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Silibinin suppresses PMA-induced MMP-9 expression by blocking the AP-1 activation via MAPK signaling pathways in MCF-7 human breast carcinoma cells

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Abstract

Matrix metalloproteinase-9 (MMP-9) plays an important role in the invasion and metastasis of cancer cells. In this study, we examined the inhibitory effect of silibinin, a flavonoid antioxidant from milk thistle (*Silybum marianum* L.) on PMA-induced MMP-9 expression in MCF-7 human breast carcinoma cells. Silibinin significantly and selectively suppressed PMA-induced MMP-9 expression in MCF-7. Silibinin has been found to inhibit PMA-induced MMP-9 gene transcriptional activity by blocking the activation of AP-1 via MAPK signaling pathways. Moreover, the Matrigel invasion assay showed that silibinin reduces PMA-induced invasion of MCF-7 cells. These results suggest that silibinin represents a potential anti-metastatic agent suppressing PMA-induced cancer cell invasion through the specific inhibition of AP-1-dependent MMP-9 gene expression.

Keywords: Silibinin; Milk thistle; MCF-7 human breast carcinoma cells; Invasion; PMA; MMP-9

Invasion and metastasis are fundamental properties of malignant cancer cells. The degradation of extracellular matrix (ECM) which exerts biochemical and mechanical barriers to cell movement has been shown to be an important biological process in the metastasis of cancer cells [1]. ECM degradation and remodeling require the action of extracellular proteinases, among which the MMPs have been shown to play an essential role. Recent reports have suggested that type IV collagenases or gelatinases (MMP-2 and -9) are critical for cell migration leading to invasion and metastasis of cancer [2,3].

The synthesis and secretion of MMP-9 can be stimulated by a variety of stimuli including cytokines and PMA during the various pathological processes such as tumor invasion, atherosclerosis, inflammation, and rheumatoid arthritis, while MMP-2 is usually expressed constitutively [2–4]. On the basis of reports from several different laboratories, it has been generally concluded that the basal levels of MMP-9 in most cancer cell lines are usually low, and that its expression can be induced by treatment of cytokines and PMA via the activation of transcription factors such as NF-κB and AP-1 [5–7]. AP-1 is a well-known key transcription factor which regulates the expression of a number of genes, the products of which are involved in metastasis, tumorigenesis, and inflammation [6,8,9]. Thus, agents able to suppress AP-1 activation represent potential drugs for the inhibition of cancer metastasis.

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Silibinin, a flavonoid antioxidant from milk thistle (*Silybum marianum* L.), is being used as a clinical agent and a dietary supplement for the treatment of various liver diseases in Asia and Europe [10]. Recently, it has become popular in the United States as a sole dietary supplement or as a component of various antioxidant mixtures. Moreover, recent studies have shown that silibinin exerts anticarcinogenic effects in different cancer models including human breast, prostate, lung, colon, and skin cancer cells in vitro or in vivo [11–15]. It has been suggested that silibinin suppresses cell growth through G0/G1 or G2 arrest and inhibition of DNA synthesis together with induction of apoptotic cell death [13,15].

In addition, it has been reported that silibinin exerts inhibitory effects on invasion and metastasis of cancer cells based on its anti-angiogenic property [14,16] and suppressive effects on urokinasetype plasminogen activator and MMP-2 [17]. However, no information is available about its potentials on the regulation of MMP-9 expression and related molecular mechanisms. Therefore, we hypothesized that the reported anti-cancer and anti-metastatic activities of silibinin may be related to its function on the regulation of MMP-9 expression. In the present study, silibinin was examined for its potential on PMA-induced MMP-9 expression in MCF-7 cells with detailed molecular mechanisms. Here, we provide evidence showing that silibinin suppresses PMA-induced MMP-9 expression by blocking the AP-1 activation via MAPK signaling pathways and the suppression of MMP-9 expression is correlated well with the inhibition of cell invasion by silibinin.

Materials and methods

Cells and materials. MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM (Gibco-BRL, Rockville, MD) supplemented with 10% fatal bovine serum (FBS) and 5% CO_2 at 37 °C. All chemicals including silibinin were obtained from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Silibinin was dissolved in dimethyl sulfoxide (DMSO) and the maximum concentration of DMSO was 0.1%. Recombinant human TNF-α was obtained from R&D Systems (Boston, MA). Polyclonal antibodies to MAPK family and phospho-MAPK family were purchased from Cell Signaling Technologies (Beverly, MA). Polyclonal NF-κB (p65) and phospho c-jun antibodies were purchased from Santa Cruz Biotechnology (California, USA). [γ^{32}-P]ATP was obtained from Amersham (Buckinghamshire, UK). Cell culture reagents were purchased from Gibco-BRL (Rockville, MD).

Cell viability assay. The cytotoxic effect of silibinin on MCF-7 cells was investigated using a commercially available proliferation kit (XTT II, Boehringer Mannheim, Mannheim, Germany) as described previously [18]. Cells were plated in 96-well culture plate at a density of 3.2×10^4 cells/well.

Gelatin zymography assay. Conditioned medium was electrophoresed in a polyacrylamide gel containing 0.1% (w/v) gelatin. The gel was then washed at room temperature for 30 min with 2.5% Triton X-100 and subsequently incubated at 37 °C for 24 h in a buffer containing 10 mM $CaCl_2$, 0.01% NaN_3, and 50 mM Tris–HCl (pH 7.5). The gel was stained with 0.2% Coomassie brilliant blue and photographed on a light box. Proteolysis was detected as a white zone in a dark blue field.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted by the RNAzol B reagent (Tel-test, Friendswood, TX, USA) according to the manufacturer's instructions. For RT-PCR, a cDNA was synthesized from 1 μg of total RNA using AMV RNA PCR Kit (Takara, Japan) according to the manufacturer's protocol. The cDNA was amplified by PCR with the following primers: MMP-9 (263 bp), 5′-CACTGTCCACCCCTCAGAGC-3′ (sense) and 5′-GCCACTTGTC GGCGATAAGG-3′ (antisense); β-actin (247 bp), 5′-CAAGAGATGGC CACGGCTGCT-3′ (sense) and 5′-TCCTTCTGCATCCTGT CGGCA-3′ (antisense). PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide.

Transient transfection and luciferase reporter assay. A 710 bp fragment from the 5′-promoter region of the MMP-9 gene was cloned and a 710 bp fragment at the 5′-flanking region of the human MMP-9 gene was amplified by PCR using specific primers from the human MMP-9 gene (GeneBank Accession No. D10051): 5′-ACATTTGCCCGAGC TCCTGAAG (forward/*Sac*I) and 5′-AGGGGCTGCCAGAAGCTTA TGGT (reverse/*Hin*dIII) as described previously [18]. Cells were plated onto 6-well plates at a density of 1×10^6 cells/well and grown overnight. Cells were cotransfected with 1 μg of MMP-9 promoter-luciferase reporter constructs and 0.5 μg of the pCMV-β-galactosidase reporter plasmid for 5 h using Lipofectamine reagent (Invitrogen, San Diego, CA, USA). After transfection, the cells were cultured in 10% FBS medium and incubated with drugs for 24 h. Luciferase and β-galactosidase activities were assayed by using the luciferase and β-galactosidase enzyme assay system (Promega). Luciferase activity was normalized with the β-galactosidase activity in the cell lysate and expressed as an average of three-independent experiments.

Electrophoretic mobility shift assay (EMSA). Nuclear extract of cells was prepared as described previously [18]. EMSA was performed using a gel shift assay system kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, double-stranded oligonucleotides containing the consensus sequence for AP-1-1 (5′-TGACCCCTGAGT CAGCACTT-3′) was end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase and used as probes for EMSA. Competition was performed using either the unlabeled AP-1-1 oligonucleotides. Nuclear extract proteins (2 μg) were preincubated with the gel shift binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl (pH 7.5), and 0.05 mg/ml poly deoxyinosine-deoxycytosine for 10 min, then incubated with the labeled probe for 20 min at room temperature. Each sample was electrophoresed in a 4% nondenaturing polyacrylamide gel in 0.5× TBE buffer at 250 V for 20 min. The gel was dried and exposed to X-ray film overnight.

Western blot analysis. Total cell lysates were prepared as described previously [18]. Aliquots of cellular proteins (30 μg/lane) were electrophoresed on 10% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to an Immobilon-P-membrane (Millipore, USA). The membrane was allowed to react with a specific antibody and detection of specific proteins was carried out by enhanced chemiluminescence following the manufacturer's instructions. Loading differences were normalized using a polyclonal β-actin antibody.

Invasion assay. Matrigel-coated filter inserts (8 μm pore size) that fit into 24-well invasion chambers were obtained from Becton–Dickinson (New Jersey, USA). MCF-7 cells to be tested for invasion were detached from the tissue culture plates, washed, and resuspended in conditioned medium (1×10^5 cells/well), then added to the upper compartment of the invasion chamber in the presence or absence of drugs. Conditioned medium (500 μl) was added to the lower compartment of the invasion chamber. The chambers were incubated at 37 °C for 24 h in 5% CO_2. After incubation, the filter inserts were removed from the wells and the cells on the upper side of the filter were removed using cotton swabs. The filters were fixed, stained, and mounted according to the manufacturer's instructions (Becton–Dickinson). The cells that invaded through the Matrigel and were located on the underside of the filter were counted. Three to five chambers were used per condition. The values obtained were calculated by averaging the total number of cells from three filters.

Statistical analysis. The results are expressed as means ± SE and differences between means for two groups were determined by unpaired Student's *t* test. The minimum significance level was set at *P* value of $\leqslant 0.05$ for all analysis. All experiments were performed at least three times.

Results

Silibinin suppresses PMA-induced MMP-9 expression in MCF-7 cells

Prior to the investigation into the pharmacological potential of silibinin on PMA-induced MMP-9 expression, we first determined the dose dependence of the cytotoxic effects of silibinin in MCF-7 cells by means of an XTT assay. Silibinin at concentrations lower than 80 μM had modest cytotoxic effect on the cells and silibinin showed about 35% decrease in cell viability at 100 μM (Fig. 1A). We next used a gelatin zymography and a Western blot assay to investigate the inhibitory effect of silibinin on PMA-induced MMP-9 secretion and protein expression. The media from control cells contained very weak proteolytic activity at 92 kDa, corresponding to MMP-9 and high proteolytic activity at 72 kDa, corresponding to MMP-2. Treatment with PMA for 24 h dramatically induced MMP-9 secretion and protein expression in a dose-dependent manner in MCF-7 cells (data not shown), while the level of MMP-2 secretion was not affected by PMA (Fig. 1B). The PMA-induced MMP-9 secretion and protein expression were dramatically inhibited in the presence of silibinin in a dose-dependent manner in the concentration range of 10–80 μM (Fig. 1B).

Silibinin suppresses PMA-induced MMP-9 secretion through inhibition of its transcriptional activity

To determine whether the inhibition of MMP-9 secretion by silibinin was due to a decreased level of transcription, we performed RT-PCR and promoter assay using transiently transfected cells with a luciferase reporter gene linked to the MMP-9 promoter sequence. Treatment of cells with silibinin decreased the levels of PMA-stimulated MMP-9 mRNA expression (Fig. 1B). As shown in Fig. 1C, luciferase activity was increased up to 5.2-fold in cells treated with PMA as compared with untreated cells. Treatment of cells with silibinin decreased PMA-stimulated luciferase activity in a dose-dependent manner. These results show that silibinin suppresses PMA-induced MMP-9 secretion through inhibition of its transcriptional activity in MCF-7 cells.

Silibinin inhibits PMA-induced MMP-9 expression by blocking the AP-1 activation

It has recently been reported that the MMP-9 promoter contains cis-acting regulatory elements for transcription factors that include two AP-1 sites (located at −79 and −533 bp) and an NF-κB site (located at −600 bp), and the AP-1 and the NF-κB elements are centrally involved in the induction of the MMP-9 gene by PMA [5,6]. To investigate which of these transcription factors are involved in the inhibition of the MMP-9 transcription by silibinin in MCF-7 cells, we first examined the effect of silibinin on the PMA-stimulated nuclear translocation of p65, a major sub-

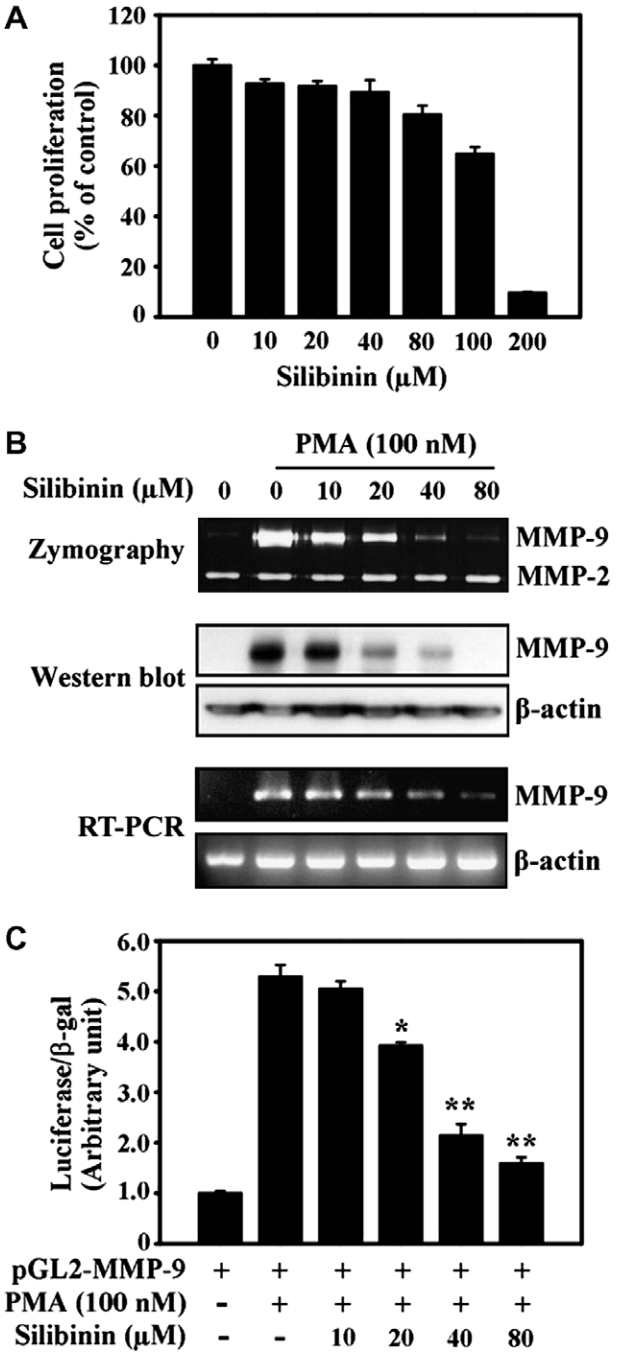

Fig. 1. Effects of silibinin on the growth of MCF-7 cells and PMA-induced MMP-9 expression. Cells were treated with the indicated concentrations of drugs for 24 h. Cell viability was determined by an XTT assay (A). Cells were treated with the indicated concentrations of drugs in the presence of PMA (100 nM) for 24 h. The conditioned medium was prepared and used for gelatin zymorgraphy. The MMP-9 protein levels were measured by Western blotting and GAPDH was used as an internal control. The MMP-9 mRNA levels were measured by RT-PCR and β-actin was used as an internal control (B). pMMP-9WT-luciferase vector was cotransfected with pCMV-β-galactosidase vector into the cells. After 5 h transfection, the cells were treated with the indicated concentrations of silibinin in the presence of PMA (100 nM) (C). Luciferase activities were normalized to β-galactosidase activity. Each value represents the mean ± SE of three-independent experiments and is expressed relative to a control; $^{*}P < 0.05$ vs PMA, $^{**}P < 0.001$ vs PMA.

unit of NF-κB and phosphorylation of c-Jun, a major subunit of AP-1, which are required for the transcriptional activities as determined by Western blot analysis. As shown in Fig. 2A, PMA induced the nuclear translocation of p65 and phosphorylation of c-Jun and silibinin inhibited the phosphorylation of c-Jun in a dose-dependent manner. However, silibinin did not inhibit the PMA-induced nuclear translocation of p65. These data suggested that silibinin regulates the transcriptional activation of MMP-9 through the inhibition of PMA-stimulated AP-1 activity, but not NF-κB activity. Futhermore, we confirmed the inhibitory effect of silibinin on the binding of AP-1 isolated from PMA-induced MCF-7 cells to oligonucleotides that contain the sequence for the AP-1 binding site from the MMP-9 promoter. MCF-7 cells were incubated with different concentrations of silibinin in the presence of PMA for 30 min, and nuclear extracts were prepared and tested by EMSA. As shown in Fig. 2B, silibinin at a concentration of 40 μM completely inhibited PMA-induced binding activity of AP-1.

MAPK signaling pathways are involved in the inhibition of PMA-induced MMP-9 expression by silibinin

MMP-9 gene expression can be activated via a number of signal transduction pathways including those involving ERK1/2, p38 MAPK, and JNK, which are the upstream modulators of AP-1 or NF-κB [5,6,19]. The subsequent experiments were designed to elucidate which of these signal transduction pathways are involved in PMA-stimulated MMP-9 expression and silibinin inhibition of the MMP-9 expression in MCF-7 cells. First, the effects of specific kinase inhibitors on the expression of MMP-9 in PMA-induced MCF-7 cells were analyzed by gelatin zymography. PMA-induced MMP-9 secretion was completely inhibited by selective inhibitor of the ERK1/2 (U0126) and partly inhibited by selective inhibitors of the p38 MAPK (SB203580) or JNK (SP600125) (Fig. 3A). Then, we investigated whether silibinin inhibited the activation of these three signal pathways. PMA induced the phosphorylation of all of three members of the MAPKs as early as 5 min, with a maximal phosphorylation at 15 min (data not shown). Silibinin showed inhibitory effects on the phosphorylation of ERK1/2, p38 MAPK, and JNK pathways in a dose-dependent manner at 15 min after PMA treatment (Fig. 3B). These results suggest that the specific inhibitions of MAPK signaling pathways are directly involved in the regulation of PMA-induced MMP-9 expression by silibinin.

Silibinin inhibits PMA-induced invasion of MCF-7 cells in vitro

It has been reported that the up-regulation of MMP-9 expression contributes to invasion of cancer cells [2,3].

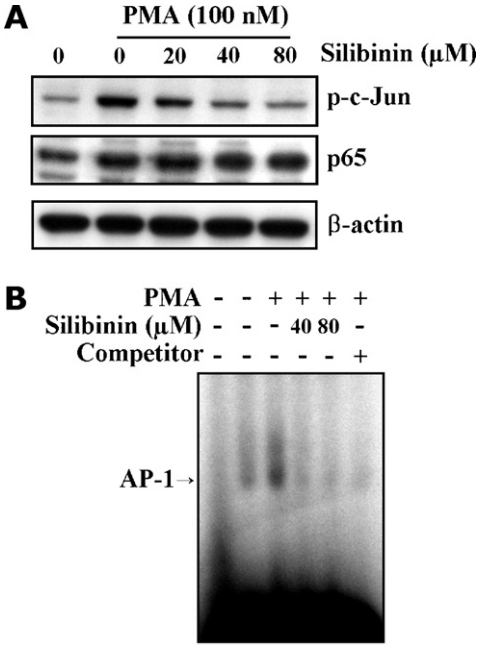

Fig. 2. Effects of silibinin on the PMA-induced AP-1 and NF-κB activations in MCF-7 cells. Cells were treated with the indicated concentrations of silibinin in the presence of PMA (100 nM). The nuclear extracts were prepared and examined for phospho-c-Jun and p65 protein expressions by Western blotting. β-Actin was used as an internal control (A). Nuclear extracts were also examined for AP-1 activation by EMSA. Competition was performed using an unlabeled AP-1 double-stranded oligonucleotides (B).

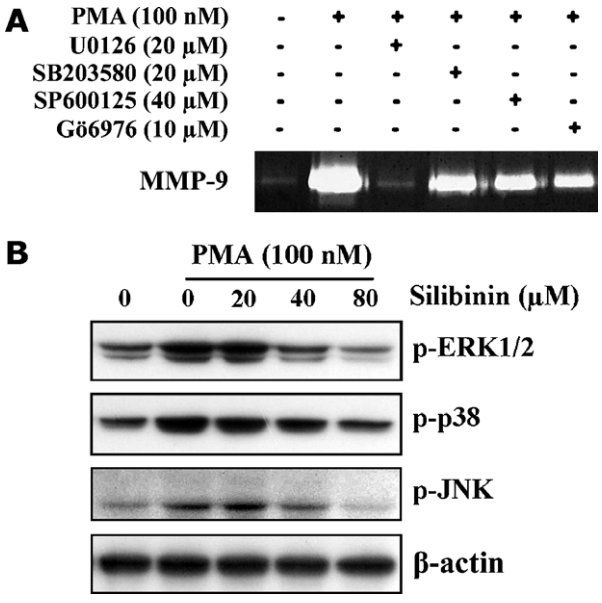

Fig. 3. Effects of silibinin on PMA-induced activation of MAPK signaling pathways in MCF-7 cells. Cells were stimulated with PMA (100 nM) for 24 h in the presence or absence of each inhibitor, and MMP-9 secretion in the conditioned medium was determined by gelatin zymorgraphy (A). Cells were treated with PMA (100 nM) for 15 min in the presence or absence of silibinin, and the phosphorylation levels of ERK1/2, p38 MAPK, and JNK were measured by Western blotting (B).

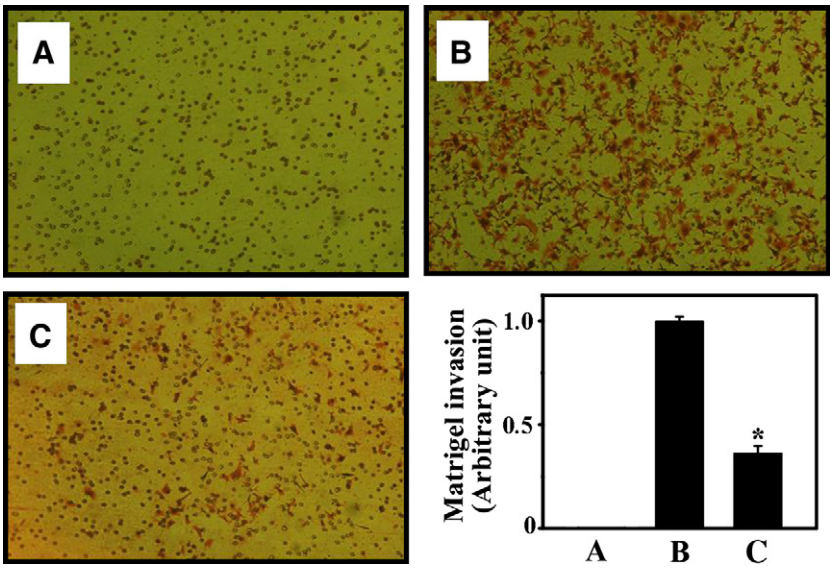

Fig. 4. Effect of silibinin on PMA-induced Matrigel invasion of MCF-7 cells. A Matrigel migration assay was carried out with silibinin in the presence of PMA (100 nM). After 24 h incubation, cells on the bottom side of filter were fixed, stained, and counted. (A) Control; (B) PMA alone; (C) PMA with silibinin (80 μM). Each value represents the mean ± SE of three-independent experiments and is expressed relative to a control; $^{*}P < 0.001$ vs PMA.

Thus, we examined whether the invasion of PMA-induced MCF-7 cells was decreased by silibinin, which previously showed a selective inhibition of PMA-induced MMP-9 expression. As shown in Fig. 4, the invasion of MCF-7 cells was increased by treatment with PMA when compared with PMA-untreated control cells, as evidenced by a Matrigel invasion assay. However, silibinin inhibited the PMA-induced migration of MCF-7 cells.

Discussion

Tumor metastasis is a multistep and complex process that includes cell proliferation, proteolytic digestion of ECM, cell migration to circulation system, and tumor growth at metastatic sites [5]. MMPs play a major role in promoting tumor metastasis and overexpression of MMP-9 has been shown to be associated with the progression and invasion of tumors including mammary tumors [20]. Thus, the inhibitory effect on its expression is important for a therapeutic experimental model of tumor metastasis.

Although many anti-cancer drugs can be clinically applicable, they are generally known to have strong cellular cytotoxicity and side effects. An essential requirement for the successful long-term treatment for cancer is that the drug has little or no toxicity [16]. Based on this suggestion, silibinin, a potential anticarcinogenic flavonoid which has been widely used as a dietary supplement to improve liver function and clinically as an antihepatotoxic drug, is a good example of such an agent. It has been reported that silibinin does not show any substantial adverse effects when it is given to human and rodents at doses as high as 1% (w/w) or 2 g/kg body weight [21,22]. Human trials of silibinin are already underway for the treatment of prostate cancer, and in the completed phase I study has displayed no toxic effects [23]. Recently, a number of studies have shown that silibinin exerts antitumor activity in animal models of different cancers. We also demonstrated here that silibinin suppresses cell invasion through inhibition of MMP-9 expression with its detailed molecular mechanisms, supporting the previous reports of its therapeutic potentials in cancer.

Our results demonstrated that silibinin inhibits the PMA-induced MMP-9 secretion in a dose-dependent manner through suppression of the transcriptional activity of MMP-9 gene in MCF-7 cells (Figs. 1 and 2). Silibinin also inhibited the enzymatic activity of the MMP-9 secreted from PMA-induced MCF-7 cells in a dose-dependent manner (Supplementary Fig. IA), suggesting that silibinin is strong candidate for treatment of tumor invasion and metastasis via dual inhibition of MMP-9 enzyme activity and gene transcription. Silibinin also significantly inhibited MMP-9 secretion in MCF-7 cells induced by TNF-α which is an important physiological inducer for MMP-9 (Supplementary Fig. IB), indicating its possible inhibitory effect in physiological relevance.

Silibinin blocked the activation of AP-1, but not NF-κB, by suppressing the interaction of AP-1 proteins with oligonucleotides that contain the sequence for the AP-1 binding site from the MMP-9 promoter (Fig. 2). On the other hand, previous studies showed that SP-1 site of MMP-9 promoter is also involved in the induction of its gene expression in response to PMA in tumor cell lines [24]. However, Hong et al. [6] reported that SP-1 binding activity is not stimulated by PMA in human renal carcinoma. Our results were consistent with their findings that a reporter vector containing reiterated SP-1site was not affected by PMA with or without silibinin (data not shown). These data clearly

indicate that silibinin blocks PMA-induced MMP-9 production mainly through inhibition of AP-1-mediated MMP-9 induction.

Several studies have identified signal transduction pathways that are involved in regulation of MMP-9 expression in tumor cells [25,26], endothelial cells [27], keratinocytes [28], and VSMC [29,30]. The role of MAP kinases in the regulation of MMP-9 expression in malignant cells is specially well understood. In this study, we found that PMA-induced MMP-9 activation was decreased by ERK1/2, p38 MAPK, JNK inhibitors, or PKC-α and -β inhibitor (Gö6976) and ERK1/2 is a major regulator of MMP-9 expression (Fig. 3A). Our observation in this experiment is in general agreement with previous reports showing that activation of ERK1/2 play a critical role in the MMP-9 expression in various cell types when they are induced by different stimulators such as PMA [6], IL-1β [31], or fibroblast growth factor-2 [32]. Here we also identified the signal pathway-mediated regulation of the MMP-9 gene in PMA-induced MCF-7 cells in response to the treatment with silibinin. The data here show that all of three members of the MAPKs are involved pathways in the silibinin-mediated inhibition of MMP-9 expression in PMA-induced MCF-7 cells (Fig. 3B). These findings suggest that silibinin regulates PMA-stimulated MMP-9 expression by suppressing the MAPK pathways in MCF-7 cells.

The data obtained from the Matrigel invasion assay showed that the selective induction of MMP-9 expression by PMA causes to the stimulation of cell invasion, however, silibinin significantly inhibits the PMA-induced invasion potential of MCF-7 cells (Fig. 4), suggesting that the inhibition of cell invasion by silibinin is correlated well with the inhibition of MMP-9 expression.

In conclusion, silibinin suppresses PMA-induced MMP-9 expression in MCF-7 cells by inhibiting the activation of AP-1 via the MAPK signaling pathways. This is the first study showing that silibinin suppresses PMA-stimulated invasion of cancer cells through inhibition of MMP-9 expression, and this new beneficial effect of silibinin may expand future researches on the regulation of tumor invasion and metastasis in vivo.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.181.

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