evaluate the effect of silibinin on TPA-induced MMP-9 and VEGF expression. The results suggest that silibinin inhibits TPA-induced ERK phosphorylation and then prevents TPA-induced MMP-9 and VEGF expression in MCF-7 human breast cancer cells. Silibinin may be an effective ingredient for anti-cancer therapy by preventing cancer metastasis through the down-regulation of MMP-9 and VEGF in breast cancer.

Materials and methods

Reagents and cell cultures

Cell culture media, antibiotics, and 10% Zymogram gel were purchased from Life Technologies (Rockville, MD, USA). Rabbit polyclonal anti-phospho-ERK and anti-JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). MMP-9 antibody was obtained from Lab Vision (Neomarker; Fremont, CA, USA). TPA was purchased from R&D Systems (Minneapolis, MN, USA). Silibinin [2,3-Dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-6-(3,5,7-trihydroxy-4-oxobenzopyran-2-yl)benzodioxin] (Fig. 1A; stock solution 50 mM in DMSO) was purchased from Sigma (St. Louis, MO, USA).[1]

The human breast cancer cell line, MCF-7, was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Each cell was maintained in culture medium supplemented without FBS for 24 h.

MTT assay

The viability of cells was monitored after various concentrations of silibinin treatment; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used for the quantification of living, metabolically active cells. Mitochondrial dehydrogenases metabolize MTT to a purple formazan dye, which was measured photometrically at 570 nm (Kim et al. 2007).

[1]Silibinin is a diastereoisomer pair of the A compound with 2R, 3R, 2′S, 3′R absolute configuration and the B-compound with the 2R, 3R, 2′S, 3′S configuration.

Chemical treatments

Cells were maintained in culture medium without FBS for 24 h and then culture media were replaced with fresh media without FBS and the cells were further incubated with inhibitors, such as UO126, SP600125, and SB203580 for 24 h. In experiments involving these inhibitors, cells were pretreated for 30 min prior to 10 nM TPA treatment.

Zymography

Zymography was performed in 10% polyacrylamide gels that had been cast in the presence of gelatin, as described previously (Kim et al. 2007). Briefly, samples (culture media) were resuspended in loading buffer and run on a 10% SDS-PAGE gel containing 0.5 mg/ml gelatin without prior denaturation. After electrophoresis, gels were washed to remove SDS and incubated for 30 min at room temperature in a renaturing buffer (50 mM Tris, 5 mM $CaCl_2$, 0.02% NaN_3, and 1% Triton X-100). The gels were then incubated for 48 h at 37 °C in a developing buffer (50 mM Tris–HCl [pH 7.8] 5 mM $CaCl_2$, 0.15 M NaCl, and 1% Triton X-100). The gels were subsequently stained with Coomassie brilliant blue G-250 and destained in 30% methanol, and 10% acetic acid to detect gelatinase secretion. Signal densities were quantified using a densitometric program (Bio 1D; Vilber Lourmat, Marne La Vallec, France).

Western blotting

Cell lysates were used in immunoblot analysis for Raf, MEK1/2, ERK, and β-actin. Proteins were boiled for 5 min in Laemmli sample buffer and electrophoresed in 10% SDS-PAGE gels. Proteins were transferred to PVDF membranes and the membranes were then blocked with 10% skim milk in TBS with 0.01% Tween-20 for 15 min. The blots were incubated with anti-phospho-Raf, MEK, and ERK antibodies (1/1000 dilution) in 1% TBS/T buffer (0.01% Tween 20 in TBS) at 4 °C overnight. Blots were washed three times in TBS with 0.01% Tween 20 and subsequently incubated in anti-rabbit peroxidase-conjugated antibody (1/2000 dilution) in TBS/T buffer. After 1 h incubation at room temperature, the blots were washed three times and ECL reagents (Amersham Bioscience, Buckinghamshire, UK) were used for development. Signal densities were quantified using a densitometric program (Bio 1D; Vilber Lourmat, Marne La Vallec, France).

ELISA

MCF-7 cells were pretreated with various concentrations of silibinin for 30 min prior to TPA treatment and then treated with 10 nM TPA for 24 h. ELISA assays were performed on culture media (400 μl) collected from the cells. Vehicle (DMSO)-treated cells were used as a control in all of the experiments to provide baseline levels of VEGF production. Protein levels of VEGF were measured using an ELISA kit for human VEGF (DuoSet ELISA Development System; R&D Systems).

Adenovirus transfer

Adenoviral human CA-MEK cDNA was gifted from Dr. Hyunil Ha (Seoul National University, Korea). For infection, recombinant adenovirus was diluted in DMEM containing 10% FBS, and added to the cells at 37 °C for 24 h. After Ad-CA-MEK transfection, the media were replaced with serum-free media for 24 h. The expression of construct was confirmed by Western blotting against phospho-ERK.

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 the significance was accepted when $p < 0.05$.

Results

The basal levels of expression of MMP-9 and VEGF are increased by TPA treatment of MCF-7 cells

The chemical structure of silibinin is shown in Fig. 1A. To verify the effect of silibinin on cell viability, we treated cells with silibinin at the concentrations indicated for 24 h. MCF-7 cell viabilities were not affected by silibinin at various concentrations (Fig. 1B). Thus, in additional experiments, we demonstrated that the reduction in TPA-induced MMP-9 and VEGF expression by silibinin was irrelevant to cell viability.

To examine the time response of TPA on MMP-9 expression, we treated cells with 10 nM TPA for the indicated times in serum-free media. We found that the level of MMP-9 expression was increased in a time-dependent manner by TPA treatment and was significantly increased from 16 to 24 h (Fig. 2A).

In addition, to investigate the dose–response of TPA, we treated cells with TPA at the concentrations indicated for 24 h in serum-free media. Our results showed that TPA increased the levels of MMP-9 and VEGF expression in a dose-dependent fashion (Figs. 2B and C). TPA-induced MMP-9 expression was significantly increased by 10,108.2 ± 1409.8% of the control level following treatment with 20 nM TPA (Fig. 2B). TPA-induced VEGF expression was also increased by

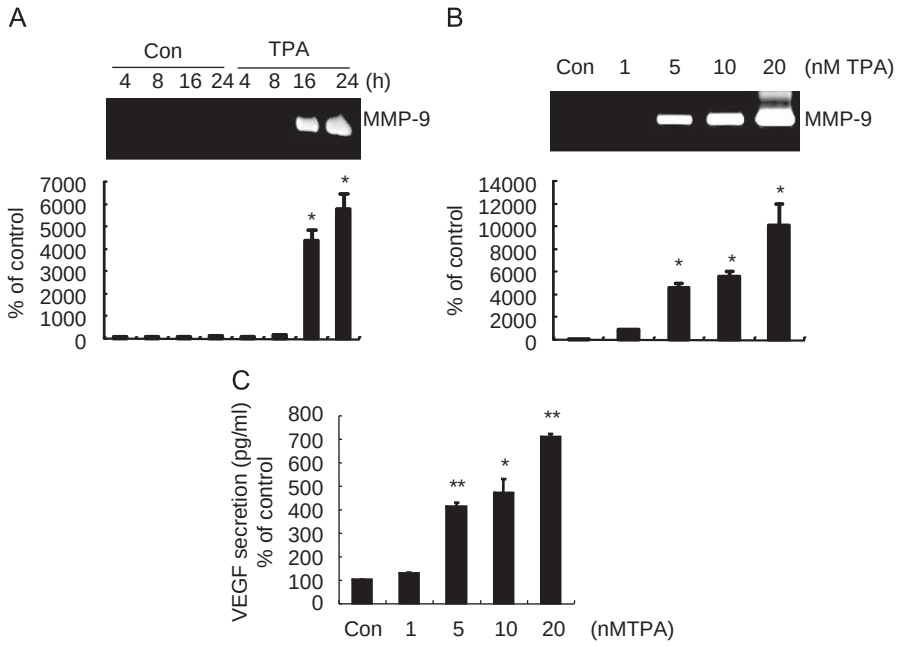

Fig. 2. The basal levels of MMP-9 and VEGF expression are increased by TPA treatment in MCF-7 cells. (A) After serum starvation for 24 h, cells were treated with 10 nM TPA for the times indicated. (B, C) After serum starvation for 24 h, cells were treated with TPA at the concentrations indicated for 24 h. MMP-9 gelatinase activity and VEGF expression were analyzed by Zymography (A, B) and ELISA (C), respectively. The results are representative of three independent experiments. The values shown are the means ± SEM; $*p<0.05$, $**p<0.01$ vs. control. Con—control.

707.2 ± 24.1% of the control level with 20 nM TPA (Fig. 2C). Therefore, we demonstrated that TPA may augment tumor metastasis and angiogenesis through up-regulation of MMP-9 expression in MCF-7 breast cancer cells.

TPA-induced MMP-9 and VEGF expression was decreased in a dose-dependent fashion by silibinin in MCF-7 cells

To investigate the effect of silibinin on TPA-induced MMP-9 and VEGF expression, we pretreated cells with various concentrations of silibinin prior to 10 nM TPA treatment in MCF-7 cells. After 24 h, culture media were harvested, subjected to Zymography and ELISA, and the expression of MMP-9 and VEGF was determined. Our results showed that the level of TPA-induced MMP-9 and VEGF expression was decreased by silibinin in MCF-7 cells in a dose-dependent manner (Figs. 3A and B). The level of MMP-9 expression was significantly increased by 9728.8 ± 342.9% of the control level following treatment with 10 nM TPA in MCF-7 cells (Fig. 3A). In contrast, TPA-induced MMP-9 expression was decreased by 324.8 ± 18.3% of the control level following treatment with 100 μM silibinin (Fig. 3A). These results were also demonstrated in SKBR3 cells (data not shown).

In addition, we detected the amount of VEGF secreted in culture media using ELISA. Our results showed that the amount of VEGF secreted was significantly increased by TPA, while TPA-induced VEGF secretion was decreased by silibinin in MCF7 cells (Fig. 3B). The level of VEGF secretion was increased by 749.8 ± 11.2% of the control level following treatment with 10 nM TPA (Fig. 3B). TPA-induced VEGF secretion, however, was decreased by 416.5 ± 21.5% of the control level following treatment with 100 μM silibinin (Fig. 3B). Therefore, we demonstrated that silibinin may be a candidate for therapy of tumor metastasis and angiogenesis through inhibition of TPA-induced MMP-9 and VEGF expression in MCF-7 breast cancer cells.

Silibinin inhibits TPA-induced ERK phosphorylation, but not JNK and p38 phosphorylation in MCF-7 cells

We next examined the effect of specific inhibitors of MEK1/2, JNK, and p38 on TPA-induced MMP-9 expression. Specifically, we pretreated MCF-7 cells with 10 μM UO126 (MEK1/2 inhibitor), SP600125 (JNK inhibitor), and SB253580 (p38 inhibitor) prior to treatment with 10 nM TPA for 30 min, and then further incubated for 24 h. Our results showed that TPA-induced MMP-9 expression was significantly inhibited

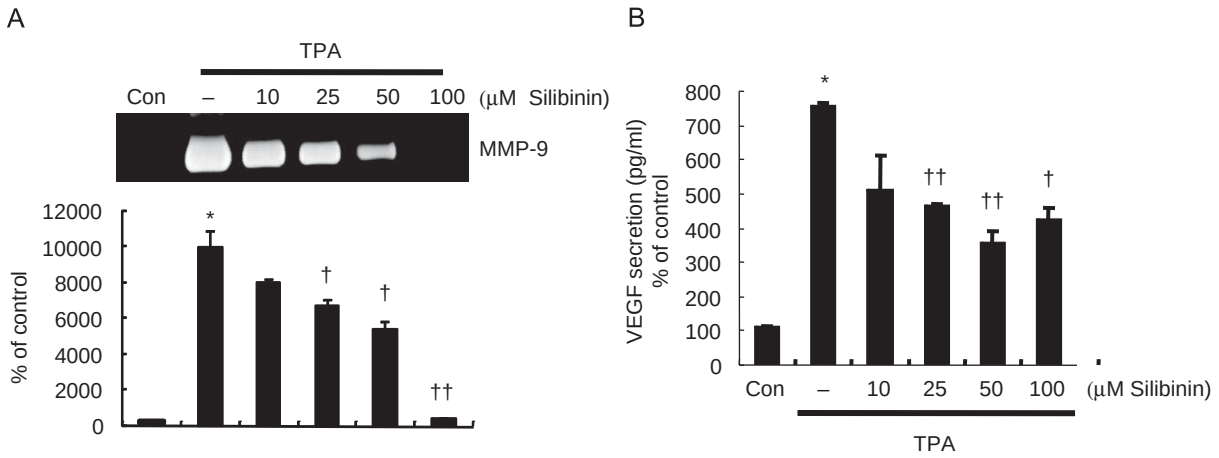

Fig. 3. TPA-induced MMP-9 and VEGF expression was decreased by silibinin in a dose-dependent fashion in MCF-7 cells. (A, B) After serum starvation for 24 h, cells were pretreated with silibinin at the concentrations indicated for 60 min and then treated with 10 nM TPA for 24 h. MMP-9 gelatinase activity and VEGF expression were analyzed by Zymography (A) and ELISA (B), respectively. These results are representative of three independent experiments. The values shown are the means ± SEM; $^{*}p < 0.01$ vs. control; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ vs. TPA-treated cells. Con; control.

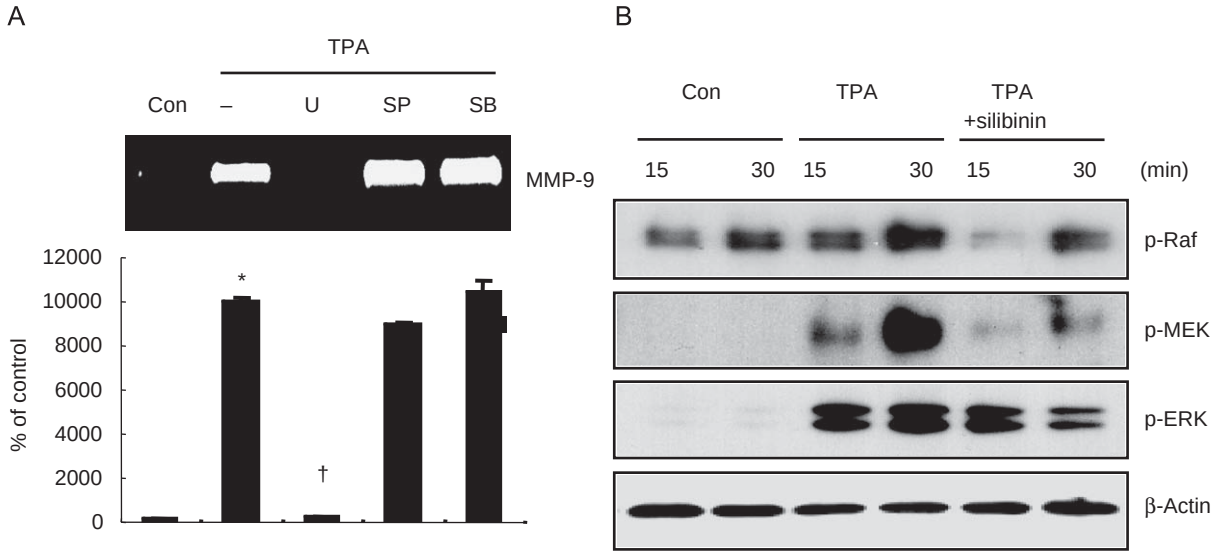

Fig. 4. Silibinin-inhibited TPA-induced ERK phosphorylation, but not JNK and p38 phosphorylation in MCF-7 cells. (A) After serum starvation for 24 h, cells were pretreated with 10 μM U (UO126, MEK inhibitor), SP (SP600125, JNK inhibitor), and SB (SB203580) for 30 min, and then treated with 10 nM TPA for 24 h. MMP-9 gelatinase activity was analyzed by Zymography in culture media. (B) After serum starvation for 24 h, cells were pretreated with 100 μM silibinin for 60 min, and then treated with 10 nM TPA for the times indicated. Using the cell lysates, the phosphorylation of Raf, MEK, and ERK was analyzed by Western blotting. These results are representative of three independent experiments. The values shown are the means ± SEM; $^{*}p < 0.01$ vs. control, $^{\dagger}p < 0.01$ vs. TPA-treated cells. Con—control.

by MEK1/2 inhibitor, but not by JNK and p38 inhibitors (Fig. 4A).

To determine the regulatory mechanism of silibinin on TPA-induced MMP-9 expression, we pretreated MCF-7 cells with 100 μM silibinin prior to treatment with 10 nM TPA for 60 min and then the incubation was continued for the times indicated. We found that the phosphorylation of Raf, MEK, and ERK was significantly increased by TPA at 30 min (Fig. 4B). As shown in Fig. 4B, TPA-induced Raf, MEK, and ERK phosphorylation was maximally decreased by silibinin at 30 min. However, TPA-induced JNK and p38 phosphorylation was not affected by silibinin (data not shown). Therefore, we demonstrated that silibinin

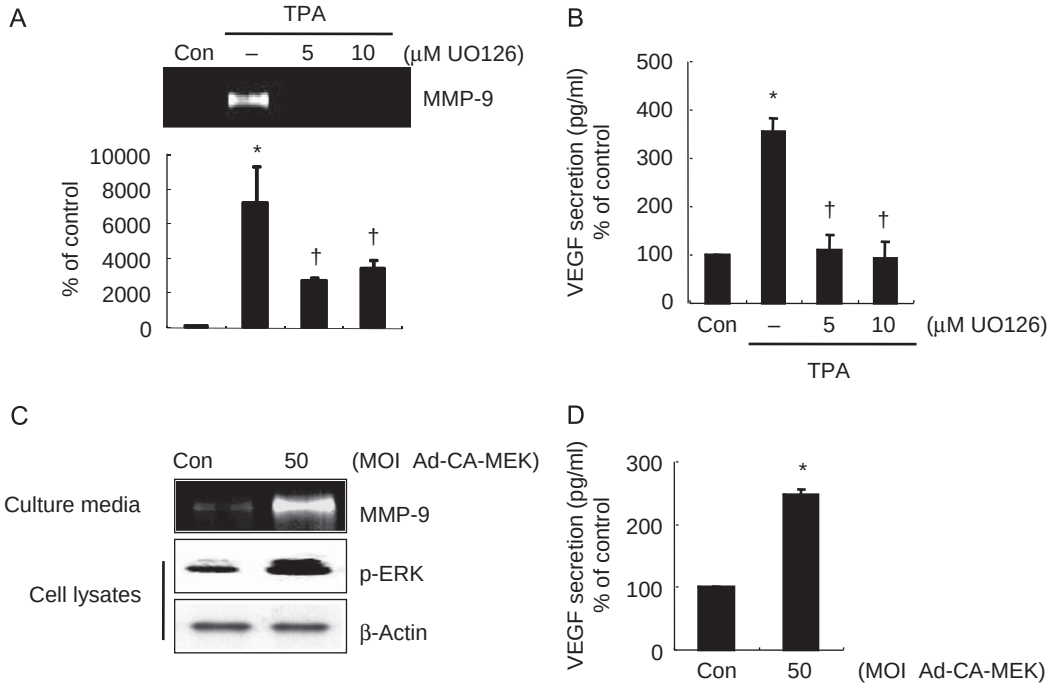

Fig. 5. TPA-induced MMP-9 and VEGF expression is increased by Ad-CA-MEK in MCF-7 cells. (A, B) After serum starvation for 24 h, cells were pretreated with 5 and 10 μM U (UO126, MEK1/2 inhibitor) for 30 min and then treated with 10 nM TPA for 24 h. (C, D) After Ad-CA-MEK transfection for 24 h, cells were incubated in serum-free media for 24 h and then fresh serum-free media were added for 24 h. MMP-9 gelatinase activity and VEGF expression was analyzed in culture media by Zymography (A, C) and ELISA (B, D), respectively. Using the cell lysates, the phosphorylation of ERK was analyzed by Western blotting (C). These results are representative of three independent experiments. The values shown are the means ± SEM; $^{*}p < 0.01$ vs. control, $^{\dagger}p < 0.05$ vs. TPA-treated cells. Con—control.

inhibited TPA-induced MMP-9 expression through inactivation of the Raf/MEK/ERK pathway in MCF-7 cells.

TPA-induced MMP-9 and VEGF expression is increased by Ad-CA-MEK in MCF-7 cells

To confirm the effect of the Raf-MEK-ERK pathway on TPA-induced MMP-9 expression and VEGF secretion, we pretreated MCF-7 cells with 5 and 10 μM UO126 for 30 min prior to treatment with 10 nM TPA and then continued the incubation for 24 h. As shown in Figs. 5A and B, TPA-induced MMP-9 and VEGF expression was significantly decreased by the MEK1/2 inhibitor.

We next investigated the effect of Ad-CA-MEK on TPA-induced MMP-9 and VEGF expression. Thus, we transfected MCF-7 cells with 50 MOI Ad-CA-MEK for 24 h and then continued incubation for 24 h in serum-free media. Our results showed that the levels of MMP-9 and VEGF expression were significantly increased by Ad-CA-MEK (Figs. 5C and D). In particular, the level of expression of VEGF was increased by 246.5 ± 14.1% of the control level in Ad-CA-MEK-transfected cells.

Discussion

Silibinin, one of the major flavanones, has a wide range of pharmacologic effects, such as inhibition of DNA synthesis, cell proliferation, cell cycle progression, and apoptosis in various cancer cell lines, including breast cancer (Bhatia et al. 1999; Sharma et al. 2003). However, the mechanism underlying the inhibitory action of silibinin on tumor metastasis and angiogenesis in breast cancer has not been completely elucidated. Therefore, we investigated whether silibinin prevents TPA-induced MMP-9 and VEGF expression, and the mechanism by which silibinin regulates MCF-7 breast cancer cells.

The enhancement of MMPs expression is involved in tumor invasion, metastasis, and angiogenesis (Naglich et al. 2001; Peng et al. 2006), and the inhibition of MMPs expression may play an important role in cancer therapy because MMPs trigger the degradation of extracellular matrix (ECM) and induce tumor invasion (Naglich et al. 2001). The transcriptional regulation of MMP-9 has been widely investigated in various cell types, including breast and lung cancer cells. The MMP-9 promoter has several transcription factor-binding motifs, including AP-1 and NF-κB (Himelstein et al. 1998).

The AP-1 transcriptional factor induces MMP-9 expression in human keratinocytes (Steinbrenner et al. 2003). Furthermore, MAP kinases, including ERK, JNK, and p38, are known to be involved in the regulation of MMP-9 transcription (Simon et al. 2001). In our previous study, we reported that TPA-induced MMP-9 expression is significantly decreased by berberine, through inactivation of the ERK pathway in human keratinocytes (Kim et al. 2008). Consistent with these reports, our results showed that TPA-induced MMP-9 expression is inhibited by the MEK1/2 inhibitor, UO126, but not by the JNK and p38 inhibitors. In addition, our results showed that silibinin prevents TPA-induced MMP-9 expression, as well as the phosphorylation of Raf, MEK, and ERK in MCF-7 cells.

Vascular endothelial growth factor (VEGF) has multifunctional activity in cancer biology, such as cell migration, vessel formation, and tumor metastasis (Jain et al. 2006). The transcriptional activity of VEGF has been shown to be induced by exogenous oxidants, cytokines, or different growth factors, such as insulin. Our results showed that the level of VEGF expression was significantly increased by TPA treatment, while TPA-induced VEGF expression was decreased by silibinin.

The VEGF promoter region contains various transcription factor-binding sites, including STAT-3, Sp1, and AP-2. VEGF expression appears to be mediated by phosphorylation of extracellular signal-regulated kinase (ERK; Berra et al. 2000; Pages and Pouyssegur 2005). It has been reported that overexpression of dominant negative Raf or Ras, which are upstream molecules of the MEK1/2 and ERK signaling pathway, inhibits ROS-induced VEGF expression (Schafer et al. 2003). In agreement with these findings, our results showed that TPA-induced VEGF expression was decreased by the MEK1/2 inhibitor, UO126, in a dose-dependent fashion, while basal levels of VEGF expression were significantly increased in Ad-CA-MEK overexpressed cells.

As shown in Fig. 6, we suggest that silibinin exhibits a novel function to prevent TPA-induced MMP-9 and VEGF expression through inhibition of the Raf/MEK/ERK pathway in MCF-7 breast cancer cells. Therefore, we suggest that silibinin may be used as an effective constituent for anti-cancer products, which can prevent the degradation of ECM proteins by MMP-9 and VEGF expression.

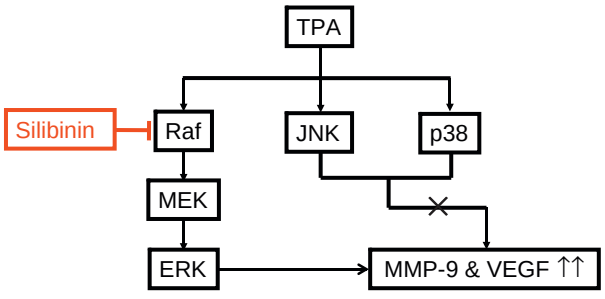

Fig. 6. A schematic model.

Acknowledgements

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