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Suppression of PAI-1 Expression Through Inhibition of the EGFR-Mediated Signaling Cascade in Rat Kidney Fibroblast by Ascofuranone

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ABSTRACT

Fibrosis in glomerulosclerosis causes progressive loss of renal function. Transforming growth factor (TGF)-β, one of the major profibrotic cytokines, induces the synthesis of plasminogen activator inhibitor (PAI)-1, a factor that plays a crucial role in the development of fibrosis. Here, we found that an isoprenoid antibiotic, ascofuranone, suppresses expression of profibrotic factors including matrix proteins and PAI-1 induced by TGF-β in renal fibroblasts. Ascofuranone selectively inhibits phosphorylation of epidermal growth factor receptor (EGFR), and downstream kinases such as Raf-1, MEK-1/2, and ERK-1/2. PAI-1 transcription also is suppressed by treatment with kinase inhibitors for MEK-1/2 or EGFR, and with small interfering RNA for EGFR. Ascofuranone inhibits cellular metalloproteinase activity, and an inhibitor of metalloproteinases suppresses EGFR phosphorylation and PAI-1 transcription. These results suggest that ascofuranone suppresses expression of profibrotic factors through the inhibition of an EGFR-dependent signal transduction pathway activated by metalloproteinases. J. Cell. Biochem. 107: 335–344, 2009.

KEY WORDS: ASCOFURANONE; FIBROSIS; PLASMINOGEN ACTIVATOR INHIBITOR-1; EPIDERMAL GROWTH FACTOR RECEPTOR; HB-EGF; RENAL FIBROBLAST

Fibrosis is a pathological process resulting from injury, and can occur in any organ including heart, lung, liver, or kidney, causing tissue dysfunction [Border and Noble, 1997; Fogo, 2003]. Renal fibrosis is the inevitable consequence of an excessive accumulation of extracellular matrix (ECM) that occurs in virtually every type of chronic renal disease [Bottinger and Bitzer, 2002;

Abbreviations used: EGFR, epidermal growth factor receptor; GPCR, G-protein-coupled receptors; HB-EGF, heparin-binding epidermal growth factor-like growth factor; MMP-9, matrix metalloproteinase-9; PAI-1, plasminogen activator inhibitor; PPAR, peroxisome proliferator-activated receptors.

Hyun-Ji Cho and Jeong-Han Kang contributed equally to this work.

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Gaedeke et al., 2004]. Several genes and proteins participate directly or indirectly in the accumulation of matrix proteins in renal fibrosis progression [Liu et al., 2006b; Qi et al., 2008]. Among them, transforming growth factor (TGF)-β has a pivotal role in the regulation of various physiological processes and is the predominant growth factor involved in tissue fibrosis as well as in the incidence and progression of cancer, providing both pro-apoptotic signals and survival signals, depending on the cell type [Cutroneo et al., 2006; Liu et al., 2006a; Ghosh et al., 2007]. Plasminogen activator inhibitor (PAI)-1, a serine protease inhibitor, plays a central role in the regulation of vascular function and tissue remodeling by modulating thrombosis, inflammation, and accumulation of ECM. PAI-1 promotes angiogenesis by regulating plasmin-mediated proteolysis or by modulating cell migration through the modulation of cell–matrix interactions [Rerolle et al., 2000]. Although normal human renal cells do not express PAI-1, its expression is induced in the development of renal disease. Because PAI-1 expression is positively correlated to fibrotic changes, including renal fibrosis, and is consistently and dramatically up-regulated in a variety of fibrotic diseases including glomerulosclerosis, PAI-1 is a good candidate for a therapeutic target in renal diseases [Loskutoff and Quigley, 2000; Higuchi et al., 2005].

Ascochlorin and ascofuranone (Fig. 1) are prenylphenol antifungal antibiotics isolated from an incomplete fungus, *Ascochyta visiae*. Although originally reported to be antiviral antibiotics [Tamura et al., 1968; Nawata et al., 1969], ascochlorin and its derivatives exhibit a variety of physiological effects including hypolipidemic activity [Hosokawa et al., 1981b], suppression of hypertension [Hosokawa et al., 1981a], amelioration of type I and II diabetes [Hosokawa et al., 1985], immunomodulation [Magae et al., 1982; Magae et al., 1986], and anti-tumor activity [Magae et al., 1982; Magae et al., 1988]. Ascochlorin and ascofuranone, one of its derivatives, suppress oxidative phosphorylation by inhibiting ubiquinone-dependent electron transport in isolated mitochondria [Takatsuki et al., 1969; Magae et al., 1993], and it has been suggested that the anti-viral activity of ascochlorin and macrophage activation by ascofuranone are caused by this inhibitory activity on mitochondrial respiration [Takatsuki et al., 1969; Magae et al., 1993; Ashikaga et al., 1998]. These compounds also modulate the activity of nuclear hormone receptors. Some derivatives of ascochlorin activate peroxisome proliferator-activated receptor (PPAR)-γ and induce differentiation of preadipocytes [Togashi et al., 2002; Togashi et al., 2003]. Ascochlorin-6 (AS-6) inhibits tumor necrosis factor-(TNF-)-α-stimulated nuclear factor-(NF-)κB activity and inflammatory molecular expression of rat vascular smooth muscle cells in a manner dependent on activation of PPAR-γ [Park et al., 2006] Ascochlorin activates human estrogen receptor [Togashi et al., 2003], suggesting that mechanisms other than those involving the respiratory chain contribute to its physiological activities.

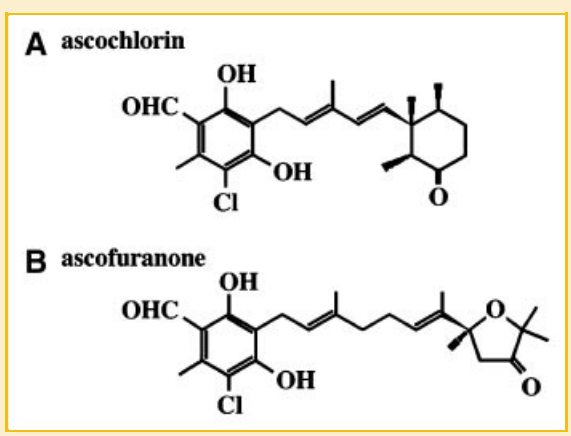

Fig. 1. Chemical structures of ascochlorin (A) and ascofuranone (B).

We recently found that ascochlorin and ascofuranone selectively suppress activator protein (AP)-1 activity of human renal carcinoma cells and its downstream targets such as matrix metalloproteinase-9 (MMP-9) through suppression of the extracellular signal-regulated kinase-1/2 (ERK-1/2) signaling pathway [Hong et al., 2005; Cho et al., 2007]. Proteome analysis of ascochlorin-treated human osteosarcoma cells shows a decrease in expression of several genes in the mitogen-activated protein kinase (MAPK) signaling cascade including epidermal growth factor receptor (EGFR) and ERK-1/2 [Kang et al., 2006]. Because MMP-9 plays an important role in the metastasis and evolution of tumors as well as in extravasation and migration of macrophages [Nabeshima et al., 2002], it is assumed that ascochlorin suppresses tumor malignancy or atherosclerosis. In fact, ascochlorin significantly suppresses the invasion of tumor cells in vitro [Hong et al., 2005; Cho et al., 2007] and oxidizes low-density lipoprotein-induced MMP-9 expression in human monocytic cells [Kang et al., 2007]. We also found that human breast cancer cell lines that are devoid of estrogen receptors exhibit higher AP-1 activity and express higher levels of c-Jun, c-Fos, and Fra-1 compared with conventional estrogen receptor-positive human breast cancer cell lines, and that ascochlorin selectively kills estrogen receptor-negative breast cancer cells and suppresses their propagation in vivo, partly due to the induction of apoptosis [Sakaguchi et al., 2005; Nakajima et al., 2007]. These results suggest that modulation of AP-1 activity and/or the MAPK cascade is involved in ascochlorin-mediated physiological activity.

Recent studies indicate that TGF-β up-regulates human PAI-1 promoter activity through the Smad 3/4 complex, which directly binds to the CAGA box of the PAI-1 promoter region [Dennler et al., 1998; Higashiyama et al., 2007]. It is proposed that the kinase cascade involving MAPK is also important for intracellular transmission initiated by TGF-β stimulation in a number of different cell types [Guo et al., 2005]. PAI-1 induction in response to TGF-β requires MEK/ERK activation [Kutz et al., 2001; Samarakoon and Higgins, 2003]. A recent finding demonstrates that TGF-β-induced PAI-1 expression requires EGFR-mediated signaling [Samarakoon et al., 2005]. It is also known that p21ras and pp60 c-src are necessary for PAI-1 induction in response to TGF-β [Uchiyama-Tanaka et al., 2002; Kutz et al., 2006]. In this study, we found that ascofuranone markedly blocked expression of profibrotic genes, including PAI-1 induced by TGF-β, in renal fibroblast cells through a mechanism involving the EGFR-mediated signaling cascade. Our findings suggest that ascofuranone can be used as a clinical drug for amelioration of fibrosis associated with human renal disease.

MATERIALS AND METHODS

CELLS AND MATERIALS

Rat kidney fibroblast cells (NRK-49F) were obtained from the American Type Culture Collection (Rockville, MD). NRK-49F cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic (Invitrogen, Carlsbad, CA). Ascofuranone was purified from a culture broth of *Ascochyta visiae* as described previously [Sasaki et al., 1973]. TGF-β was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

CELL PROLIFERATION ASSAY

1×10^4 cells/well were incubated for 24 h, and 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) (Roche Molecular Biochemical, Indianapolis, IN) was added 4 h prior to termination of the culture.

GELATIN SUBSTRATE GEL ZYMOGRAPHY

Rat kidney fibroblast cells were plated at 1×10^6 cells/ml in 35-mm diameter dishes and incubated until they reached 80% confluence. Fresh serum-free medium was added to each dish, followed by further culturing for 6 h. The resultant supernatant was subjected to SDS–PAGE containing 10% polyacrylamide and 1 mg/ml of gelatin. After electrophoresis, run at 4°C, the gels were washed several times with 2.5% Triton X-100 for 30 min at room temperature and incubated overnight at 37°C in buffer containing 5 mM $CaCl_2$ and 1 μM $ZnCl_2$. The gels were stained with Coomassie Brilliant Blue R-250 (0.1% Coomassie Brilliant Blue R-250, 45.5% methanol, 9% acetic acid) (Bio-Rad Laboratories, Inc., Hercules, CA) for 30 min, and then destained for 1 h in a solution of 10% acetic acid and 10% methanol.

WESTERN BLOT ANALYSIS

Cells (1×10^4/ml) were suspended in 40 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) at 4°C for 30 min, followed by centrifugation at 14,000 rpm for 5 min. The cell extract was subjected on SDS–PAGE, electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Western blotting was carried out as described previously [Hong et al., 2005; Cho et al., 2007]. Detection of specific proteins was carried out by enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK), following the manufacturer's instructions. The PAI-1, fibronectin, collagen type I, Raf-1, phospho-Raf-1 (Ser 338), MEK-1/2, phospho-MEK-1/2 (Ser 218/Ser 222), ERK-1/2, phospho-ERK-1/2 (Tyr 204), p38, phospho-p38 (Tyr 182), JNK1, phospho-JNK (Thr 183 and Tyr 185), TGFβRII, phospho-TGFβRII (Tyr 259), EGFR, and phospho-EGFR (Tyr 1173)-specific antibodies were purchased from Santa Cruz Biotechnology. Phosphospecific EGFR (Tyr 1173) antibody was purchased from Cell Signaling Technology, Inc. (Beverly, MA).

RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesized from 500 ng of total RNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The PCR primers are described in Table I. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

PLASMID TRANSFECTION AND LUCIFERASE GENE ASSAYS

p800neo-luc (PAI-1 luciferase promoter construct) [Ahn et al., 2004] was used in transient transfection assays. Cells were plated onto six-well dishes at a density of 1×10^4 cells/ml and allowed to grow overnight. The cells were cotransfected with 2 μg of various plasmid constructs and 1 μg of the pCMA-β-galactosidase plasmid for 12 h with Lipofectamine reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. After 12 h incubation in fresh medium, the enzyme activities of luciferase and β-galactosidase were determined using commercial kits (Promega), according to the manufacturer's protocol. Luciferase activity was calculated as luciferase activity normalized with β-galactosidase activity in each cell lysate.

RNA INTERFERENCE

Stealth™ RNA oligonucleotides (Invitrogen) are 25-bp double-stranded RNA oligonucleotides with extensive chemical modification that decreases nonspecific binding [Huang et al., 2006]. Stealth™ siRNA were designed using BLOCk-iT™ RNAI Designer (Invitrogen), to target sequences of EGFR (sense strand: 5′-UGA UCU GUC ACC ACA UAA UUA CGG G-3′; antisense strand: 5′-CCC GUA AUU AUG UGG UGA CAG AUC A-3′). Detailed descriptions of siRNA transfection techniques have been described previously [Magae et al., 1993]. Briefly, rat kidney fibroblast cells growing in 10% FBS–DMEM–low glucose were maintained in humidified 95% air, 5% CO_2 in an incubator at 37°C. For transfection experiments with siRNA directed against EGFR, the medium for rat kidney fibroblast cells was changed to fresh DMEM, and cells were transfected for 24 h with

TABLE I. Sequences of the Amplification Primers for RT-PCR

Gene	Primer sequence (5′–3′)	No. of cycles	Annealing temp.	Linear cycle range
PIA-1	Forward: tgtcctcggtgctggctatgctg Reverse: tcagacttgtggaacaggcgctg	27	72	25–30
Fibronectin	Forward: gcatcagggcagggaaagcaa Reverse: gttggttgggggagacagcgg	26	69	25–30
Collagen type 1	Forward: tggggcaagacagtcatcgaataca Reverse: ggcagcatttggggtatcataag	25	68	20–27
EGFR	Forward: cccactcatgctctacaacc Reverse: gccggtatgatttctaggt	28	51	25–30

50 ng/ml of siRNA by Trans IT-TKO (Mirusbio, Madison, WI) according to the manufacturer's instructions. After 24 h, the medium was changed to fresh serum-free DMEM, cells were treated with 2 ng/ml TGF-β for 6 h and total RNA in the cells was extracted for RT-PCR.

STATISTICAL ANALYSIS

Results are presented as means ± SE. Statistical differences between experimental and control groups were determined by analysis of variance, and a value of $P < 0.05$ by Student's t-test was considered statistically significant.

RESULTS

ASCOFURANONE INHIBITS EXPRESSION OF PROFIBROTIC GENES IN RAT KIDNEY FIBROBLAST CELLS

Because TGF-β signaling is critical in the development and progression of fibrosis associated with renal disease [Gaedeke et al., 2004], we analyzed the effect of ascofuranone on the expression of fibrosis marker genes in rat fibroblast cells activated by TGF-β. TGF-β (2 ng/ml) significantly stimulated PAI-1 protein expression after 6 h of incubation (Fig. 2). The stimulatory effect was dose-dependent, and maximum activity was attained with 5 ng/ml TGF-β. The treatment of cells with ascofuranone ranging from 1 to 30 μM was not cytotoxic and showed mild growth inhibitory activity with a 10% decrease in cell proliferation at 10 μM (Fig. 3). In spite of a weak effect on cell growth, ascofuranone suppressed the transcription of fibronectin, type I collagen, and PAI-1 induced by TGF-β (Fig. 4A). A similar suppressive effect on PAI-1 expression by ascofuranone was also confirmed by Western blot (Fig. 4B). These results suggest that ascofuranone suppresses the profibrotic activity of TGF-β in rat kidney fibroblast cells and thereby suppresses the accumulation of ECM proteins.

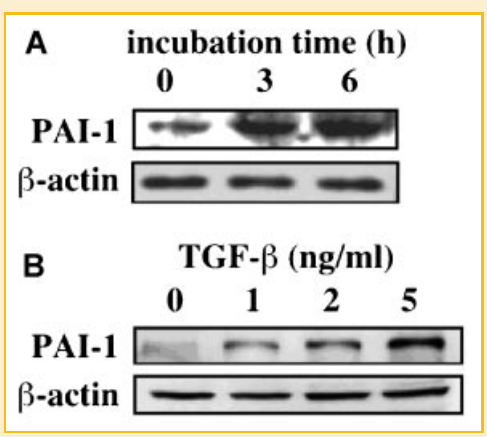

Fig. 2. Induction of PAI-1 expression by TGF-β. Rat kidney fibroblasts (1×10^4 cells/ml) were treated with TGF-β (2 ng/ml) for the indicated time (A), or with the indicated concentration of TGF-β for 6 h (B). PAI-1 protein expression was analyzed by Western blot. Results are means ± SE of three separate experiments, and representative data are shown.

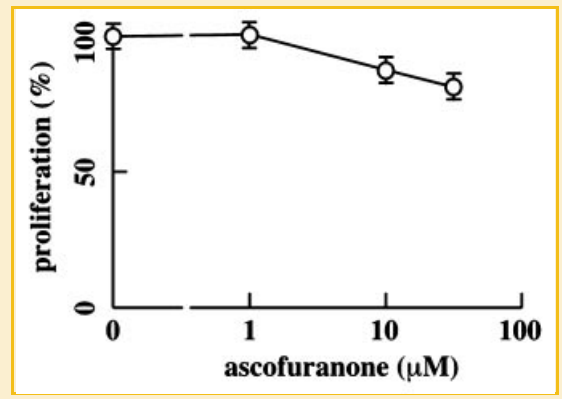

Fig. 3. Effect of ascofuranone on the proliferation of rat kidney fibroblasts. Rat kidney fibroblast cells (1×10^4 cells/ml) were treated with ascofuranone in the absence of serum for 24 h. Cell proliferation was determined by WST-1 assay. Results shown are means ± SE of three separate experiments.

EGFR/MEK SIGNALING PATHWAY IS INVOLVED IN SUPPRESSION OF TGF-β-INDUCED PAI-1 TRANSCRIPTION BY ASCOFURANONE

To investigate the mechanism for suppression of PAI-1 transcription by ascofuranone, we examined the effect of ascofuranone on PAI-1 promoter activity by transiently transfected luciferase reporter plasmid containing the PAI-1 promoter sequence. As shown in Figure 5, ascofuranone significantly inhibited PAI-1 promoter activity induced by TGF-β in a dose-dependent manner. Ascofuranone suppressed PAI-1 expression to the basal level at 10 μM. We further determined the signaling pathway involved in TGF-β-

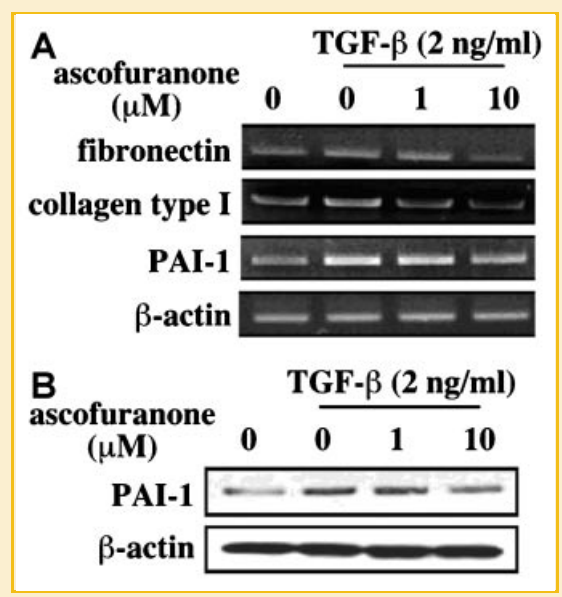

Fig. 4. Suppression of TGF-β-induced profibrotic factors by ascofuranone. Rat kidney fibroblasts (1×10^4 cells/ml) were incubated with 2 ng/ml of TGF-β for 30 min, followed by treatment with ascofuranone for 6 h. mRNA expression (A) and protein expression (B) were analyzed by RT-PCR and Western blot. Results are means ± SE of three separate experiments, and representative data are shown.

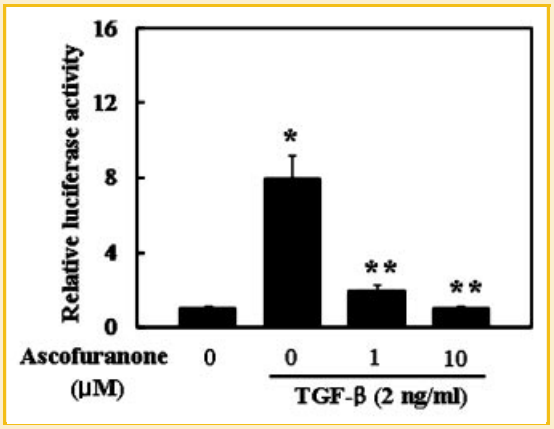

Fig. 5. Suppression of TGF-β-activated PAI-1-promoter by ascofuranone. Rat kidney fibroblasts (1×10^4 cells/ml) were transfected with PAI-1 promoter-containing reporter vector (p800-luc) and incubated with various concentrations of ascofuranone in the absence or presence TGF-β (2 ng/ml). Luciferase activity was measured 6 h after transfection. Results shown are means ± SE of three separate experiments. $^{*}P < 0.01$ versus controls, $^{**}P < 0.05$ versus TGF-β-stimulated cells.

induced PAI-1 induction, utilizing specific kinase inhibitors for protein kinase C (PKC, GO6978), phosphatidylinositide-3′-kinase (PI3K, wortmannin), p38 kinase (SB98059), c-Jun N-terminal kinase (JNK, SP600125), MEK (PD98059), and EGFR inhibitor (AG1478). Among these inhibitors, PD98059 and AG1478, like ascofuranone, inhibited TGF-β-induced PAI-1 mRNA accumulation to the basal level, whereas inhibitors for PKC, PI3K, p38, and JNK showed no suppressive effect (Fig. 6). These results suggest that ascofuranone suppresses induction of PAI-1 expression induced by TGF-β through the EGFR/MEK signaling pathway.

ASCOFURANONE DOWN-REGULATES PAI-1 TRANSCRIPTION THROUGH THE SPECIFIC SUPPRESSION OF EGFR PHOSPHORYLATION

To investigate ascofuranone-mediated modulation of the EGFR/MEK signaling pathway in rat fibroblasts, we assessed the activation

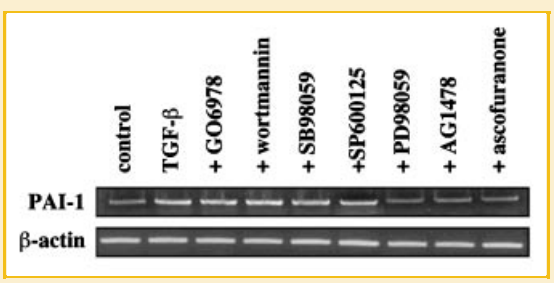

Fig. 6. Suppression of TGF-β-induced PAI-1 mRNA expression by inhibitors of EGFR-mediated signaling. Rat kidney fibroblast cells (1×10^4 cells/ml) were treated with inhibitors of PKC (GO6978, 10 μM), PI3K (wortmannin, 50 nM), p38 (SB98059, 20 μM), JNK (SP600125, 20 μM), MEK (PD98059, 50 μM) or EGFR (AG1478, 2.5 μM), or with ascofuranone (10 μM) for 24 h. PAI-1 mRNA expression was analyzed by RT-PCR.

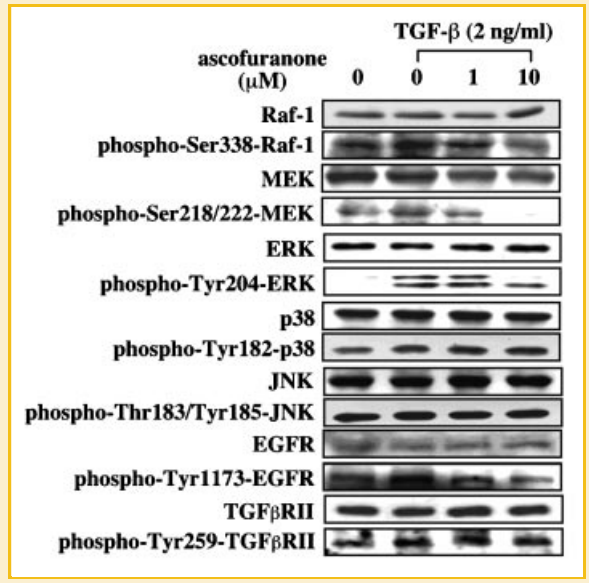

Fig. 7. Suppression of EGFR downstream signaling by ascofuranone. Rat kidney fibroblasts (1×10^4 cells/ml) were incubated with 2 ng/ml of TGF-β for 30 min, followed by treatment with ascofuranone for 6 h. Cellular protein was analyzed by Western blot. Results are means ± SE of three separate experiments, and representative data are shown.

of these pathways using immunoblot analysis of the phosphorylation status of kinases involved in this cascade. Treatment with TGF-β increased expression levels of phospho-Raf, phospho-MEK1/2, and phospho-ERK-1/2 without affecting the phosphorylation of p38 and JNK. Ascofuranone effectively attenuated expression levels of phospho-Raf-1, phospho-MEK1/2, and phospho-ERK-1/2, but not phospho-JNK and phospho-p38 (Fig. 7). We recently found, through proteome analysis in human osteosarcoma cells, that ascochlorin suppresses EGFR expression [Kang et al., 2007]. EGF regulates the expression of PAI-1 [Paugh et al., 2008]. TGF-β-induced PAI-1 expression is E box/USF-dependent and requires EGFR signaling [Kutz et al., 2006]. Therefore, we also analyzed the effect of ascofuranone on the phosphorylation of EGFR and type II TGF-β receptor (TGFβRII). Western blotting showed that phosphorylation of EGFR stimulated with TGF-β, but not with TGFβRII, was significantly reduced by ascofuranone (Fig. 7). Ascofuranone had no effect on the total protein expression of these receptors in spite of significant reduction of EGFR in osteosarcoma cells [Kang et al., 2007]. These results suggest that ascofuranone regulates EGFR-mediated signaling, leading to the down-regulation of PAI-1 expression. Indeed, ascofuranone efficiently blocked EGF-induced phosphorylation of EGFR and resultant PAI-1 expression (Fig. 8A). To confirm the involvement of EGFR in ascofuranone-mediated suppression of PAI-1 transcription, we transiently transfected rat kidney fibroblast cells with siRNA targeted for EGFR. The transfection markedly reduced the EGFR expression as well as PAI-1 expression in rat kidney fibroblast cells (Fig. 8B). Taken together, these results suggest that ascofuranone suppresses PAI-1 expression through the attenuation of EGFR phosphorylation and the resultant suppression of the MEK/ERK signaling pathway.

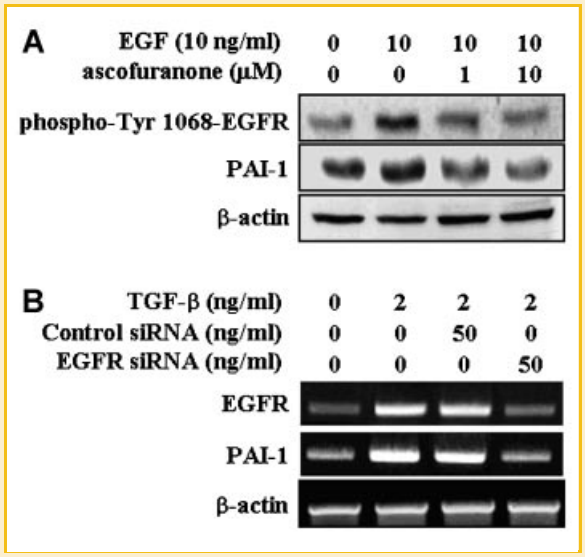

Fig. 8. Suppression of TGF-β-mediated PAI-1 expression by ascofuranone is dependent on EGFR. A: Rat kidney fibroblasts (1×10^4 cells/ml) were incubated with ascofuranone for 1 h, followed by treatment with 10 ng/ml of EGF for 15 min. EGFR phosphorylation and PAI-1 expression were analyzed by Western blot. B: Rat kidney fibroblasts (1×10^4 cells/ml) were transfected with 50 nM EGFR-targeting siRNA and cultured with 2 ng/ml of TGF-β for 6 h. mRNA expression was analyzed by RT-PCR.

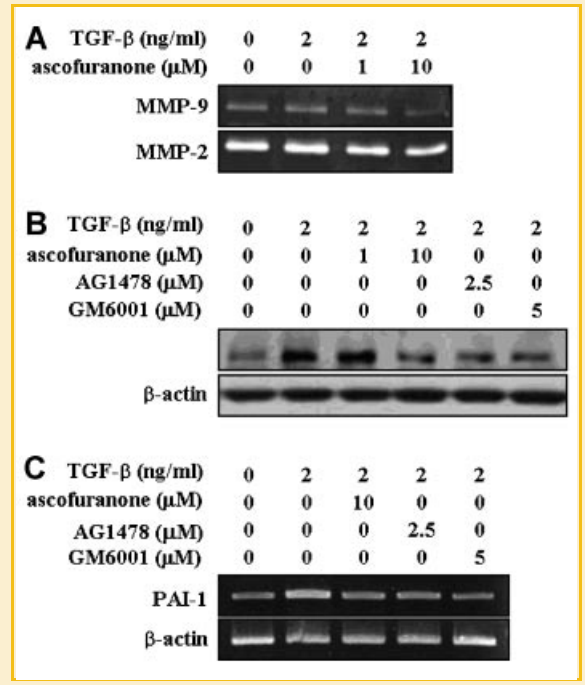

Fig. 9. Suppression of MMP expression by ascofuranone. A: Rat kidney fibroblasts (1×10^4 cells/ml) were treated with TGF-β for 30 min and cultured in the presence of ascofuranone for 6 h. The conditioned medium was collected and analyzed by gelatin zymography. B: Rat kidney fibroblasts (1×10^4 cells/ml) were incubated with 2 ng/ml of TGF-β for 30 min, followed by the treatment with ascofuranone for 6 h. Phospho-EGFR was analyzed by Western blot. C: Rat kidney fibroblasts (1×10^4 cells/ml) were incubated with 2 ng/ml of TGF-β for 30 min, followed by the treatment with ascofuranone, AG1478 or GM6001 for 6 h. mRNA expression was analyzed by RT-PCR.

SUPPRESSION OF MMP ACTIVITY IS INVOLVED IN DOWN-REGULATION OF EGFR SIGNALING UPSTREAM IN RAT KIDNEY FIBROBLAST CELLS TREATED WITH ASCOFURANONE

The activation of EGFR-dependent signaling pathways upon stimulation of G-protein-coupled receptors (GPCR) involves heparin-binding epidermal-like growth factor (HB-EGF) and MMP activity [Prenzel et al., 1999]. To investigate the involvement of this system in ascofuranone-mediated suppression of EGFR signaling, we assessed the expression and activities of MMP-2 and -9, the MMPs involved in HB-EGF processing [Lucchesi et al., 2004], in TGF-β-treated rat fibroblast cells by zymography assay. Although MMP activity was not affected by TGF-β, ascofuranone significantly suppressed enzymatic activity of MMP-2 and -9 (Fig. 9A). Moreover, a specific inhibitor for MMP, GM6001, like the kinase inhibitor for EGFR, AG1478, and ascofuranone, suppressed phosphorylation of EGFR as well as PAI-1 transcription induced by TGF-β (Fig. 9B,C). These results suggest that MMP down-regulation by ascofuranone plays an essential role in the suppression of TGF-β-induced EGFR phosphorylation and PAI-1 expression.

DISCUSSION

Recent observations demonstrate that high-level expression of PAI-1 in renal carcinoma is associated with a poor prognosis for survival [Ohba et al., 2005]. PAI-1 not only inhibits fibrinolysis but also has complex interactions with matrix and plays a crucial role in the development of fibrosis in renal disease [Fogo, 2003]. Because the induction of PAI-1 by TGF-β, one of the critical cytokines involved in the development of fibrosis, is regulated by an EGFR-mediated signaling pathway [Kutz et al., 2001, 2006; Uchiyama-Tanaka et al., 2002; Samarakoon and Higgins, 2003; Guo et al., 2005; Samarakoon et al., 2005], we studied the effect of a prenylphenol antibiotic, ascofuranone, which suppresses expression of EGFR and the signaling pathway involving MEK/ERK [Cho et al., 2007; Hong et al., 2005; Kang et al., 2006]. We found that ascofuranone reduced the expression of ECM proteins, including fibronectin and collagen type I, and inhibited PAI-1 expression in rat kidney fibroblast cells. Ascofuranone suppressed TGF-β-induced transcription of PAI-1 and phosphorylation of kinases involved in the EGFR-initiated signaling pathway including EGFR, Raf-1, MEK-1/2, and ERK-1/2. Inhibitors of the phosphorylation of ERK-1/2 and EGFR, and a neutralizing siRNA against EGFR suppressed TGF-β-induced PAI-1 expression. Ascofuranone inhibited EGF-induced EGFR phosphorylation as well as PAI-1 transcription induced by EGF. These results suggest that ascofuranone inhibits TGF-β-induced PAI-1 expression by interfering the EGFR-mediated signaling pathway. Moreover, ascofuranone inhibited the expression of metalloproteinases that are crucial in processing proHB-EGF [Suzuki et al., 1997; Izumi et al., 1998; Prenzel et al., 1999; Uchiyama-Tanaka et al., 2002], and an inhibitor of metalloproteinase suppressed PAI-1 expression induced by TGF-β. These results suggest that ascofuranone suppresses TGF-β-induced PAI-1 expression through the down-regulation of metalloproteinases, which subsequently represses the EGFR-

mediated signaling pathway that leads to activation of the PAI-1 promoter.

TGF-β usually initiates its cellular response by binding to its distinct receptors [Massague, 1998]. TGF-β binds to a constitutively active type II receptor, which recruits and phosphorylates the type I receptor. Smad 2/3 are recruited to the activated type I receptor, where Smad 2/3 are phosphorylated. They form a heterooligomeric complex with Smad 4 that translocates into the nucleus and regulates the transcription of target genes. Smad 7 associates with the activated type I receptor and inhibits receptor binding of Smad 2/3 as well as subsequent signaling events. Smad-dependent signaling is crucial in the development of fibrosis because Smad 7 prevents accumulation of matrix proteins induced by TGF-β as well as angiotensin II and high glucose, and it attenuates renal fibrosis in several animal models [Wang et al., 2005]. TGF-β also activates the MAPK cascade, including p38 and ERK-1/2. While TGF-β activates p38 through TGF-β-activated kinase-1, a member of the MAPK kinase kinase family [Yamaguchi et al., 1995; Hanafusa et al., 1999], ERK is activated by the EGFR signaling pathway. TGF-β stimulates metalloproteinase-mediated processing and the release of membrane-bound proHB-EGF, and the cleavage product binds EGFR to activate the ERK signaling pathway [Prenzel et al., 1999; Uchiyama-Tanaka et al., 2002]. Phosphorylation of ERK-1/2, induced by TGF-β, is inhibited by specific inhibitors for metalloproteinases and EGFR, suggesting that processing of proHB-EGF and its binding to EGFR is crucial to activating ERK-1/2 and the subsequent activation of PAI-1 transcription in TGF-β-stimulated rat fibroblasts. Several specific responsive elements have been defined in the PAI-1 promoter. A duplicate E box sequence between −740 and −528 mediates the response to TGF-β, and pharmacological or dominant-negative inhibition of EGFR, Ras, MEK, or c-src virtually eliminates TGF-β-augmented PAI-1 promoter activity [Kutz et al., 2006].

HB-EGF belongs to the EGF family of proteins, which encompasses a number of structurally homologous mitogens including EGF and TGF-β. HB-EGF is synthesized as a membrane-anchored protein of 208 amino acids, composed of signal peptide, HB-EGF-like, transmembrane and cytoplasmic domains [Higashiyama et al., 1991]. The membrane-anchored form of HB-EGF (proHB-EGF) is cleaved from the cell surface to yield a soluble growth factor of 75–86 amino acids [Goishi et al., 1995]. Metalloproteinases are endopeptidases that contain an active Zn^{2+} site and are divided into subfamilies based on evolutionary relationships and the structure of the catalytic domain [Ra and Parks, 2007]. The metzincin subfamily of metalloproteinases is characterized by a 3-histidine zinc-binding motif and a conserved methionine turn following the active site. Members of the metzincin family include ADAM (a disintegrin and metalloproteinase), serralysins, astacins, and MMPs. It is reported that metalloproteinases, such as MDC-9, ADAM proteins, and MMP-3, are responsible for HB-EGF shedding [Suzuki et al., 1997; Izumi et al., 1998]. PKC activation is involved in MDC-9-mediated shedding of HB-EGF [Izumi et al., 1998], while HB-EGF shedding induced by ionomycin, or GPCR-mediated EGFR phosphorylation are resistant to PKC inhibitors [Dethlefsen et al., 1998; Prenzel et al., 1999], suggesting that several pathways are responsible for the induction of metalloproteinase-mediated cleavage of proHB-EGF. In our system, using rat fibroblast cells, TGF-β-stimulated PAI-1 induction is resistant to PKC inhibitor.

MMP expression is modulated by transcription factors including AP-1, SP-1, and NF-κB through the MAPK signaling pathway [Gum et al., 1997; Eberhardt et al., 2000; Arai et al., 2003; Qureshi et al., 2005]. Specific inhibitors of MMP-2 and -9 suppress EGFR phosphorylation, HB-EGF processing, and pressure-induced myogenic tone in mouse mesenteric resistance arteries [Lucchesi et al., 2004]. Thus, it is possible that activation of the EGFR signaling pathway induces MMP-2 and -9 transcription, which subsequently promote processing of HB-EGF and activation of EGFR signaling. Ascofuranone might prevent this positive feedback loop for activation of EGFR-mediated signaling. Ascofuranone inhibits AP-1-dependent transcription activation of MMP-9 induced by phorbol ester [Cho et al., 2007]. Ascofuranone specifically suppresses binding activity of nuclear extract to the AP-1-motif. Phosphorylation of specific amino acid residues by protein kinases such as MAPK modulates AP-1 activity including transcription activation, protein stability, and the intracellular localization of AP-1 proteins [Karin, 1995]. Ascofuranone specifically inhibits phosphorylation of the ERK-signaling factors ERK-1/2, Ras, Raf-1, and MEK-1/2 without affecting PMA-induced phosphorylation of p38 and JNK, suggesting that the primary signaling target for suppression of AP-1 activity in the ERK-signaling pathway is upstream of Ras phosphorylation [Cho et al. 2007]. EGFR is a strategic candidate for targeting by ascofuranone because ascofuranone inhibits EGF-induced phosphorylation of EGFR. Ascofuranone might inhibit kinase activity of EGFR-1 directly or indirectly through the modulation of associating molecules. However, we did not determine the effect of ascofuranone on kinase activity in vitro. EGF-induced phosphorylation of EGFR is inhibited by NO in neural stem cells [Torroglosa et al., 2007]. Because inhibitors of

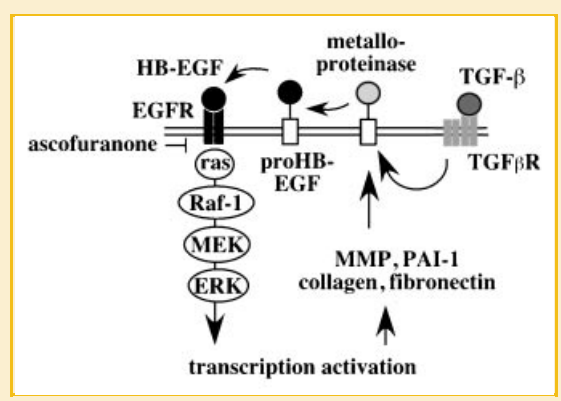

Fig. 10. Schematic model for suppression of profibrotic factor production in rat kidney fibroblasts by ascofuranone. TGF-β stimulates EGFR through the release of HB-EGF cleaved by metalloproteinase(s), which further activates the ERK signaling pathway, resulting in transcription of profibrotic factors, including MMP. MMP enhances EGFR activation through the cleavage of proHB-EGF, forming a positive feedback loop for the amplification of EGFR signaling. Ascofuranone blocks the feedback amplification loop through the suppression of EGFR phosphorylation. See text for detail.

mitochondrial respiratory chain generate ROS [Li et al., 2003], ascofuranone could modulate phosphorylation of EGFR through the modulation of cellular redox potential.

In conclusion, the present study demonstrates that TGF-β-induces PAI-1 transcription through the metalloproteinase-mediated processing of proHB-EGF, which activates the ERK-MAPK signaling pathway, with subsequent activation of the E-box in the PAI-1 promoter region. TGF-β also stimulates metalloproteinase activity in part through activation of the ERK signaling pathway and AP-1. Ascofuranone suppresses TGF-β-mediated PAI-1 transcription through the inhibition of EGFR activation, and thereby inhibits ERK signaling, which prevents subsequent metalloproteinase activation induced by TGF-β (Fig. 10). Our results suggest that EGFR and its downstream signaling pathway are good targets for fibrosis therapy, and ascofuranone provides a new therapeutic strategy for diseases associated with elevated PAI-1 synthesis.

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REFERENCES

Ahn JD, Morishita R, Kaneda Y, Kim HJ, Kim YD, Lee HJ, Lee KU, Park JY, Kim YH, Park KK, Chang YC, Yoon KH, Kwon HS, Park KG, Lee IK. 2004. Transcription factor decoy for AP-1 reduces mesangial cell proliferation and extracellular matrix production in vitro and in vivo. Gene Ther 11:916–923.

Arai K, Lee SR, Lo EH. 2003. Essential role for ERK mitogen-activated protein kinase in matrix metalloproteinase-9 regulation in rat cortical astrocytes. Glia 43:254–264.

Ashikaga T, Honma M, Munemura K, Kataoka T, Endo T, Yamasaki M, Magae J, Nagai K. 1998. Selective induction of interleukin-1 production and tumor killing activity of macrophages through apoptosis by the inhibition of oxidative respiration. Biosci Biotechnol Biochem 62:1115–1121.

Border WA, Noble NA. 1997. TGF-beta in kidney fibrosis: A target for gene therapy. Kidney Int 51:1388–1396.

Bottinger EP, Bitzer M. 2002. TGF-beta signaling in renal disease. J Am Soc Nephrol 13:2600–2610.

Cho HJ, Kang JH, Kwak JY, Lee TS, Lee IS, Park NG, Nakajima H, Magae J, Chang YC. 2007. Ascofuranone suppresses PMA-mediated matrix metalloproteinase-9 gene activation through the Ras/Raf/MEK/ERK and Ap1-dependent mechanisms. Carcinogenesis 28:1104–1110.

Cutroneo KR, White SL, Chiu JF, Ehrlich HP. 2006. Tissue fibrosis and carcinogenesis: Divergent or successive pathways dictate multiple molecular therapeutic targets for oligo decoy therapies. J Cell Biochem 97:1161–1174.

Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. 1998. Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. EMBO J 17:3091–3100.

Dethlefsen SM, Raab G, Moses MA, Adam RM, Klagsbrun M, Freeman MR. 1998. Extracellular calcium influx stimulates metalloproteinase cleavage and secretion of heparin-binding EGF-like growth factor independently of protein kinase C. J Cell Biochem 69:143–153.

Eberhardt W, Huwiler A, Beck KF, Walpen S, Pfeilschifter J. 2000. Amplification of IL-1 beta-induced matrix metalloproteinase-9 expression by superoxide in rat glomerular mesangial cells is mediated by increased activities of NF-kappa B and activating protein-1 and involves activation of the mitogen-activated protein kinase pathways. J Immunol 165:5788–5797.

Fogo AB. 2003. Renal fibrosis: Not just PAI-1 in the sky. J Clin Invest 112:326–328.

Gaedeke J, Noble NA, Border WA. 2004. Curcumin blocks multiple sites of the TGF-beta signaling cascade in renal cells. Kidney Int 66:112–120.

Ghosh AK, Mori Y, Dowling E, Varga J. 2007. Trichostatin A blocks TGF-beta-induced collagen gene expression in skin fibroblasts: Involvement of Sp1. Biochem Biophys Res Commun 354:420–426.

Goishi K, Higashiyama S, Klagsbrun M, Nakano N, Umata T, Ishikawa M, Mekada E, Taniguchi N. 1995. Phorbol ester induces the rapid processing of cell surface heparin-binding EGF-like growth factor: Conversion from juxtacrine to paracrine growth factor activity. Mol Biol Cell 6:967–980.

Gum R, Wang H, Lengyel E, Juarez J, Boyd D. 1997. Regulation of 92 kDa type IV collagenase expression by the jun aminoterminal kinase- and the extracellular signal-regulated kinase-dependent signaling cascades. Oncogene 14:1481–1493.

Guo B, Inoki K, Isono M, Mori H, Kanasaki K, Sugimoto T, Akiba S, Sato T, Yang B, Kikkawa R, Kashiwagi A, Haneda M, Koya D. 2005. MAPK/AP-1-dependent regulation of PAI-1 gene expression by TGF-beta in rat mesangial cells. Kidney Int 68:972–984.

Hanafusa H, Ninomiya-Tsuji J, Masuyama N, Nishita M, Fujisawa J, Shibuya H, Matsumoto K, Nishida E. 1999. Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression. J Biol Chem 274:27161–27167.

Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsbrun M. 1991. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. Science 251:936–939.

Higashiyama H, Yoshimoto D, Kaise T, Matsubara S, Fujiwara M, Kikkawa H, Asano S, Kinoshita M. 2007. Inhibition of activin receptor-like kinase 5 attenuates bleomycin-induced pulmonary fibrosis. Exp Mol Pathol 83:39–46.

Higuchi C, Tanihata Y, Nishimura H, Naito T, Sanaka T. 2005. Effects of glucose and plasminogen activator inhibitor-1 on collagen metabolism in the peritoneum. Ther Apher Dial 9:173–181.

Hong S, Park KK, Magae J, Ando K, Lee TS, Kwon TK, Kwak JY, Kim CH, Chang YC. 2005. 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. J Biol Chem 280:25202–25209.

Hosokawa T, Okutomi T, Sawada M, Ando K, Tamura G. 1981a. Unusual concentration of urine and prevention of polydipsia by fungal prenylphenols in DOCA hypertensive rats. Eur J Pharmacol 69:429–438.

Hosokawa T, Sawada M, Ando K, Tamura G. 1981b. Alteration of cholesterol metabolism by 4-O-methylascochlorin in rats. Lipids 16:433–438.

Hosokawa T, Ando K, Tamura G. 1985. An ascochlorin derivative, AS-6, reduces insulin resistance in the genetically obese diabetic mouse, db/db. Diabetes 34:267–274.

Huang Y, Wongamorntham S, Kasting J, McQuillan D, Owens RT, Yu L, Noble NA, Border W. 2006. Renin increases mesangial cell transforming growth factor-beta1 and matrix proteins through receptor-mediated, angiotensin II-independent mechanisms. Kidney Int 69:105–113.

Izumi Y, Hirata M, Hasuwa H, Iwamoto R, Umata T, Miyado K, Tamai Y, Kurisaki T, Sehara-Fujisawa A, Ohno S, Mekada E. 1998. A metalloprotease-disintegrin, MDC9/meltrin-gamma/ADAM9 and PKCdelta are involved in

TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor. EMBO J 17:7260–7272.

Kang JH, Park KK, Lee IS, Magae J, Ando K, Kim CH, Chang YC. 2006. Proteome analysis of responses to ascochlorin in a human osteosarcoma cell line by 2-D gel electrophoresis and MALDI-TOF MS. J Proteome Res 5:2620–2631.

Kang JH, Kim JK, Park WH, Park KK, Lee TS, Magae J, Nakajima H, Kim CH, Chang YC. 2007. Ascochlorin suppresses oxLDL-induced MMP-9 expression by inhibiting the MEK/ERK signaling pathway in human THP-1 macrophages. J Cell Biochem 102:506–514.

Karin M. 1995. The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 270:16483–16486.

Kutz SM, Hordines J, McKeown-Longo PJ, Higgins PJ. 2001. TGF-beta1-induced PAI-1 gene expression requires MEK activity and cell-to-substrate adhesion. J Cell Sci 114:3905–3914.

Kutz SM, Higgins CE, Samarakoon R, Higgins SP, Allen RR, Qi L, Higgins PJ. 2006. TGF-beta 1-induced PAI-1 expression is E box/USF-dependent and requires EGFR signaling. Exp Cell Res 312:1093–1105.

Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, Melendez JA, Robinson JP. 2003. Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. J Biol Chem 278:8516–8525.

Liu X, Hu H, Yin JQ. 2006a. Therapeutic strategies against TGF-beta signaling pathway in hepatic fibrosis. Liver Int 26:8–22.

Liu X, Sun SQ, Hassid A, Ostrom RS. 2006b. cAMP inhibits transforming growth factor-beta-stimulated collagen synthesis via inhibition of extracellular signal-regulated kinase 1/2 and Smad signaling in cardiac fibroblasts. Mol Pharmacol 70:1992–2003.

Loskutoff DJ, Quigley JP. 2000. PAI-1, fibrosis, and the elusive provisional fibrin matrix. J Clin Invest 106:1441–1443.

Lucchesi PA, Sabri A, Belmadani S, Matrougui K. 2004. Involvement of metalloproteinases 2/9 in epidermal growth factor receptor transactivation in pressure-induced myogenic tone in mouse mesenteric resistance arteries. Circulation 110:3587–3593.

Magae J, Hosokawa T, Ando K, Nagai K, Tamura G. 1982. Antitumor protective property of an isoprenoid antibiotic, ascofuranone. J Antibiot (Tokyo) 35:1547–1552.

Magae J, Suzuki S, Nagai K, Yamasaki M, Ando K, Tamura G. 1986. In vitro effects of an antitumor antibiotic, ascofuranone, on the murine immune system. Cancer Res 46:1073–1078.

Magae J, Hayasaki J, Matsuda Y, Hotta M, Hosokawa T, Suzuki S, Nagai K, Ando K, Tamura G. 1988. Antitumor and antimetastatic activity of an antibiotic, ascofuranone, and activation of phagocytes. J Antibiot (Tokyo) 41:959–965.

Magae J, Munemura K, Ichikawa C, Osada K, Hanada T, Tsuji RF, Yamashita M, Hino A, Horiuchi T, Uramoto M, et al. 1993. Effects of microbial products on glucose consumption and morphology of macrophages. Biosci Biotechnol Biochem 57:1628–1631.

Massague J. 1998. TGF-beta signal transduction. Annu Rev Biochem 67:753–791.

Nabeshima K, Inoue T, Shimao Y, Sameshima T. 2002. Matrix metalloproteinases in tumor invasion: Role for cell migration. Pathol Int 52:255–264.

Nakajima H, Mizuta N, Sakaguchi K, Fujiwara I, Mizuta M, Furukawa C, Chang YC, Magae J. 2007. Aberrant expression of Fra-1 in estrogen receptor-negative breast cancers and suppression of their propagation in vivo by ascochlorin, an antibiotic that inhibits cellular activator protein-1 activity. J Antibiot (Tokyo) 60:682–689.

Nawata Y, Ando K, Tamura G, Arima K, Iitaka Y. 1969. The molecular structure of ascochlorin. J Antibiot (Tokyo) 22:511–512.

Ohba K, Miyata Y, Kanda S, Koga S, Hayashi T, Kanetake H. 2005. Expression of urokinase-type plasminogen activator, urokinase-type plasminogen activator receptor and plasminogen activator inhibitors in patients with renal cell carcinoma: Correlation with tumor associated macrophage and prognosis. J Urol 174:461–465.

Park KG, Lee KM, Chang YC, Magae J, Ando K, Kim KB, Kim YN, Kim HS, Park JY, Lee KU, Lee IK. 2006. The ascochlorin derivative, AS-6, inhibits TNF-alpha-induced adhesion molecule and chemokine expression in rat vascular smooth muscle cells. Life Sci 80:120–126.

Paugh BS, Paugh SW, Bryan L, Kapitonov D, Wilczynska KM, Gopalan SM, Rokita H, Milstien S, Spiegel S, Kordula T. 2008. EGF regulates plasminogen activator inhibitor-1 (PAI-1) by a pathway involving c-Src, PKC{delta}, and sphingosine kinase 1 in glioblastoma cells. FASEB J 22:455–465.

Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, Ullrich A. 1999. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 402:884–888.

Qi W, Chen X, Poronnik P, Pollock CA. 2008. Transforming growth factor-beta/connective tissue growth factor axis in the kidney. Int J Biochem Cell Biol 40:9–13.

Qureshi HY, Sylvester J, El Mabrouk M, Zafarullah M. 2005. TGF-beta-induced expression of tissue inhibitor of metalloproteinases-3 gene in chondrocytes is mediated by extracellular signal-regulated kinase pathway and Sp1 transcription factor. J Cell Physiol 203:345–352.

Ra HJ, Parks WC. 2007. Control of matrix metalloproteinase catalytic activity. Matrix Biol 26:587–596.

Rerolle JP, Hertig A, Nguyen G, Sraer JD, Rondeau EP. 2000. Plasminogen activator inhibitor type 1 is a potential target in renal fibrogenesis. Kidney Int 58:1841–1850.

Sakaguchi K, Nakajima H, Mizuta N, Furukawa C, Ozawa S, Ando K, Chang YC, Yamagishi H, Magae J. 2005. Selective cytotoxicity of ascochlorin in ER-negative human breast cancer cell lines. Biochem Biophys Res Commun 329:46–50.

Samarakoon R, Higgins PJ. 2003. Pp60c-src mediates ERK activation/nuclear localization and PAI-1 gene expression in response to cellular deformation. J Cell Physiol 195:411–420.

Samarakoon R, Higgins CE, Higgins SP, Kutz SM, Higgins PJ. 2005. Plasminogen activator inhibitor type-1 gene expression and induced migration in TGF-beta1-stimulated smooth muscle cells is pp60(c-src)/MEK-dependent. J Cell Physiol 204:236–246.

Sasaki H, Hosokawa T, Sawada M, Ando K. 1973. Isolation and structure of ascofuranone and ascofranol, antibiotics with hypolipidemic activity. J Antibiot (Tokyo) 26:676–680.

Suzuki M, Raab G, Moses MA, Fernandez CA, Klagsbrun M. 1997. Matrix metalloproteinase-3 releases active heparin-binding EGF-like growth factor by cleavage at a specific juxtamembrane site. J Biol Chem 272:31730–31737.

Takatsuki A, Tamura G, Arima K. 1969. Antiviral and antitumor antibiotics. XIV. Effects of ascochlorin and other respiration inhibitors on multiplication of Newcastle disease virus in cultured cells. Appl Microbiol 17:825–829.

Tamura G, Suzuki S, Takatsuki A, Ando K, Arima K. 1968. Ascochlorin, a new antibiotic, found by the paper-disc agar-diffusion method. I. Isolation, biological and chemical properties of ascochlorin (Studies on antiviral and antitumor antibiotics. I). J Antibiot (Tokyo) 21:539–544.

Togashi M, Masuda H, Kawada T, Tanaka M, Saida K, Ando K, Tamura G, Magae J. 2002. PPARgamma activation and adipocyte differentiation induced by AS-6, a prenyl-phenol antidiabetic antibiotic. J Antibiot (Tokyo) 55:417–422.

Togashi M, Ozawa S, Abe S, Nishimura T, Tsuruga M, Ando K, Tamura G, Kuwahara S, Ubukata M, Magae J. 2003. Ascochlorin derivatives as ligands for nuclear hormone receptors. J Med Chem 46:4113–4123.

Torroglosa A, Murillo-Carretero M, Romero-Grimaldi C, Matarredona ER, Campos-Caro A, Estrada C. 2007. Nitric oxide decreases subventricular zone

stem cell proliferation by inhibition of epidermal growth factor receptor and phosphoinositide-3-kinase/Akt pathway. Stem Cells 25:88–97.

Uchiyama-Tanaka Y, Matsubara H, Mori Y, Kosaki A, Kishimoto N, Amano K, Higashiyama S, Iwasaka T. 2002. Involvement of HB-EGF and EGF receptor transactivation in TGF-beta-mediated fibronectin expression in mesangial cells. Kidney Int 62:799–808.

Wang W, Koka V, Lan HY. 2005. Transforming growth factor-beta and Smad signalling in kidney diseases. Nephrology (Carlton) 10:48–56.

Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K. 1995. Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science 270:2008–2011.