THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 26, Issue of July 1, pp. 25202–25209, 2005
Printed in U.S.A.

Ascochlorin Inhibits Matrix Metalloproteinase-9 Expression by Suppressing Activator Protein-1-mediated Gene Expression through the ERK1/2 Signaling Pathway

INHIBITORY EFFECTS OF ASCOCHLORIN ON THE INVASION OF RENAL CARCINOMA CELLS*

Received for publication, December 13, 2004, and in revised form, April 15, 2005
Published, JBC Papers in Press, April 29, 2005, DOI 10.1074/jbc.M413985200

SaHyun Hong‡, Kwan-Kyu Park‡, Junji Magae§¶, Kunio Ando¶, Tae-Sung Lee‖, Taeg Kyu Kwon, Jong-Young Kwak‡‡, Cheorl-Ho Kim§§, and Young-Chae Chang‡¶¶**

*From the ‡Department of Pathology and ‖Department of Obstetrics and Gynecology, College of Medicine, Catholic University of Daegu, Daegu, 705-034, Korea, §Department of Biotechnology, Institute of Research and Innovation, Kashiwa 277-0861, Japan, ¶NRL Pharma Inc., Kawasaki 213-0012, Japan, **Department of Immunology, Keimyung University School of Medicine, Daegu 700-712, Korea, ‡‡Medical Research Center for Cancer Molecular Therapy, Dong-A University, Busan 602-714, Korea, and §§National Research Laboratory for Glycobiology, Korean Ministry of Science and Technology, College of Oriental Medicine, Dongguk University, Kyungbuk 780-714, Korea*

The expression of matrix metalloproteinases (MMPs) has been implicated in the invasion and metastasis of cancer cells. Here we examined the effect of ascochlorin, a prenyl-phenol anti-tumor compound from the fungus *Ascochyta viciae*, on the regulation of signaling pathways that control MMP-9 expression in human renal carcinoma (Caki-1) cells. Ascochlorin reduced the invasive activity of Caki-1 cells and inhibited phorbol 12-myristate 13-acetate-induced increases in MMP-9 expression and activity in a dose-dependent manner. Reporter gene, electrophoretic mobility shift, kinase inhibitor assays, and *in vitro* kinase assay showed that ascochlorin inhibits MMP-9 gene expression by suppressing activation of the nuclear transcription factor activator protein-1 (AP-1) via the extracellular signal-regulated kinase 1 and 2 pathway. The AP-1 family member most specifically affected by ascochlorin was Fra-1. Ascochlorin did not affect the activation of the c-Jun N-terminal or p38 kinase pathways. Moreover, transfection of Caki-1 cells with AP-1 decoy oligodeoxynucleotides resulted in the suppression of phorbol 12-myristate 13-acetate-induced MMP-9 expression and invasion. In conclusion, ascochlorin represents a unique natural anti-tumor compound that specifically inhibits MMP-9 activity through suppression of AP-1-dependent induction of MMP-9 gene expression.

Ascochlorin (see Fig. 1*A*), a prenyl-phenol compound isolated from the fungus, *Ascochyta viciae*, was originally found to have antiviral antibiotic activity (1). In addition to its antiviral and anti-fungal activity, natural and synthetic derivatives of ascochlorin reduce serum cholesterol and triglyceride levels suppress hypertension and tumor development and ameliorate type I and II diabetes (2–9). Moreover, several ascochlorin derivatives have been reported to be potent agonists of nuclear hormone receptors, including peroxisome proliferator-activated receptor γ, suggesting that the structure of ascochlorin would be useful in designing modulators of nuclear receptors (6, 10, 11, 13).

The most well known extracellular matrix (ECM)[1]-degrading enzymes are the matrix metalloproteinases (MMPs). MMPs are a family of zinc-dependent endoproteinases that are capable of degrading all the components of the ECM. MMPs are structurally and functionally homologous proteins, with at least 20 known members. MMPs can be divided into four families based on structure and substrate specificity: collagenases, gelatinases, stromelysins, and membrane-associated MMPs. Among the previously reported human MMPs, gelatinase-A (MMP-2) and gelatinase-B (MMP-9) are key enzymes that degrade type IV collagen (14, 15). These two gelatinases share structural and catalytic similarities, but transcription of the MMP-2 and MMP-9 genes is independently regulated due to distinct arrays of regulatory elements in the gene promoters. Regulated expression of MMP-9 has been implicated in renal development, macrophage differentiation, atherosclerosis, inflammation, rheumatoid arthritis, and tumor invasion (16–17). In contrast, MMP-2 is usually expressed constitutively.

Recent studies have reported a positive correlation between the expression of MMP-9 and tumor metastasis in colorectal cancer and several other types of epithelial cancers (18, 19), suggesting an important functional role for these proteinases in metastasis. Metastasis of cancer cells requires several sequential steps, including changes in cell-ECM interactions, dissociation of intercellular adhesion complexes, separation of single cells from the solid tumor mass, degradation of the ECM, and locomotion of tumor cells into the ECM (20, 21).

The mechanisms of MMP-9 gene activation in human cancer cells are not well defined. The production of MMP-9 can be induced by a number of factors, including the inflammatory cytokine tumor necrosis factor-α. Tumor necrosis factor-α also serves as an autocrine regulator of the phorbol 12-myristate 13-acetate (PMA)-induced expression of MMP-9. It is known that the human MMP-9 promoter contains several cis-acting

* This work was supported by Korea Research Foundation Grant KRF-2002-005-C00016. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶¶ To whom correspondence should be addressed: Dept. of Pathology, College of Medicine, Catholic University of Daegu, 3056-6, Daemyung-4-Dong, Nam-gu, Daegu 705-034, Korea. Tel.: 82-53-650-4848, Fax: 82-53-650-4834, E-mail: ycchang@cu.ac.kr.

[1] The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; NF-κB, nuclear factor κB; PMA, phorbol myristate acetate; AP, activator protein; ERK1/2, extracellular signal-regulated kinase; WT, wild type; ASC, ascochlorin; MTT, 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; PKC, protein kinase C; DTT, dithiothreitol; JNK, c-Jun NH_2-terminal kinase; SAPK, stress-activated protein kinase; RT, reverse transcription; mt-, mutant; TIMP, tissue inhibitor of metalloproteinase; ODN, oligodeoxynucleotide.

regulatory elements that participate in the regulation of the MMP-9 gene expression, including sites that bind the transcription factors AP-1 (located at −79 and −533 bp), NF-κB (−600 bp), and Sp1 (−588 bp) (22, 23).

We hypothesized that the anti-tumor and anti-metastatic activity of ASC may function to regulate MMP-9 in renal diseases. Renal cell carcinoma is a common renal malignancy, and no specific therapy presently is available for treating advanced renal cell carcinoma or tumor recurrence. It has been shown that MMP-9 expression correlates with invasion and metastasis of renal carcinoma cells, but the mechanisms regulating expression in this and other tumors are poorly understood (24, 25). Because early diagnosis of renal cell carcinoma would enhance treatment, we are interested in the expression and activity of markers like MMPs for the detection and treatment of renal cell carcinoma. In the studies described here, a human renal carcinoma cell line (Caki-1) was used to investigate the pathways that regulate MMP-9 expression. When Caki-1 cells are stimulated by PMA, expression of MMPs, particularly MMP-9, is enhanced greatly. Here we describe the molecular mechanism by which ASC blocks expression of MMP-9 in Caki-1 cells.

EXPERIMENTAL PROCEDURES

Cells and Materials—Caki-1 (human renal carcinoma), Chang (human liver cells), MDA-MB-231 (breast adenocarcinoma), and U2OS (osteosarcoma) cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum, 20 mM HEPES buffer, and 100 μg/ml gentamicin. ASC produced by *A. viciae* was purified as described previously (1). Lipofectamine reagent was obtained from Invitrogen. Luciferase and β-galactosidase assay systems were obtained from Promega (Madison, WI). PMA, dexamethasone and α-lipoic acid were purchased from Sigma. Specific PKC, mitogen-activated protein kinase kinase (MEK)-1, c-Jun N-terminal kinase, and p38 inhibitors (Go6976, PD98059, SP600125, and SB203580, respectively) were obtained from Calbiochem.

Cytotoxicity Assays—Reduction of 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) (Roche Applied Science) assays were performed as described in the supplier's protocol to evaluate the cytotoxicity of ASC.

Cell Invasion Assays—5×10^4 cells/chamber were used for each invasion assay. The upper parts of the Transwell (Corning Costar, Cambridge, MA) were coated with 30 μl of a 1:2 mixture of Matrigel: phosphate-buffered saline. Cells were plated on the Matrigel-coated Transwells in the presence of various concentrations of ASC. The inserts were incubated for 24 h at 37 °C. Cells that had invaded the lower surface of the membrane were fixed with methanol and stained with hematoxylin and eosin. Random fields were counted by light microscopy under a high power field (×200).

Gelatin Substrate Gel Zymography—To determine the effect of ASC on PMA-induced MMP-9 activity, cells were treated with various concentrations of ASC in the presence of 50 nM PMA, and MMP-9 expression was evaluated by zymography. Zymography was performed using a previously described the procedure with minor modifications (26). Caki-1 cells were suspended in medium containing 10% fetal bovine serum and plated at 3×10^5 cells/35-mm dish. The dishes were incubated until the cultures were 80% confluent; the medium then was changed to fresh serum-free medium with or without ASC compounds. Supernatants were collected after incubation for 24 h. The medium was subjected to SDS-PAGE in 10% polyacrylamide gels that had been copolymerized with 1 mg/ml gelatin. After electrophoresis, the gels were washed several times in 2.5% Triton X-100 for 1 h at room temperature, then incubated for 24 h at 37 °C in buffer containing 5 mM $CaCl_2$ and 1 μM $ZnCl_2$. Gels were stained with Coomassie Brilliant Blue R250 (0.25%) (Bio-Rad) for 1 h and then destained. Proteolytic activity was evidenced as clear bands against the blue background of the stained gelatin.

Western Blot Analysis—Cellular lysates were prepared by suspending 3×10^5 cells/35-mm dish in 30 μl of lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.5% Nonidet P-40, 100 μM phenylmethylsulfonyl fluoride, 20 μM aprotinin, and 20 μM leupeptin, adjusted to pH 8.0). The cells were disrupted and extracted at 4 °C for 30 min. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corp., Bredford, MA). Detection of specific proteins was carried out by enhanced chemiluminescence following the manufacturer's instructions (Amersham Biosciences). MMP-9, MMP-2, p38, PKC, JNK/stress-activated protein kinase (SAPK), ERK1/2, c-Fos, c-Jun, Fra-1, and ATF2 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific ERK1/2, p38, and JNK/SAPK antibodies were purchased from New England Biolabs (Beverly, MA).

RT-PCR—Total RNA was extracted by RNA ZolBee (Invitrogen). For reverse transcription reaction, cDNA was synthesized from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Promega). PCR primers are described below. PCR products were analyzed by agarose gel and visualized by ethidium bromide. Used primers in RT-PCR are described in Ahn *et al.* (30).

Plasmid Transfection and Luciferase Reporter Gene Assays—MMP-9 wild type (pGL2-MMP-9WT), AP-1 site-mutated MMP-9 (pGL2-MMP-9mAP-1–1 and pGL2-MMP-9mAP-1–2), NF-κB site-mutated MMP-9 luciferase promoter constructs (pGL2-MMP-9mNF-κB), MMP-2 promoter construct (26, 27), and SP-1 reporter constructs (28) were used in transient transfection assays as described previously. The AP-1 and NF-κB reporter constructs were purchased from Clontech (Palo Alto, CA). Cells were plated at a density of 3×10^5 cells/35-mm dish and grown overnight. Cells were cotransfected with 2 μg of various plasmid constructs and 1 μg of the pCMV-β-galactosidase plasmid for 5 h using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. After transfection, the cells were cultured in 10% fetal bovine serum medium with vehicle (Me_2SO) or ASC for 24 h. Luciferase and β-galactosidase activities were assayed according to the manufacturer's protocol (Promega). Luciferase activity was normalized for β-galactosidase activity in cell lysates and expressed as the average of three independent experiments.

Electrophoretic Mobility Shift Assay—Cultured cells were collected by centrifugation, washed, and suspended in buffer A (10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride). After 15 min on ice, the cells were vortexed in the presence of 0.5% Nonidet P-40. The nuclear pellet was then collected by centrifugation and extracted with buffer B (20 mM Hepes (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) for 15 min at 4 °C. The nuclear extracts (10 μg) were preincubated at 4 °C for 30 min with a 100-fold excess of an unlabeled oligonucleotide consensus sequence of AP-1, NF-κB, and SP-1. After this time the reaction mixtures were incubated at 4 °C for 30 min in 25 mM Hepes buffer (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 0.05 M NaCl, and 2.5% glycerol with 1 μg of poly(dI/dC) and 5 fmol (2×10^4 cpm) of a probe end-labeled with [γ-^{32}P]ATP. Probes included 30-mer oligonucleotides encompassing the consensus sequences for AP-1, NF-κB, and SP-1. The reaction mixtures were resolved by electrophoresis at 4 °C in 6% polyacrylamide gels using a TBE (89 mM Tris, 89 mM boric acid, and 1 mM EDTA) running buffer. Gels were rinsed with water, dried, and exposed to x-ray film overnight.

In Vitro Kinase Assay of ERK and JNK—Cells ($5–10 \times 10^6$ cells) detached from a dish were washed with 0.9% NaCl, centrifuged for 5 min at 3000 rpm at 4 °C, and lysed with a buffer containing 50 mM Tris, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1% Triton X-100, 0.5% Nonidet P-40, 10% glycerol, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate, 2 mM *p*-nitrophenylphosphate, and 0.3 units/ml aprotinin. The cell lysate was centrifuged for 10 min at $10{,}000 \times g$ at 4 °C and stored at −80 °C. The immunoprecipitates were immobilized on protein G-Sepharose beads (Amersham Biosciences) by incubation for 4 h at 4 °C overnight. The pellet was washed twice with the lysis buffer, suspended in kinase assay buffer (25 mM Hepes, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.3 units/ml aprotinin, and 2 mM *p*-nitrophenylphosphate), and mixed with either 20 μg of c-Jun (for JNK assay) or 20 μg of Elk-1 protein (for ERK-1 assay), 5 μCi of [^{32}P]ATP and incubated at 30 °C for 30 min. The reaction was stopped by the addition of 2× Laemmli buffer, and the sample was boiled, centrifuged, and separated by electrophoresis. The gels were washed, dried, and analyzed by a phosphorimaging analyzer.

Synthesis of AP-1 Decoy ODNs and Selection of Target Sequences—ODNs included the transcriptional factor AP-1 binding sequence (underlined below (in this paragraph)) at the central site and dummy sequences upstream and downstream of the binding sequence. As described (29, 30), double-stranded ODNs were prepared and designated as the AP-1 decoy. A mutant AP-1 (mt-AP-1) decoy with ODNs that were mutated at two bases (small letters) of the AP-1 binding sequence was used as a control. Inspection of the resulting nucleotide sequences in transcriptional regulation databases revealed no homology with other known transcription factors. AP-1 decoy, single-stranded sequence, 5′-TGTC<u>TGACTCA</u>TGTC-3′; mt-AP-1 decoy, 5′-TGTCTctCTgATGTC-3′. 100 nM decoy ODN was

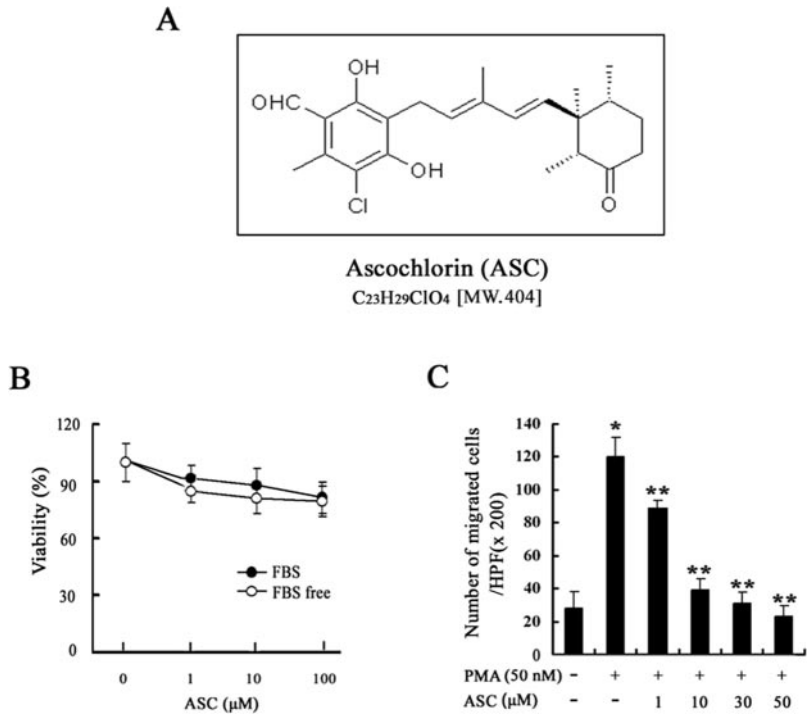

FIG. 1. **Chemical structure of ASC and the effects of ASC on the viability and *in vitro* invasion of Caki-1 cells.** *A*, chemical structure and molecular weight of ASC. *B*, effects of ASC on cell viability. Caki-1 cells were treated with ASC in the presence (*open circle*) or absence of serum (*closed circle*), and cell viability was tested by MTT assay after 24 h. *C*, ASC inhibits cell invasion. For invasion assays, the upper parts of Transwells were coated with Matrigel, and Caki-1 cells with various concentrations of PMA and ASC were added. After 24 h, cells on the bottom side of filter were fixed, stained, and counted. Data represent the mean ± S.E. of at least three independent experiments. Results were statistically significant (*, $p < 0.05$ compared with control; **, $p < 0.01$ compared with PMA only treatment) using Student's *t* test. *FBS*, fetal bovine serum.

transferred to cells by Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions.

RESULTS

Effect of ASC on Proliferation and Invasion of Caki-1 Cells—The structure of ASC used in this study is shown in Fig. 1*A*. Because it was known to have antibiotic activity, we first tested the cytotoxic effects of ASC in Caki-1 cells in serum-free and 10% serum-containing medium using an MTT assay. Treatment of cells with ASC ranging in concentration from 1 to 100 μM showed a 10–12% decrease in cell viability in serum containing medium and a 12–15% decrease in cell viability in serum-free medium (Fig. 1*B*). We next used a cell invasion assay to begin analysis of the anti-tumor activity of ASC. As shown in Fig. 1*C*, treatment of Caki-1 cells with PMA stimulated invasion about 5-fold. ASC inhibited PMA-dependent invasion in a dose-dependent manner, with inhibition in ASC-treated cells reaching the levels of control cells by 10–30 μM. These results indicated that ASC counteracts the effects of PMA on induction of invasive activity in Caki-1 cells.

ASC Inhibits MMP-9 Activity in a Dose-dependent Manner—Reduced invasion activity by ASC prompted us to examine the effect of ASC on proMMP-9 activity induced by PMA. Caki-1 cells, which release proMMP-2 and proMMP-9 when cultured in serum-free medium, were treated with various concentrations of PMA for 24 h. The level of proMMP-2 expression was not significantly altered by PMA (50 nM), but the expression of proMMP-9 was induced, as evidenced by gelatin zymography (Fig. 2*A* and data not shown). This concentration of PMA had no effect on cell growth. From 1 to 50 μM ASC caused dose-dependent decreases in PMA-induced proMMP-9 activity; the activity of proMMP-2 also was decreased but only at higher doses.

To determine whether the inhibitory effect of ASC on proMMP-9 activity is a general phenomenon, Chang, MDA-MB-231, and U2OS cells were tested for sensitivity to ASC. Chang cells secrete MMP-2 constitutively in a manner similar to Caki-1 cells, whereas MDA-MB-231 and U2OS cells constitutively secrete primarily MMP-9. Increased activity of proMMP-9 in response to PMA (50 nM) was dramatically decreased by ASC in each cell line (Fig. 2*A*). As for Caki-1 cells, the effect on proMMP-2 activity was less dramatic. These results indicated that the ASC effect on proMMP-9 activity is not cell type-specific.

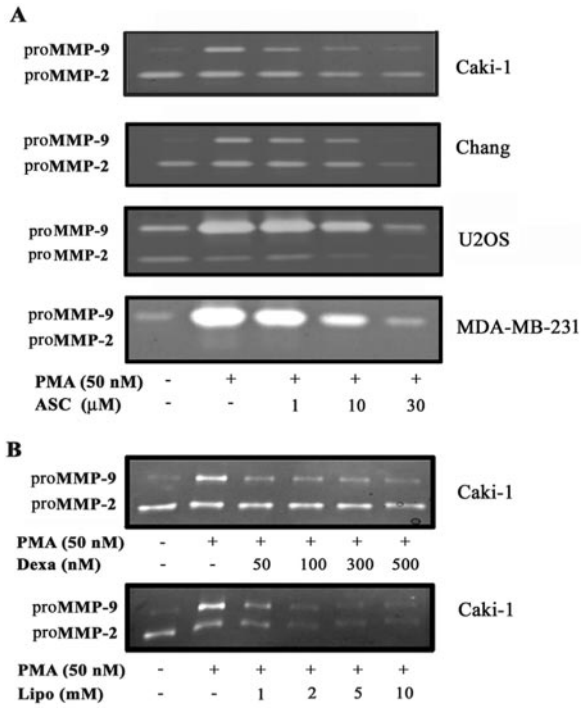

FIG. 2. **Effect of ASC, dexamethasone, and α-lipoic acid on proMMP-9 activity.** Caki-1, Chang, MDA-MB-231, and U2OS cells were treated with various concentrations of ASC (*A*), dexamethasone (*Dexa*, *B*), or α-lipoic acid (*Lipo*, *C*) in the presence of PMA (50 nM). Conditioned medium was collected from cultures after 24 h and analyzed by gelatin zymography.

We next compared the effects of ASC to other known MMP-9 inhibitors that suppress AP-1 activity. Caki-1 cells were treated with dexamethasone in the presence of PMA, and proMMP-9 expression was evaluated by zymography as described previously (21, 31). Treatment with dexamethasone decreased proMMP-9 activity in a dose-dependent manner; proMMP2 activity was not

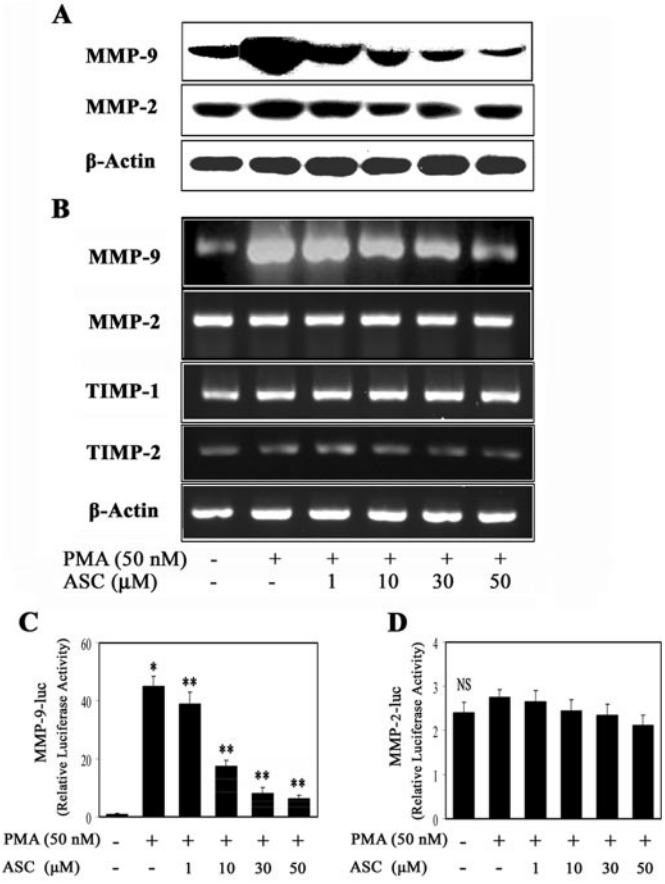

FIG. 3. **ASC inhibits PMA-induced MMP-9 expression.** *A*, expression of MMP-9 and MMP-2 in Caki-1 cells treated with vehicle and ASC in the presence of PMA for 24 h was evaluated by Western blot analysis using anti-MMP-9 and MMP-2 antibodies. Expression of β-actin in cell lysates was used as a control. *B*, using RT-PCR, the levels of MMP-9, MMP-2, and TIMP-1 and TIMP-2 mRNA from cells treated as indicated were determined; a representative result from three independent experiments is shown. *C*, Caki-1 cells were transfected with WT-MMP-9 promoter-containing reporter vector and incubated with various concentrations of ASC in the absence or presence of PMA (50 nM) as indicated. Luciferase activity was measured 24 h after transfection. Values represent the mean of at least three independent experiments; *bars,* ±S.D. Results were statistically significant (*, $p < 0.05$ compared with control; **, $p < 0.01$ compared with PMA only treatment) using Student's *t* test. *D*, Caki-1 cells were transfected with WT-MMP-2 reporter vector, and luciferase activity was measured 24 h after transfection. Values represent the mean of at least three independent experiments; *bars,* ± S.D.

affected. α-Lipoic acid, another inhibitor of MMP-9 (32), decreased both proMMP-9 and proMMP-2 activity in a dose-dependent manner (Fig. 2*B*). These results suggest that ASC is more selective for MMP-9 than these known inhibitors.

ASC Inhibits Expression of MMP-9—The levels of MMP-9 and MMP-2 proteins were determined by Western blot analysis (Fig. 3*A*). The expression levels of MMP-9 protein gradually decreased in a dose-dependent manner, indicating that reduced proMMP-9 enzyme activity was the result of decreased amounts of MMP-9 protein. As before, ASC had less dramatic effects on expression of MMP-2.

To determine whether the inhibition of MMP-9 enzyme expression by ASC was due to a decreased level of transcription, we performed RT-PCR and reporter gene analyses. In RT-PCR analysis, the treatment of Caki-1 cells with ASC decreased the levels of PMA-stimulated MMP-9 mRNA expression (Fig. 3*B*). Because the activities of MMP-9 are also tightly regulated by endogenous inhibitors, tissue inhibitor of metalloproteinases (TIMPs) (16, 33), the levels of expression of TIMP-1 and -2 RNA were assessed by RT-PCR. ASC had no effect on TIMP-1 and TIMP-2 mRNA expression at the concentrations tested (Fig. 3*B*).

The effect of ASC on MMP-9 promoter activity was also investigated using Caki-1 cells that had been transiently transfected with a luciferase reporter gene linked to the MMP-9 promoter sequence. As shown in Fig. 3*C,* luciferase gene expression was activated up to 50-fold in cells treated with PMA as compared with untreated cells. Treatment of cells with ASC (10 μM) decreased PMA-mediated luciferase activity in a dose-dependent manner, indicating that ASC inhibits MMP-9 expression at the transcriptional level and that the MMP-9 promoter contains ASC response elements. ASC had little effect on a luciferase reported gene containing the MMP-2 (Fig. 3*D*).

Effects of ASC on AP-1 and NF-κB Activities—The MMP-9 promoter contains cis-acting regulatory elements for transcription factors that include two AP-1 sites (located at −79 bp and −533 bp) and an NF-κB site (located at −600 bp). To test which of these transcription factors may regulate the MMP-9 gene in Caki-1 cells, cells were transiently transfected with reporter genes that included the wild type (WT) MMP-9 promoter or promoters with mutations in one or both AP-1 sites or the NF-κB site (Fig. 4*A*). The mutation of the AP-1 binding site at −533 bp drastically decreased induction by PMA, and mutation of both AP-1 binding sites completely abolished the activation of the MMP-9 promoter by PMA. Mutation of the NF-κB binding site also decreased PMA-induced MMP-9 reporter gene activity but not to the extent achieved by mutation of the AP-1 sites. Treatment with 10 μM ASC in the presence of PMA also decreased MMP-9 activity in reporters with AP-1 and NF-κB site mutations.

In subsequent experiments cells were transiently transfected with reporter vectors that included the tandem repeat of AP-1, NF-κB, or SP-1 binding sites. Luciferase activity in the cells with the AP-1 construct was significantly reduced by treatment with ASC at 1 to 50 μM, whereas luciferase activity in cells containing the NF-κB construct showed no statistically significant changes in the presence of ASC (Fig. 4*C*). Vectors containing reiterated SP-1 sites were not affected by ASC with or without PMA (data not shown). These results show that both the AP-1 and NF-κB binding sites in the MMP-9 promoter contribute to promoter activity and that the AP-1 sites represent the primary sites for regulating responses to ASC during PMA-dependent activation of the MMP-9 promoter.

ASC Decreases Transcription Factor Binding to AP-1 Motifs—To examine the reporter gene results further, electrophoretic mobility shift assays were performed using oligonucleotides containing the consensus sequences for AP-1, NF-κB, and SP-1 as probes. Caki-1 cells were incubated in the presence of PMA with different concentrations of ASC for 24 h, and nuclear extracts then were prepared and analyzed for AP-1 and NF-κB DNA binding activity (34). The results indicated that AP-1 is induced by PMA, as expected, but this activity decreased dramatically when cells were treated with ASC (Fig. 5*A*). In contrast, NF-κB binding activity was slightly increased (Fig. 5, *A* and *B*). These data were consistent with the reporter gene analysis and suggest that ASC blocks MMP-9 expression at least in part by decreasing the expression or DNA binding activity of members of the AP-1 transcription factor family. Although NF-κB contributed to PMA-dependent MMP-9 reporter gene expression, activation of the NF-κB pathway does not appear to be involved directly in the ASC-mediated inhibition.

The PKC and ERK1/2 Mitogen-activated Protein Kinase Signaling Pathways Are Involved in PMA-dependent Modulation of MMP-9 Expression—The electrophoretic mobility shift assay results suggest that the AP-1 transcription factor complex

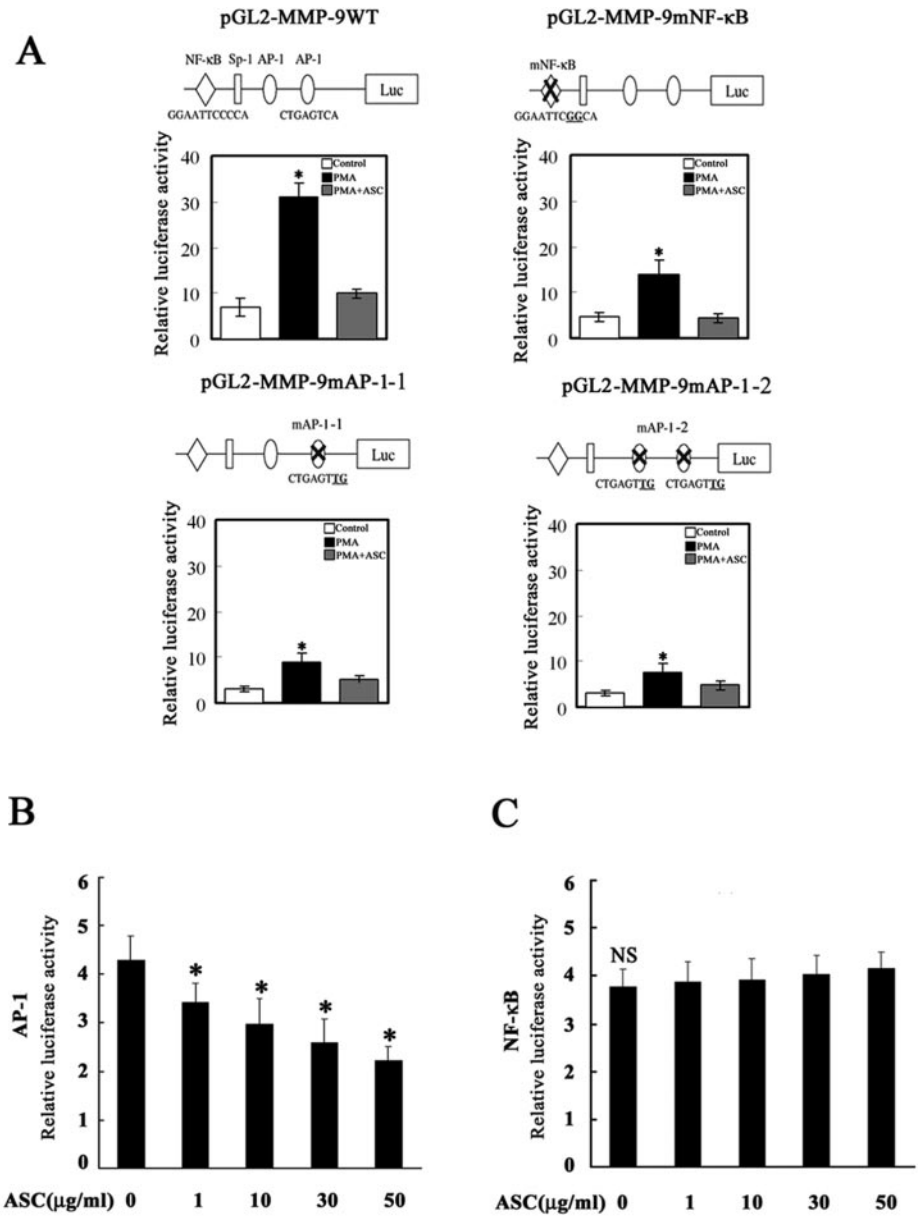

FIG. 4. **Effects of ASC on the activities that function through AP-1 and NF-κB binding sites.** *A*, mutations were introduced in the NF-κB or AP-1 binding sites of pGL2-MMP-9WT by 2-bp changes. Caki-1 were transfected with pGL2-MMP-9WT or pGL2-MMP-9mNF-κB or pGL2-MMP-9mAP-1 reporter plasmids and then cultured with PMA (50 nM) and ASC (10 μM). Caki-1 cells transfected with the indicated reporters were cultured with ASC (10 μM) and/or PMA (50 nM) for 24 h, and luciferase activity in cell extracts was then determined. Values represent the mean of at least three independent experiments; *bars,* ± S.D. Results were statistically significant (*, $p < 0.05$ compared with control) using Student's *t* test. *B* and *C*, Caki-1 cells were transfected with reporter vectors containing binding sites for AP-1 (*B*) or NF-κB (*C*) and cultured in the presence of ASC for 24 h, and luciferase activity was measured. Values represent the mean of at least three independent experiments; *bars,* ± S.D. Results were statistically significant (*, $p < 0.05$ compared with control) using Student's *t* test.

plays an essential role in stimulation of MMP-9 promoter activity. In its active form the AP-1 complex may be composed of homodimers of c-Jun or heterodimers between c-Fos, c-Jun, and ATF2 (35–38). To determine whether PKC is involved in PMA-dependent MMP-9 expression, MMP-9 expression was analyzed by gelatin zymography in cell cultures treated with Go6976, a general PKC-α and -β inhibitor. Treatment of Caki-1 cells with Go6976 (1–10 μM) abolished PMA-induced MMP-9 secretion in a dose-dependent manner (Fig. 6*A*), demonstrating that activation of PKC is critical for induction of MMP-9 secretion in response to PMA.

It is known that activation of one or more mitogen-activated protein kinase pathways is important for the MMP-9 induction by PMA in various cell types (27). The experiments described below were designed to elucidate the signaling cascades that activate expression of the MMP-9 gene in Caki-1 cells in response to stimulation by PMA and how ASC influences information flow through these pathways. Hence, the effects of specific kinase inhibitors on the expression of MMP-9 in Caki-1 cells were analyzed by zymography. PD98059 is known to selectively block the activity of mitogen-activated protein kinase kinase (MEK), an activator of ERK1/2 kinases. SP600125 is known to inhibit the c-Jun N-terminal kinase (JNK)/(SAPK). Before stimulation with PMA, cells were incubated with various concentration of PD98059 (10 μM) or SP600125 (10 μM) for 1 h and then incubated for 30 min in the presence of PMA. PD98059 caused a dose-dependent decrease in PMA-dependent induction of proMMP-9 activity in culture medium, whereas SP600125 had no effect to proMMP-9 activity (Fig. 6*A* and data not shown). We also tested other kinase inhibitors, including SB203580, a specific inhibitor of p38 kinase. SB203580 (100 nM) had no effect on MMP-9 activity (Fig. 6*A*).

To further evaluate the effects of ASC on these signaling cascades, we used antibodies against the phosphorylated forms of the three mitogen-activated protein kinases ERK1/2, JNK/SAPK, and p38. As shown in Fig. 6*B*, ERK1/2 phosphorylation was induced by PMA and was decreased as the result of ASC treatment; the levels of phosphorylated JNK and p38 remained unchanged (Fig. 6*B*). We confirmed that the concentrations of inhibitors used in these studies effectively reduced the phosphorylation of the corresponding target kinases (Fig. 6*C*). These results suggest that ASC specifically inhibits ERK1/2 activity. This result was further confirmed by *in vitro* kinase assay for endogenous ERK-1 and JNK. ERK-1 and JNK were

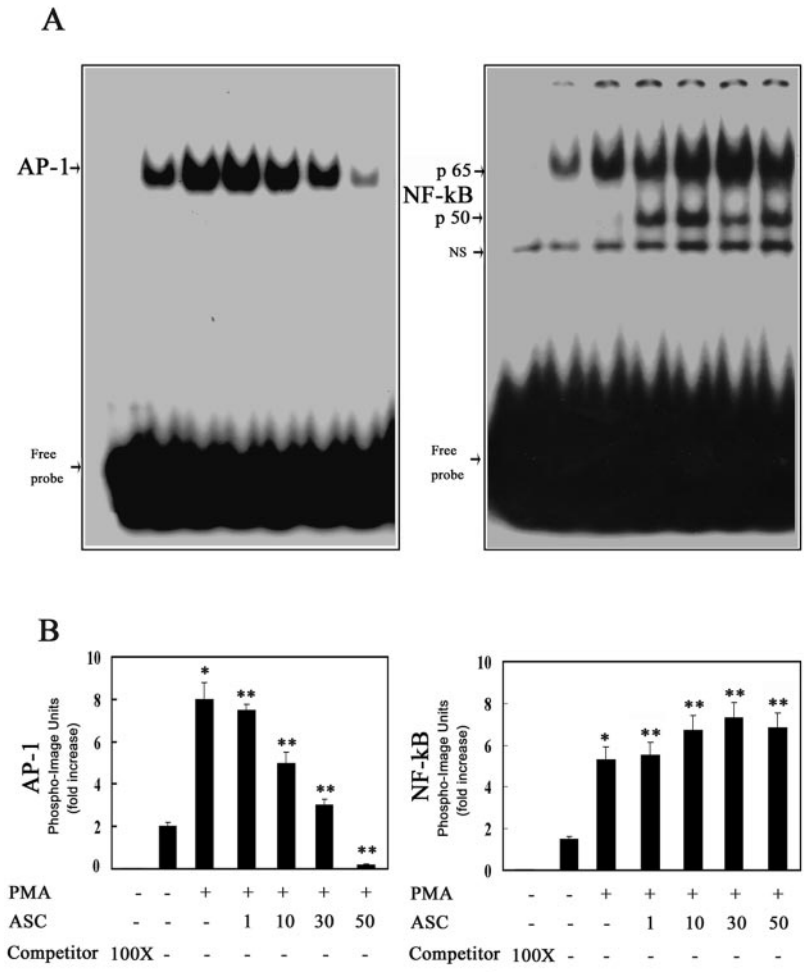

FIG. 5. **Transcription factor binding to the MMP-9 AP-1 motif.** Nuclear extract (10 μg) prepared from control or Caki-1 cells treated for 24 h with 50 nM PMA and ASC was mixed with radioactive oligonucleotide containing AP-1 and NF-κB motif. Bound complexes were analyzed by electrophoresis (*A*). The results are expressed as the mean ± S.D. of three independent experiments (*B*). *NS*, nonspecific signal. Statistical significance was determined as: *, $p < 0.05$ compared with control; **, $p < 0.01$ compared with PMA only treatment using Student's *t* test.

immunoprecipitated with specific antibodies and incubated with and without Elk-1 and c-Jun, specific substrates for ERK-1 and JNK, in the presence of [γ-^{32}P]ATP. Although PMA activated both ERK-1 and JNK activities, ASC specifically decreased ERK-1 activity induced by PMA stimulation without affecting JNK activity (Fig. 7). Western blotting of immunoprecipitates showed that ASC did not alter protein expression of ERK and JNK after PMA treatment. Taken together, these results indicate an essential role for PKC/ERK1/2 in activation of MMP-9 expression in Caki-1 cells.

Next we examined the effects of ASC on expression of c-Jun, c-Fos, Fra-1, and ATF2 by immunoblotting using the same culture and treatment conditions as before. PMA (50 nM) treatment greatly induced expression of c-Fos and Fra-1 and induced phosphorylation of c-Jun as expected (Fig. 8). PMA had no discernible effect on ATF2. In the presence of PMA, ASC treatment (1 to 30 μM) decreased expression of c-Fos and Fra-1 but did not influence expression or phosphorylation of c-Jun. Again, ATF2 expression was not affected (Fig. 8).

AP-1 Decoys Partially Inhibit PMA-induced MMP-9 mRNA Expression—We next used transfection of decoy AP-1 binding sites to examine the requirement for AP-1 on PMA-induced expression of MMP-9 in Caki-1 cells. Cells were transfected with wild type or mutant AP-1 decoys stimulated with PMA (50 nM) for 24 h and then subjected to RT-PCR analysis. Caki-1 cells treated with Lipofectamine alone were used as controls. Treatment with the wild type AP-1 decoy reduced PMA-induced MMP-9 mRNA expression but not to control levels (Fig. 9*A*). In contrast, transfection of the mt-AP-1 decoy or treatment with Lipofectamine had little inhibitory effect on PMA-induced MMP-9 mRNA.

We also examined whether PMA-induced cell invasion was inhibited by AP-1 decoy transfection. Caki-1 cells were transfected with wild type AP-1 decoy or mt-AP-1 decoy as before, and invasion was assayed after treatment with 50 μM PMA. Enhanced invasion in response to PMA in Caki-1 cells transfected with wild type AP-1decoy was reduced to about 60% that of the untransfected control (Fig. 9*B*). In contrast, transfection with mt-AP-1 decoy treatment only had minimal effect on invasiveness. Because the transfection efficiency of decoy oligonucleotides was not measured directly in these experiments, we are unable to conclude if these effects represent a modest decrease in expression of MMP-9 in most cells or more substantial decreases in a fraction of the cell population. However, these results reinforce those described above that link the inhibitory effects of ASC on PMA-dependent MMP-9 expression to activation of AP-1 by the ERK1/2-signaling pathway.

DISCUSSION

ASC is a potent antiviral and antifungal compound, and its derivatives are known to modulate a variety of physiological activities, anti-tumor, anti-metastatic, and anti- diabetic activities in animals (1–9). A variety of phenotypic changes are associated with cancer cells, including increased proliferation, migration, and invasion. ASC appeared to have little effect on cell viability but did affect cell invasion as assessed by the Matrigel assay (Fig. 1). Decreased invasion activity by treatment of Caki-1 cells with ASC prompted us to survey its relationship to expression of MMP-9, a protease implicated in tumor invasion in a number of settings.

Using a renal carcinoma cell line, our results indicate that ASC is a potent inhibitor of PMA-induced MMP-9 expression.

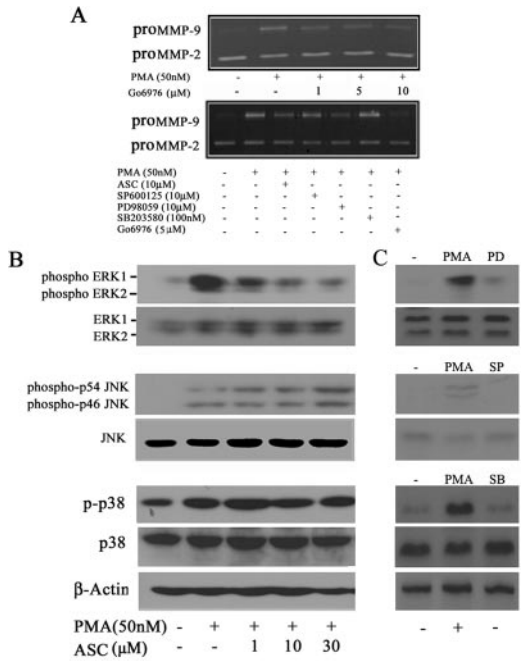

FIG. 6. **Effects of various kinase inhibitors on the PMA mediated stimulation of proMMP-9 production.** *A*, Caki-1 cells were stimulated with PMA (50 nM) in the presence or absence of several inhibitors as indicated for 24 h, and proMMP-2 and -9 production in culture medium was determined by zymography. *B*, ASC affects the levels of phosphorylated mitogen-activated protein kinase. Caki-1 cells were incubated with ASC and/or PMA as indicated, and the levels of phospho-JNK/SAPK, -ERK1/2, and -p38 were determined by Western blotting with phospho-specific antibodies. Protein expression levels of β-actin in cell lysates were used as a control. *C*, the phosphorylation level of ERK1/2, JNK/SAPK, and p38 in cells incubated with PD98059 (*PD*, 10 μM), SP600125 (*SP*, 10 μM), and SB203580 (*SB*, 100 nM) in the presence or absence of PMA were analyzed by Western blotting. Protein expression levels of β-actin in cell lysates was used.

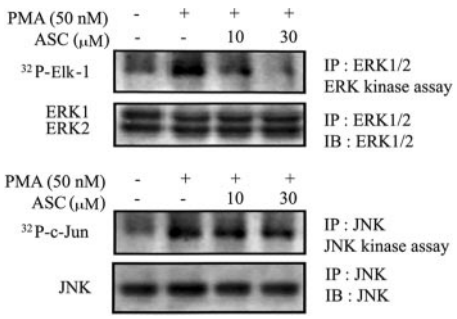

FIG. 7. **ASC Inhibits activation of nuclear ERK.** Caki-1 cells were treated with ASC and/or PMA for 24 h, and activities of nuclear ERK-1 and JNK immunoprecipitated with specific antibodies were determined by *in vitro* kinase assay using Elk-1 and c-Jun as a specific substrate for ERK-1 and JNK, respectively. Total ERK1/2 and JNK in immunoprecipitates (*IP*) were also shown by Western blotting (*IB*).

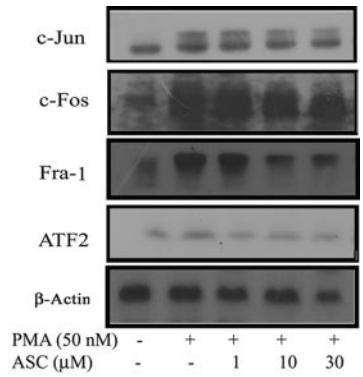

FIG. 8. **Selective suppression of Fos-family proteins by ASC.** Caki-1 cells were treated with ASC and/or PMA for 24 h, and the expression of c-Jun, c-Fos, Fra-1 and ATF2 was analyzed by Western blotting. Expression of β-actin was used as a loading control.

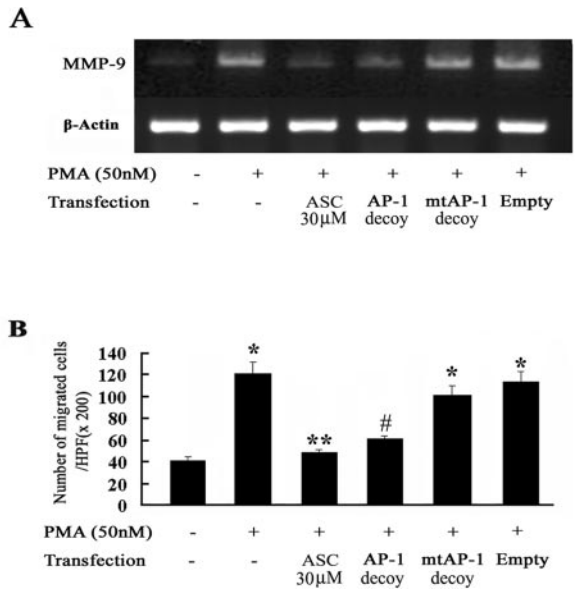

FIG. 9. **Competition for AP-1 binding activity inhibits MMP-9 expression and the invasiveness of Caki-1 cells.** *A*, Caki-1 cells transfected with AP-1 decoy or mt-AP-1 decoy or treated with Lipofectamine alone (*Empty*) were incubated in serum-free Dulbecco's modified Eagle's medium with 50 nM PMA for 24 h. Total RNA was extracted, and the expression levels of proMMP-9 and β-actin mRNA was determined by RT-PC. *B*, Caki-1 cells were transfected with AP-1 decoy or mt-AP-1 decoy oligonucleotides as above or treated with Lipofectamine alone (*Empty*). Matrix penetration was assayed as described in the legend for Fig. 1. Values represent the mean ± S.E. of at least three independent experiments. Results were statistically significant (*, $p < 0.05$ compared with control; **, $p < 0.01$ compared with PMA only treatment; #, $p < 0.01$ compared with PMA only treatment) using Student's *t* test.

The ASC effect on MMP-9 expression was also observed in other cell lines (Chang, MDA-MB-231, and U2OS), suggesting that the ability of ASC to inhibit MMP-9 expression may be a general phenomenon. The ultimate target of the inhibitory effect was on transcription of the MMP-9 gene, as evidenced by RT-PCR and reporter gene assays. BecauseTIMP-1 is a major inhibitor of MMP-9, and TIMP-1 and -2 are differentially regulated *in vivo* as well as cell in a culture systems (39, 40), we ruled out the effects of ASC on TIMP-1 and -2.

The molecular mechanism by which ASC inhibits PMA-mediated expression involves inhibition of AP-1 activation through the ERK1/2-signaling pathway. ASC also influences NF-κB DNA binding activity, and increases in this pathway may lead to p50 homodimers that inhibit expression of MMP-9, a possibility that deserves further investigation. However, our reporter gene and inhibitors studies indicate the ASC acts primarily through AP-1, which agrees with previous reports that MMP-9 is regulated by AP-1 (22, 23). In addition, the inhibition of invasion activity of Caki-1 cells by AP-1 decoys in this study supports a role for AP-1 in regulation of MMP-9 expression and that ASC acts through AP-1 to inhibit MMP-9 expression.

The expression of proteases such as MMP-9 is regulated by diverse growth factors, cytokines, and xenobiotics such as PMA. The role of mitogen-activated protein kinases in the regulation of MMP-9 expression in malignant cells is well understood. Extracellular signal-regulated kinase and JNK/SAPK

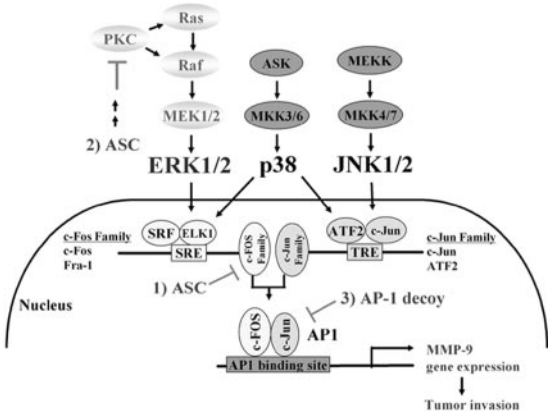

FIG. 10. **Schematic model for inhibition of invasion of renal carcinoma cells by ASC.** PMA activates AP-1 and NF-κB transcription factors through stimulation of PKC and ERK signaling pathways in renal carcinoma cells. Among other genes, AP-1 increases expression of MMP-9, which induces the invasiveness and metastatic potential of renal cancer cells. We propose that ASC suppresses expression of MMP-9 by blocking ERK1/2-dependent activation of AP-1 activation by reducing the expression or stability of c-Fos family proteins, most notably Fra-1. ASC may inhibit ERK1/2 activity by interfering with upstream signaling involving PKC. AP-1 decoy oligonucleotides effectively suppress MMP-9 expression by directly blocking DNA binding of AP-1. *MEK*, mitogen-activated protein kinase kinase; *MEKK*, MEK kinase. *SRE*, serum response element; *SRF*, serum response factor; *TRE*, TPA response elements; *MKK*, MAP kinase kinase.

are well known mitogen-activated protein kinase pathways in mammalian cells that induce the up-regulation of MMP-9 (41–46). PMA stimulation of MMP-9 expression was also reported to involve the p38 pathway, since the p38 inhibitor SB203580 suppressed MMP-9 expression in SCC cells (47). In this study we surveyed MMP-9 expression by various kinase inhibitors and found that PMA-nduced MMP-9 activation was decreased by ERK1/2 inhibitors but not p38, JNK/SAPK, or phosphatidylinositol 3-inase inhibitors. From these findings, AP-1 activation through the PKC and ERK1/2 pathways appears to be required for the induction of MMP-9 expression in Caki-1 cells.

ERK1/2 is a major regulator of AP-1 activity. ERK1/2 rapidly phosphorylates Elk-1, which subsequently stimulates transcription activity of serum response elements on the promoter region of the c-*fos* gene (48). Activation of ERK1/2 protects Fra1 from proteasomal degradation (12, 49). ASC suppressed expression of c-Fos and Fra1, whereas the phosphorylation of c-Jun that correlates with transcriptional activation was not affected by treating cells with doses of ASC that inhibit MMP-9 expression. This result suggests that the major AP-1 family members that are affected by ASC are c-Fos and Fra1. We also observed that inhibitors of PKC and ERK had a selective inhibitory effect on MMP-9 expression and that ASC suppressed ERK1/2 phosphorylation, and its kinase activity was detected by *in vitro* kinase assay without affecting JNK activity. Taken together, it can be assumed that ASC inhibits AP-1 activity through the suppression of the signaling pathway involving PKC, which subsequently inhibits ERK1/2 activity and expression of c-Fos and Fra-1 (Fig. 10).

We, therefore, suggest the anti-tumor activity of ASC is in part due to the inhibition of MMP-9 through down-regulation of AP-1. Small molecular weight inhibitors like ASC which target pathways that regulate MMP-9 expression could improve our understanding of these pathways. Considering the overwhelming evidence for a role of this type IV collagenase in tumor cell invasion, inhibitors of MMP-9 might be useful for preventing cancer. ASC may prove to be highly effective in reducing the *in vivo* invasiveness of cancers.

REFERENCES

1. Tamura, G., Suzuki, S., Takatsuki, A., Ando, K., and Arima, K. (1968) *J. Antibiot.* (*Tokyo*) **21,** 539–544
2. Sawada, M., Hosokawa, T., Okutomi, T., and Ando, K. (1973) *J. Antibiot.* (*Tokyo*) **26,** 681–686
3. Hosokawa, T., Sawada, M., Ando, K., and Tamura, G. (1981) *Lipids* **16,** 433–438
4. Hosokawa, T., Ando, K., and Tamura, G. (1985) *Diabetes* **34,** 267–274
5. Hosokawa, T., Ando, K., and Tamura, G (1985) *Biochem. Biophys. Res. Commun.* **126,** 471–476
6. Masuda, H., Kawada, T., Tanaka, M., Saida, K., Ando, K., Tamura, G., Magae, J., and Hosokawa, T. (2002) *J. Antibiot.* (*Tokyo*) **55,** 417–422
7. Magae, J., Hosokawa, T., Ando, K., Nagai, K., and Tamura, G (1982) *J. Antibiot.* (*Tokyo*) **35,** 1547–1552
8. Magae, J., Suzuki, S., Nagai, K., Yamasaki, M., Ando, K., and Tamura, G. (1986) *Cancer Res.* **46,** 1073–1078
9. Magae, J., Hayasaki, J., Matsuda, Y., Hotta, M., Hosokawa, T., Suzuki, S., Nagai, K., Ando, K., and Tamura, G. (1988) *J. Antibiot.* (*Tokyo*) **41,** 959–965
10. Hirosaki, S., Tamura, K., Taya, T., and Togashi, M. (1984) *Environ. Res.* **33,** 96–105
11. Wrana, J. L., Sodek, J., and Togashi, M. (1989) *J. Biol. Chem.* **264,** 1860–1869
12. Casalino, L., De Cesare, D., and Verde, P. (2003) *Mol. Cell. Biol.* **23,** 4401–4415
13. Togashi, M., Ozawa, S., Abe, S., Nishimura, T., Tsuruga, M., Ando, K., Tamura, G., Kuwahara, S., Ubukata, M., and Magae, J. (2003) *J. Med. Chem.* **46,** 4113–4123
14. Chambers, A. F., and Matrisian, L. M. (1997) *J. Natl. Cancer Inst.* **89,** 1260–1270
15. Lelongt, B., Trugnan, G., Murphy, G., and Ronco, P. M. (1997) *J. Cell Biol.* **136,** 1363–1373
16. Stetler-Stevenson, W. G., Hewitt, R., and Corcoran, M. (1996) *Semin. Cancer Biol.* **7,** 147–154
17. Nabeshima, K., Inoue, T., Shimao, Y., and Sameshima, T (2002) *Pathol. Int.* **52,** 255–264
18. Zucker, S., Lysik, R. M., Zarrabi, M. H., and Moll, U. (1993) *Cancer Res.* **53,** 140–146
19. Lakka, S. S., Gondi, C. S., Yanamandra, N., Dinh, D. H., Olivero, W. C., Gujrati, M., and Rao, J. S. (2003) *Cancer Res.* **63,** 2454–2461
20. Bernhard, E. J., Gruber, S. B., and Muschel, R. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4293–4297
21. Yoon, S. O., Kim, M. M., and Chung, A. S. (2001) *J. Biol. Chem.* **276,** 20085–20092
22. Sato, H., Endo, Y., Okada, Y., Mai, M., Sasaki, T., and Seiki, M. (1993) *Cancer Res.* **53,** 1397–1402
23. Sato, H., Kita, M., and Seiki, M. (1993) *J. Biol. Chem.* **268,** 23460–23468
24. Lein, M., Jung, K., Laube, C., Hubner, T., Winkelmann, B., Stephan, C., Hauptmann, S., Rudolph, B., Schnorr, D., and Loening, S. A. (2000) *Int. J. Cancer* **85,** 801–804
25. Cho, N. H., Shim, H. S., Rha, S. Y., Kang, S. H., Hong, S. H., Choi, Y. D., Hong, S. J., and Cho, S. H. (2003) *Eur. Urol.* **44,** 560–566
26. Chung, T. W., Lee, Y. C., and Kim, C. H. (2004) *FASEB J.* **18,** 1123–1125
27. Woo, J. H., Lim, J. H., Kim, Y. H., Suh, S. I., Min, do S, Chang, J. S., Lee, Y. H., and Kwon, T. K. (2004) *Oncogene* **23,** 1845–1853
28. Chae, Y. M., Park, K. K., Magae, J., Lee, I. S., Kim, C. H., Kim, H. C., Hong, S. H., Lee, J. G., Choi, I. J., Kim, H. S., Min, K. S., Lee, I. K., and Chang, Y. C. (2004) *Biochem. Biophys. Res. Commun.* **319,** 550–555
29. Shiratsuchi, T., Ishibashi, H., and Shirasuna, K. (2002) *J. Cell. Physiol.* **193,** 340–348
30. Ahn, J. D., Morishita, R., Kaneda, Y., Kim, H. S., Chang, Y. C., Lee, K. U., Park, J. Y., Lee, H.W., Kim, Y.H., and Lee, I.K. (2002) *Gene Ther.* **9,** 1682–1692
31. Wei liu, Andrew, G. H., and Jeffrey, M. H. (1995) *Mol. Biol. Cell* **15,** 1005–1013
32. Woo, J. H., Park, J. W., Lee, S. H., Kim, Y. H., Lee, I. K., Gabrielson E, Lee, S. H., Lee, H. J., Kho, Y. H., and Kwon, T.K. (2003) *Cancer Res.* **63,** 3430–3434
33. Kazes, I., Elalamy, I., Sraer, J. D., Hatmi, M., and Nguyen, G. (2000) *Blood* **96,** 3064–3069
34. Han, Y., Meng, T., Murray, N. R., Fields, A. P., and Brasier, A. R. (1999) *J. Biol. Chem.* **274,** 939–947
35. Fleenor, D. L., Pang, I. H., and Clark, A. F. (2003) *Investig. Ophthalmol. Vis. Sci.* **44,** 3494–3501
36. Karin, M., Liu, Z. G., and Zandi, E. (1997) *Curr. Opin. Cell Biol.* **9,** 240–246
37. Smeal, T., Angel, P., Meek, J., and Karin, M. (1989) *Genes Dev.* **3,** 2091–2100
38. Whisler, R. L., Chen, M., Beiqing, L., and Carle, K. W. (1997) *Cell. Immunol.* **175,** 41–50
39. Stetler-Stevenson, W. G., Brown, P. D., Onisto, M., Levy, A. T., and Liotta, L. A. (1990) *J. Biol. Chem.* **265,** 13933–13938
40. De Clerck, Y. A., Darville, M. I., Eeckhout, Y., and Rousseau, G. G. (1994) *Gene* (*Amst.*) **139,** 185–191
41. Burch, P. M., Yuan, Z., Loonen, A., and Heintz, N. H. (2004) *Mol. Cell. Biol.* **24,** 4696–4709
42. Shishodia, S., Majumdar, S., Banerjee, S., and Aggarwal, B. B. (2003) *Cancer Res.* **63,** 4375–4383
43. Gum, R., Lengyel, E., Juarez, J., Chen, J. H., Sato, H., Seiki, M., and Boyd, D. (1996) *J. Biol. Chem.* **271,** 10672–10680
44. Gum, R., Wang, H., Lengyel, E., Juarez, J., and Boyd, D. (1997) *Oncogene* **14,** 1481–1493
45. Moon, S. K., Kim, H. M., Lee, Y. C., and Kim, C. H. (2004) *J. Biol. Chem.* **279,** 33063–33070
46. Lai, W. C., Zhou, M., Shankavaram, U., Peng, G., and Wahl, L. M. (2003) *J. Immunol.* **170,** 6244–6249
47. Simon, C., Goepfert, H., and Boyd, D. (1998) *Cancer Res.* **58,** 1135–1139
48. Karin, M. (1995) *J. Biol. Chem.* **270,** 16483–16486
49. Vial, E., and Marshall, C. J. (2003) *J. Cell Sci.* **116,** 4957–4963

Ascochlorin Inhibits Matrix Metalloproteinase-9 Expression by Suppressing Activator Protein-1-mediated Gene Expression through the ERK1/2 Signaling Pathway: INHIBITORY EFFECTS OF ASCOCHLORIN ON THE INVASION OF RENAL CARCINOMA CELLS

SaHyun Hong, Kwan-Kyu Park, Junji Magae, Kunio Ando, Tae-Sung Lee, Taeg Kyu Kwon, Jong-Young Kwak, Cheorl-Ho Kim and Young-Chae Chang

J. Biol. Chem. 2005, 280:25202-25209.
doi: 10.1074/jbc.M413985200 originally published online April 29, 2005
