Isofraxidin, a Coumarin Component from *Acanthopanax senticosus*, Inhibits Matrix Metalloproteinase-7 Expression and Cell Invasion of Human Hepatoma Cells

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7-Hydroxy-6,8-dimethoxy-2*H*-1-benzopyran-2-one (isofraxidin) is a major coumarin component isolated from the stem bark of *Acanthopanax senticosus*, a widely used Chinese medicinal herb. We investigated isofraxidin in its anti-tumor effects on human hepatoma cell lines HuH-7 and HepG2. Isofraxidin significantly inhibited hepatoma cell invasion, without affecting cell attachment or growth. Expression of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced matrix metalloproteinase-7 (MMP-7) in hepatoma cells was inhibited by isofraxidin at the both mRNA and protein levels. This inhibition tended to be greater in cells inoculated at low density than in those at high density. Isofraxidin showed an inhibitory effect on the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in hepatoma cells, whereas activator protein-1 (AP-1) DNA binding activity, nuclear factor-kappa B (NF-κB) nuclear translocation, and inhibitory kappa B (IκB) degradation were affected very little. These results indicate that isofraxidin inhibits expression of MMP-7 and *in vitro* cell invasion at a non-toxic level through inhibiting ERK1/2 phosphorylation in hepatoma cell lines, which suggest isofraxidin might become an effective agent for suppressing hepatoma cell invasion.

Key words isofraxidin; matrix metalloproteinase-7; hepatoma cell; *Acanthopanax senticosus*; *in vitro* invasion; extracellular signal-regulated kinase 1/2

Invasion and metastasis of cancer cells are the major causes of high motality in cancer patients. Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes capable of degrading the components of extracellular matrix (ECM), including type IV collagen, gelatin and fibronectin. Cancer cells destruct surrounding tissues by enzymatic action of MMPs, leading to cancer invasion and metastasis. Among several MMPs, MMP-2, -7 and -9 are considered to be involved in cancer invasion and metastasis. In particular, MMP-7 is frequently found to overexpress in the invasive edge of cancer cells and play important roles in invasion or metastasis of digestive cancer.[1,2] Recently, the attempt to search for MMP inhibitors from plants is being performed, and several investigators have reported the inhibitory effects of epigallocatechin,[3] caffeic acid,[4] and proanthocyanin[5] on gene expression or enzymatic activity of MMP-2 and -9. However, except for the papers by Kawabata *et al.*,[6—8] little attention has been given to the development of MMP-7 inhibitors.

Isofraxidin occurs in a variety of plants including *Acansopanax senticosus* HARMs (Japanese name: Ezoukogi). As the major compounds of *Acanthopanax senticosus*, eleutheroside B, B1, E, isofraxidin, and chlorogenic acid have been identified, isolated, and reported to possess several biological activities, such as anti-stress, anti-fatigue, anti-gastric ulcer, anti-depressive, immuno-enhancing and anti-inflammatory effects.[9—16] We previously examined the effect of isofraxidin on the expression of inflammatory mediator cyclooxygenase-2 (COX-2) mRNA and found inhibitory effects at a significant level on human synovial sarcoma cell line SW982 cells.[11—16] In this study, we investigated isofraxidin in its anti-tumor effects on human hepatoma cell lines in cellular and molecular terms.

MATERIALS AND METHODS

Cell Culture Human hepatoma cell lines HuH-7, HepG2 and HLE and a human lung fibroblast cell line IMR90 were obtained from Japanese Collection of Research Bioresources (JCRB). Cells were grown in RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Phenolic Compounds Isofraxidin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Eleutheroside E was purchased from Phytochem Referenzsubstanzen GbRmb (Ichenhausen, Germany). The compounds were added to the culture medium at appropriate concentrations as a dimethylsulfoxide (DMSO) solution (the final DMSO concentration was 0.5% in all cultures), and the same amount of vehicle was added to the control cultures.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium Bromide (MTT) Assay For cell attachment study, cells were plated in 96-well plates at 3.0×10^4 cells/cm^2 (low cell density) in 0.1 ml of serum-free RPMI-1640 medium containing or not containing isofraxidin and incubated for 24 h. For cell growth study, cells were plated in 96-well plates at 3.0×10^4 cells/cm^2. After 2 d, the medium was replaced with 0.1 ml of RPMI-1640 medium supplemented with 10% FBS containing or not containing isofraxidin and incubated for 2—4 d. Cell attachment and growth were determined using MTT (Wako) assay, as previously reported.[16] Briefly, MTT (10 μl, 5 mg/ml) was added to each well 4 h before culture termination. The supernatant was discarded and 100 μl DMSO was added to each well. When the crystals were dissolved, the optical density (OD) values were read at 570 nm using microplate reader.

Cell Invasion Assay Cell invasion assay was performed using BioCoat Matrigel Invasion Chambers (Becton Dickin-

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son Bioscience, Bedford, MA, U.S.A.). Cells were inoculated into the culture inserts at 3.0×10^4 cells/cm^2 (low cell density). Next day, cells were treated with 80 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA, Sigma-Aldrich Co., St. Louis, MO, U.S.A.) and different concentrations of isofraxidin for 24 h in serum-free RPMI-1640 medium. After the treatment, the lower surfaces of the membrane were fixed with 100% methanol and stained with Giemsa solution. Cells invaded to the lower surface of the membranes were counted under a microscope.

Total RNA Extraction and Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR) Cells were plated into 60-mm dishes at 3.0×10^4 cells/cm^2 (low cell density), and 7.0×10^4 cells/cm^2 (high cell density). Next day, cells were treated with 80 nM TPA and different concentrations of isofraxidin for 24 h in serum-free RPMI-1640 medium.

After the treatment, total RNA was extracted from cells, as described previously.[17] The cDNA was synthesized from 2 μg total RNA using a first-strand cDNA kit (ReverTraAce-α, Toyobo Co., Ltd., Osaka, Japan). PCR amplification was performed with a 5 μl aliquot of the RT reaction product in a total volume of 50 μl containg 0.2 mM deoxynucleotide triphosphate (dNTP), 0,25 μM random primers, and 1.25 U *Taq* DNA polymerase (Fermentas Inc., Hanover, MD, U.S.A.). The PCR conditions were denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. We tested 30—35 cycles of PCR to determine optimum cycle numbers. The PCR primers used for amplification were as follows: MMP-7: sense 5′-gtggtcacctacaggatcgta-3′, anti-sense 5′-ctgaagtttctatttctttcttga-3′; MMP-9: sense 5′-cggagcacggagacgggtat-3′, anti-sense 5′-tgaaggggaagacgcacagc-3′; glyceraldehyde 3-phosphate dehydrogenase (G3PDH): sense 5′-accacagtccatgccatcac-3′, anti-sense 5′-tccaccaccctgttgctgta-3′. G3PDH was used as an internal control. Gene expression was normalized to the level of G3PDH using Scion Image Software (Scion Corp., Frederick, MD, U.S.A.).

Preparation of Nuclear Extracts and Electrophoretic Mobility Assay (EMSA) Cells were plated into 60-mm at 3.0×10^4 cells/cm^2 (low cell density). Next day, cells were pretreated with different concentrations of isofraxidin for 2 h in serum-free RPMI-1640 medium.

Cells were then treated again with isofraxidin in the presence of 80 nM TPA for 30 min. After the treatment, cells were harvested, and washed twice with cold phosphate buffered saline (PBS), and resuspended with 400 μl of buffer A [20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) (pH 7.6), 1.5 mM $MgCl_2$, 10 mM NaCl, 0.2 mM ethylenediamine tetraacetic acid (EDTA) (pH 8.0), 1 mM dithiothretiol (DTT), 0.1% NP-40, complete protease inhibitor cocktail set, one tablet (Roche Diagnostics, Tokyo, Japan), and 20% glycerol]. The samples were incubated for 15 min at 4 °C and then centrifugated at 100×***g*** for 5 min to sediment the nuclei. The nuclear pellets were extracted for 30 min in 100 μl of buffer B [20 mM Hepes (pH 7.6), 1.5 mM $MgCl_2$, 0.5 M NaCl, 0.2 mM EDTA (pH 8.0), 1 mM DTT, 0.1% NP-40, complete protease inhibitor cocktail set, one tablet, and 20% glycerol] at 4 °C and then centrifuged at 12000×***g*** for 20 min. The supernatants were used as nuclear extracts for EMSA. Protein concentration was measured by BCA™ Protein Assay Kit (Pierce, Rockford, IL, U.S.A.). Nuclear extracts (15 μg) were incubated with digoxigenin (Dig)-labelled probes in 5×binding buffer [100 mM Hepes (pH 7.6), 5 mM EDTA, 50 mM $(NH4)_2SO_4$, 5 mM DTT, 10% (w/v) Tween 20 and 150 mM KCl] at room temperature for 20 min with Dig-labelled probes. DNA–protein complexes were separated by electrophoresis in nondenaturing polyacrylamid gel using 0.25×Tris-borate-EDTA as a running buffer. After electrophoresis, gels were transferred onto nylon membranes, and detected chemiluminescently. The sequence of the activator protein-1 (AP-1) probe was 5′-CTAGTGATGAGTCAGCCGGATC-3′. The probe was labeled with digoxigenin-labeled dideoxy uridine triphosphate (Dig-ddUTP) using DIG Oligonucleotide 3′-end labeling kit (Roche Diagnostics) and used as Dig-labelled probe as mentioned above.

Immunoblot Analysis Cells were plated into 60-mm at 3.0×10^4 cells/cm^2 (low cell density). Next day, cells were pretreated with different concentrations of isofraxidin for 2 h in serum-free RPMI-1640 medium. The cells were then treated again with isofraxidin in the presence of 80 nM TPA for 30 min. After the treatment, cells were harvested and washed twice with cold PBS, and resuspended with RIPA buffer [150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate-Na, 1 mM phenyl-methylsulfonyl fluoride]. The samples were incubated for 30 min at 4 °C and then centrifugated at 12000×***g*** for 5 min. The supernatants were used as cell lysates. Culture supernatants were collected and incubated with 60% trichloro acetic acid (final, 3.75% v/v) at 4 °C over night. After centrifugation at 9000×***g*** for 10 min, the pellets were washed with ice-cold diethyl ether, and used as concentrated culture supernatants. Protein concentration was measured by BCA™ Protein Assay Kit. Cell lysates, nuclear extracts, and concentrated culture supernatants (15 μg) in SDS sample buffer were separated by 10% SDS-polyacrylamide gels, and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. After the membranes were blocked, they were incubated with a 1 : 1000 dilution of anti-phospho (P)-p44/42 mitogen-activate protein (MAP) kinase (extracellular signal-regulated kinase (ERK)1/2), anti-phospho (P)-c-Jun NH_2-terminal kinase (JNK), anti-phospho(P)-p38 MAPK (Cell Signaling Technology, Beverly, MA, U.S.A.), or anti-actin (Sigma) antibodies for 24 h at 4 °C, and then incubated with a 1 : 2000 dilution of anti-rabbit immunoglobulin G (IgG) coupled with peroxidase (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) for 1 h at room temperature. Detection was performed using ECL Plus Western Blotting Detection System (Applied Biosystems, Foster City, CA, U.S.A.). U0126 (Cell Signaling Technology) was used as a MAP kinase/ERK (MEK)1/2 inhibitor.

Statistical Analysis Values are mean±S.D. Statistical analysis was carried out using Dunnett's *post hoc* test.

RESULTS

Expression of MMPs mRNA in HepG2, HuH7, HLE and IMR90 We initially examined expression of MMPs (MMP-7 and -9) mRNA in human hepatoma (HepG2, HuH-7 and HLE) and lung fibroblast (IMR-90) cell lines by RT-PCR (Fig. 1). Under TPA-unstimulated conditions, HuH-7 and HLE cells expressed MMP-7 mRNA, whereas HepG2 and

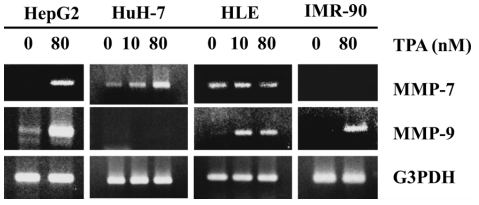

Fig. 1. Expression of MMP-7 and -9 mRNA in Several Cell Lines

HepG2, HuH-7, HLE and IMR-90 cells were treated with 10 and/or 80 nM TPA for 24 h. Untreated cells were used as controls. Total RNA was extracted from cell lysates and MMP-7 and MMP-9 mRNA were determined by RT-PCR. Expression of G3PDH was assayed as an internal loading control. Data shown are the representative of three independent experiments.

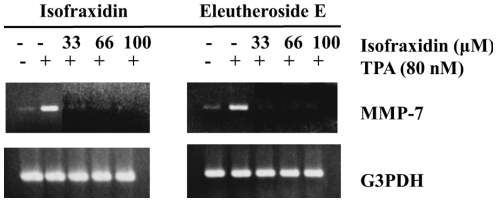

Fig. 2. Effect of Isofraxidin and Eleutheroside E on the Expression of MMP-7 mRNA

HuH-7 cells were treated with 80 nM TPA and various concentrations of isofraxidin or eleutheroside E for 24 h. Untreated cells were used as controls. Total RNA was extracted from cell lysates and MMP-7 mRNA was determined by RT-PCR. Expression of G3PDH was assayed as an internal loading control. Data shown are the representative of three independent experiments.

IMR-90 cells did not express this mRNA. Addition of TPA induced expression of MMP-7 mRNA in HepG2 and HuH-7 cells, but not in HLE and IMR-90 cells. Expression of MMP-9 mRNA was induced by TPA in HepG2, HLE and IMR-90 cells. HuH-7 cells did not express MMP-9 mRNA, regardless of the presence of TPA. The present observation was compatible with the previous reports that human MMP-7 expression appears to be more widespread in the epithelial cells, whereas other MMPs are expressed mainly in stroma cells.[18] Thus, HuH-7 and/ or HepG2 cells were used to examine the effect of isofraxidin on MMP-7 expression and cell invasion, as shown below.

Comparison of Isofraxidin with Eleutheroside E As the major compounds of *Acanthopanax senticosus*, eleutheroside B, B1, E, isofraxidin, and chlorogenic acid have been identified.[9] Among these compounds, eleutheroside E exhibited most effective inhibitory action on the expression of MMP-1 and -2 mRNA in SW982 cells.[16] In this experiment we studied the effect of isofraxidin and eleutheroside E on the expression of MMP-7 mRNA in HuH-7 cells by RT-PCR (Fig. 2). We found that these two compounds similarly suppressed TPA-induced MMP-7 mRNA expression at 33—100 μM. Since eleutheroside E is chemically unstable compared to isofraxidin, we focused on the effect of isofraxidin on MMP-7 expression and cell invasion.

Effect of Isofraxidin on Cell Attachment and Growth No significant difference in cell attachment was observed for HuH-7 cells cultured in the presence or absence of isofraxidin, as shown in Fig. 3A. Cell growth was little inhibited by treatment of HuH-7 or HepG2 cells with isofraxidin for 2 d. When HuH-7 cells were exposed to 100 μM isofraxidin for 4 d, cell growth was only weakly inhibited. Conversely, isofraxidin rarely affected growth of HepG2 cells (Fig. 3B). In addition, no morphologic changes were observed in 33 or 100 μM isofraxidin-treated HuH-7 and HepG2 cells (data not shown). These results indicate that isofraxidin is non-toxic to both HuH-7 and HepG2 cells at 33—100 μM.

Effect of Isofraxidin on Cell Invasion We examined the effect of isofraxidin on HuH-7 cell invasion by cell invasion assay. The addition of TPA significantly induced cell invasion at 80 nM (Table 1). Treatment of HuH-7 cells with isofraxidin significantly inhibited TPA-induced cell invasion at 66 and 100 μM (Table 1, Fig. 4).

Effect of Isofraxidin on MMPs Expression at Different Cell Densities Treatment with isofraxidin at 33—100 μM significantly inhibited the expression of TPA-induced MMP-7 mRNA in HuH-7 cells inoculated at both high and low cell densities (Fig. 5A). The inhibition was a little stronger at low cell density than at high density; the inhibition percent, as compared with TPA treatment only, was 94.8% (33 μM), 98.7% (66 μM), and 99.9% (100 μM) in the former, and 85.1% (33 μM), 90.9% (66 μM), and 90.1% (100 μM) in the

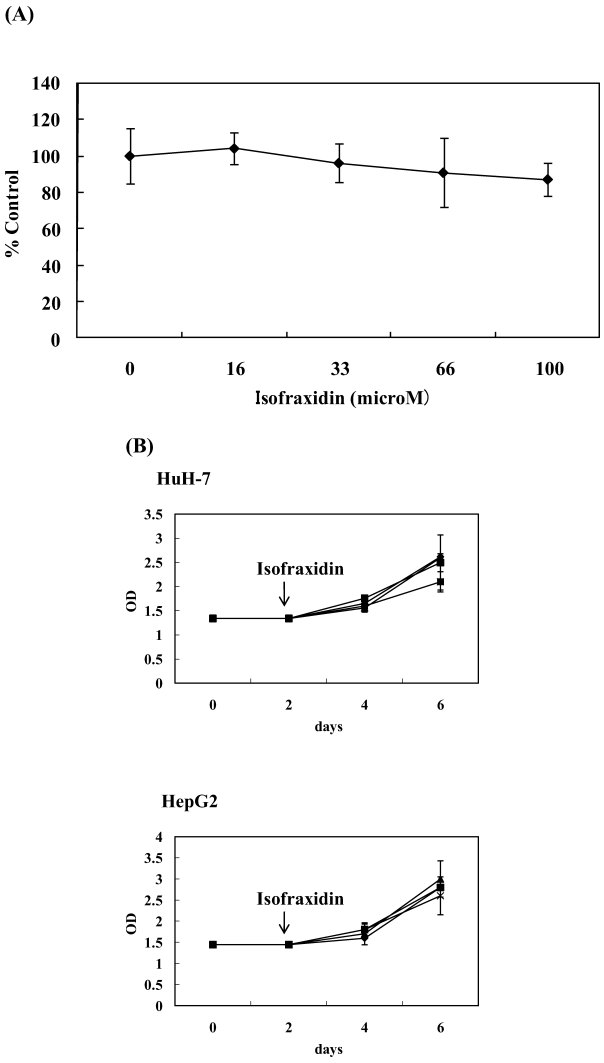

Fig. 3. Effect of Isofraxidin on Cell Attachment and Growth

(A) For cell attachment, HuH-7 cells were plated at low density (3.0×10^4 cells/cm^2), incubated for 24 h, and treated with various concentrations of isofraxidin for 24 h. (B) For cell growth, HuH-7 and HepG2 cells were plated at low density, incubated for 48 h, and treated with various concentrations of isofraxidin for 48 and 96 h (♣ 0, ■ 33, ▲ 66, ● 100 μM). Cell attachment and growth were determined by an MTT assay. OD, optical density absorbance at 570 nm. Data are shown as mean±S.D. from triplicate experiments.

Table 1. Effect of Isofraxidin on Cell Invasion

	Cell number/0.65 mm^2 (mean±S.D.)			
TPA (80 nM)	−	+	+	+
Isofraxidin (μM)	0	0	66	100
	34.6±5.6*	110±56.3	55.7±26.0*	5.9±1.2*

Results were statistically analyzed with Dunnett's *post hoc* test (*$p<0.01$ compared with the TPA treatment only) ($n=8$).

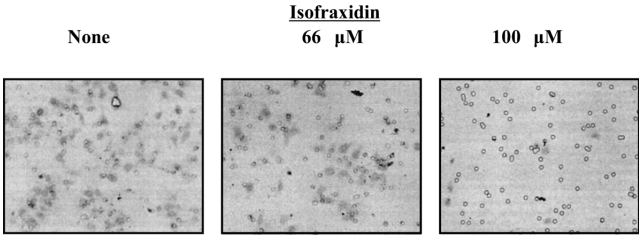

Fig. 4. Effect of Isofraxidin on Cell Invasion

After inoculation into BioCoat Matrigel Invasion Chambers, HuH-7 cells were treated with 80 nM TPA and 66 or 100 μM isofraxidin for 24 h. Untreated cells were used as controls. After treatment, the membrane was fixed with methanol and stained with Giemsa. Data shown are the representative of three independent experiments. Original magnification, 200×.

latter (Fig. 5A). Treatment with isofraxidin at 16—66 μM suppressed TPA-induced protein expression of latent MMP-7 (28 kDa) and active MMP-7 (18 kDa) forms in HuH-7 culture media (Fig. 5B). In HepG2 cells, treatment with isofraxidin significantly inhibited the expression of TPA-induced MMP-7 mRNA at 66 and 100 μM at high density and at 33—100 μM at low density, indicating that the inhibition was greater at low density than at high density; the inhibition percent, as compared with TPA-treatment only, was 90.9% (33 μM), 90.8% (66 μM), and 96.5% (100 μM) in the former, and 34.2% (33 μM), 31.2% (66 μM), and 79% (100 μM) in the latter (Fig. 5C). Inhibition of TPA-induced MMP-9 mRNA expression in HepG2 cells was also greater with isofraxidin at low cell density conditions than at high cell density (Fig. 5C).

Effects of Isofraxidin on Transcription Factors AP-1 DNA Binding Activity, Nuclear Factor kappa β (NF-κB Nulear Translocation, Inhibitory kappa B (IκB) Degradation and Phosphorylation of MAP Kinases The promoter regions of MMPs contain binding sites for transcription factors AP-1 and NF-κB, which are involved in transcriptional activation of MMPs.[19] To confirm whether isofraxidin suppresses the expression of MMP-7 through inhibiting AP-1 and NF-κB activities, we examined the effects of isofraxidin on transcription factors AP-1 DNA binding activity, NF-κB (p65) nulear translocation, IκB degradation and phosphorylation of MAP kinases (ERK1/2, SAPK-JNK, and p38) in TPA-stimulated HuH-7 cells. EMSA analysis shows that isofraxidin had little inhibitory effects on TPA-induced AP-1 DNA binding activity, NF-κB (p65) nulear translocation, and IκB degradation (Fig. 6A).

Our preliminary data showed that phosphorylation of MAPK occurred at 20—40 min after TPA treatment in HuH-7 cells (data not shown). Thus, the effect of isofraxidin on ERK1/2, SAPK-JNK and p38 phosphorylation was examined in cells stimulated by 80 nM TPA for 30 min. As shown in Fig. 6B, isofraxidin suppressed TPA-induced ERK1/2 phosphorylation in HuH-7 cells at 33 μM as well as at 100 μM. In contrast, stress-activated protein kinase (SAPK-JNK) and p38 phosphorylation were not inhibited with isofraxidin even at 100 μM. The levels of actin used as protein loading control were not affected with isofraxidin treatment (Fig. 6B).

Activation of ERK1/2 occurs by a single upstream MAP kinase kinase MEK 1/2. Whether the phosphorylation of ERK 1/2 is involved in the expression of MMP-7 can be investigated using a specific MEK inhibitor U0126. Treatment of HuH-7 cells with U0126 suppressed the expression of TPA-induced MMP-7 mRNA as well as ERK1/2 phosphorylation (Fig. 7).

DISCUSSION

Overexpression of MMP-7 in hepatocellular carcinoma (HCC) suggested that MMP-7 may be involved in the progression of HCC.[18] MMPs including MMP-7 are thought to be involved in the invasion and metastasis of a variety of malignant tumors, including HCC.[20] Thus, suppressants or inhibitors of MMP-7 are considered to be potential agents for chemoprevension and chemotherapy strategies on HCC. However, studies for MMP-7 suppressants or inhibitors are limited. In this study, we focused on the effect of isofraxidin on MMP-7 expression, cell invasion, and underlying molecular mechanisms.

Many metabolic functions as well as growth are regulated by cell density in rat hepatocytes.[21] Growth-related functions including DNA syntheses are stimulated by low cell density. In contrast, hepatocyte-specific functions are stimulated by high cell density.[22] The present study shows that MMP-7 expression is significantly inhibited by isofraxidin at the both protein and mRNA levels in human hepatoma cells and also shows that the inhibition tends to be greater at low cell density (3×10^4/cm^2) than at high cell density (7×10^4/cm^2), suggesting that isofraxidin may down-regulate MMP-7 expression more effectively when cells are in the growth phase than when they are in the stationary phase.

Isofraxidin did not affect cell attachment or growth of HuH-7 and/or HepG2 cells, but inhibited the invasive capacity of HuH-7 cells. *In vitro* invasion assay was found to correspond to metastatic potential *in vivo* within a wide variety of cell systems and the assay is widely used for testing anti-neoplastic agents.[22] Some anti-neoplastic agents might inhibit cell proliferation during the invasion assay and consequently reduce the number of cells capable of invasion.[23] The present result excludes the possibility that the reduction in the invasion rate observed after isofraxidin administration is attributable to reduced proliferation of cancer cells and rather suggests a specific effect of isofraxidin on the invasion process itself.

The process of metastasis involves a restructuring of the cytoskeleton as well as cell–cell and cell–matrix adhesions.[24] Rho and its effector protein ROCK signaling possibly contribute to the metastatic behavior of some tumor cells. Rho signaling inhibitor Y-27632 disrupts cytoskeleton structure of F-actin and reduces tumor cell invasion, confirming that ROCK inhibitor has a direct effect on reduction of tumor

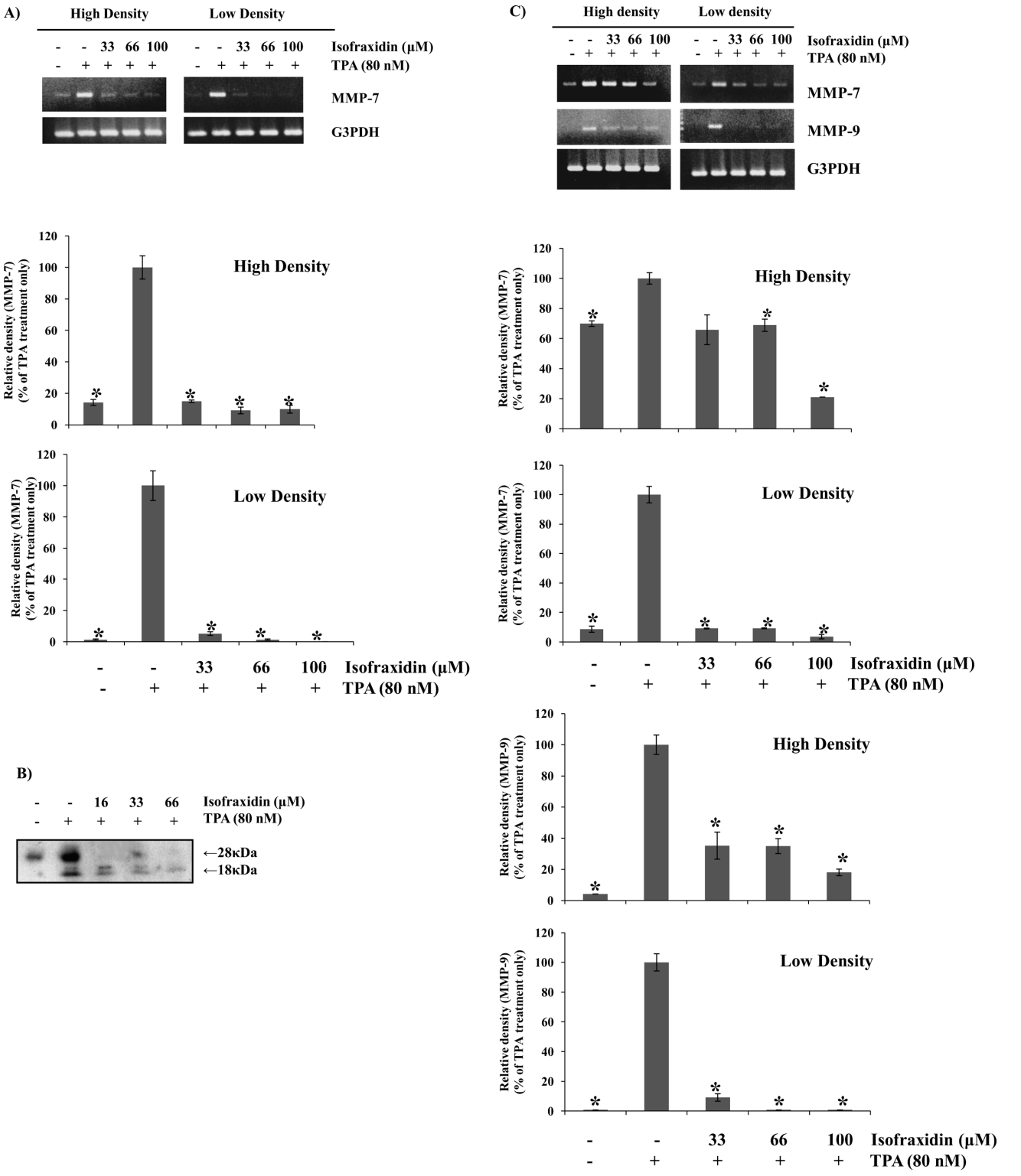

Fig. 5. Effect of Isofraxidin on MMPs Expression at Different Cell Densities

HuH-7 (A and B) and HepG2 (C) cells at low (3.0×10^4 cells/cm^2) and/or high (7.0×10^4 cells/cm^2) cell densities were treated with 80 nM TPA and various concentrations of isofraxidin for 24 h. Untreated cells were used as controls. For mRNA, total RNA was extracted from cell lysates and MMP-7 and/or MMP-9 mRNA were determined by RT-PCR (A and C). G3PDH was used as an internal control. The PCR products were quantified by densitometric analysis with that of TPA treated being 100%. Values represent mean±S.D. from three independent experiments. Results were statistically analyzed with Dunnett's *post hoc* test. (* $p<0.01$ compared with the TPA treatment only). For MMP-7 protein, conditioned media were concentrated and analyzed by immunoblot using anti-MMP-7 antibody (B).

metastasis.[25] Whether isofraxidin has similar effects to Y-27632 remains to be determined.

The promoter region of various MMPs contains many binding sites for transcription factors, and the transcriptional activation of MMPs is mainly regulated by AP-1 and NF-κB.[19] AP-1 is phosphorylated by MAP kinases family consists of ERK1/2, SAPK/JNK and p38. To clarify the molecular mechanisms by which isofraxidin suppresses expression

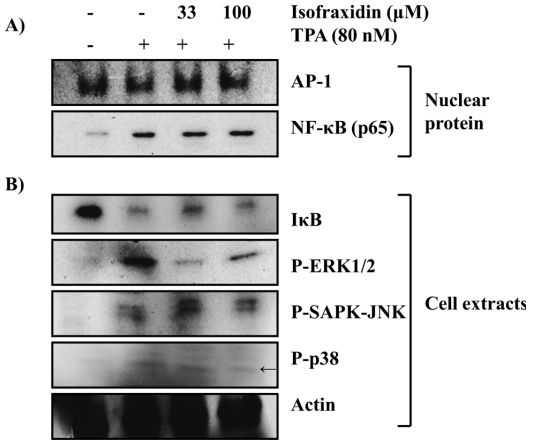

Fig. 6. Effect of Isofraxidin on Transcription Factors AP-1 DNA Binding Activity, NF-κB Nulear Translocation, IκB Degradation and Phosphorylation of MAP Kinases

HuH-7 cells were pretreated with 33 or 100 μM isofraxidin for 2 h. Cells were then treated again with isofraxidin in the presence of 80 nM TPA for 30 min. Untreated cells were used as controls. (A) For AP-1 activity and NF-κB nulear translocation, nuclear extracts were prepared and AP-1 activity and NF-κB nulear translocation were detected by elcetrophoretic mobility shift assay. (B) IκB degradation and phosphorylation of MAP kinases were analyzed by immunoblot using actin as the protein loading control. Data shown are the representative of three independent experiments.

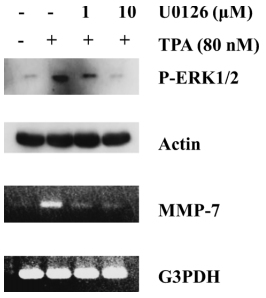

Fig. 7. Effect of a MEK1/2 Inhibitor on ERK1/2 Phosphorylation and MMP-7 Expression

HuH-7 cells were treated with 80 nM TPA and 1 or 10 μM a MEK1/2 inhibitor U0126 for 30 min. Untreated cells were used as controls. For P-ERK1/2 activation, cell extracts were prepared and analyzed by immunoblot using anti-P-ERK1/2 antibody. For MMP-7 mRNA, total RNA was extracted from cell lysates and MMP-7 mRNA was determined by RT-PCR. Expression of actin and G3PDH was assayed as internal loading controls. Data shown are the representative of three independent experiments.

of MMP-7, we examined the effects of isofraxidin on AP-1 DNA binding activity, NF-κB (p65) nulear translocation, IκB degradation and MAP kinases (ERK1/2, SAPK-JNK, p38) phosphorylation in TPA-stimulated HuH-7 cells. Of these molecules examined, isofraxidin suppressed only ERK1/2 phosphorylation. Furthermore, the treatment with MEK1/2 inhibitor U0126 inhibited TPA-induced ERK1/2 phosphorylation and expression of MMP-7 mRNA. These results suggested that the inhibitory effects of isofraxidin on the expression of MMP-7 are closely related to inhibition of ERK1/2 phosphorylation. ERK signaling pathway is known to be up-regulated in cancer tissues, and be considered to be associated with the invasion and metastasis of cancer cells. Therefore, brockade of this pathway is expected to be one of the most important target for the development of anti-cancer drugs.[26] Nobiletin, a citrus flavonoid, has been found inhibiting MMP-7 expression *via* reduction of AP-1 DNA binding activity in a human colorectal cancer cell line.[8] In the present study we found that isofraxidin suppresses MMP-7 expression, but does not affect AP-1 activity activated by ERK1/2 in hepatoma cells. Ets-1 is one of transcription factors activated by ERK1/2 signaling pathway.[27] The correlation between MMP-7 and Ets-1 expression has been observed in HCC tissues, and the forced expression of Ets-1 significiantly induced MMP-7 expression in HepG2 cells.[18] We are currently investigating whether Ets-1 is associated with isofraxidin-mediated inhibition of MMP-7 expression.

We found that isofraxidin inhibited the phosphorylation of ERK, but not JNK and p38. In this study a specific ERK inhibitor U0126 was used as a positive control. In addition, we investigated the effect of U0126 on MMP-7 expression to elucidate the involvement of ERK in isofraxidin's inhibitory effect on MMP-7 expression. The result indicates that U0126 inhibits MMP-7 expression, similar to the effect of isofraxidin. Further studies including the effect of U0126 on AP-1 DNA binding activity, NF-κB nulear translocation, and IκB degradation are needed to mimic the actions of isofraxidin in HuH-7 cells.

In conclusion, using TPA-stimulated human hepatoma cell system, we showed that isofraxidin is an efficient inhibitor of MMP-7 expression and cell invasion. It is believed that ERK1/2 is involved in the up-regulation of MMP-7 expression and cell invasion in TPA-treated hepatoma cells and serves as the molecular target for isofraxidin in its anti-invasiveness effect. *In vivo* efficacy of isofraxidin will be further addressed in the near future.

REFERENCES

1) Zeng Z. S., Shu W. P., Cohen A. M., Guillem J. G., *Clin. Cancer Res.*, **8**, 144—148 (2002).
2) Miyata Y., Iwata T., Ohba K., Kanda S., Nishikido M., Kanetaka H., *Clin. Cancer Res.*, **12**, 6998—7003 (2006).
3) Cung T. W., Moon S. K., Chang Y. C., Ko J. H., Lee Y. C., Cho G., Kim S. H., Kim J. G., Kim C. H., *FASEB J.*, **18**, 1670—1681 (2004).
4) Kim H. S., Kim M. H., Jeong M., Hwang Y. S., Lim S. H., Shin B. A., Ahn B. W., Jung Y. D., *Anticancer Res.*, **24**, 747—754 (2004).
5) Vayalil P. K., Mittal A., Katiyar S. K., *Carcinogenesis*, **25**, 987—995 (2004).
6) Kawabata K., Murakami A., Ohigashi H., *Biosci. Biotechnol. Biochem.*, **69**, 307—3314 (2005).
7) Kawabata K., Murakami A., Ohigashi H., *FEBS Lett.*, **589**, 5288—5294 (2006).
8) Kawabata K., Murakami A., Ohigashi H., *Biosci. Biotechnol. Biochem.*, **70**, 3062—3065 (2006).
9) Nishibe S., Kinoshita H., Takeda H., Okano G., *Chem. Pharm. Bull.*, **38**, 1763—1765 (1990).
10) Deyama T., Nishibe S., Nakazawa Y., *Acta Pharmacol. Shin.*, **22**, 1057—1070 (2001).
11) Tokiwa T., Yamazaki T., Sakurai S., *FFI Journal*, **211**, 576—582 (2006).
12) Yamazaki T., Yokoyama T., Akatsu H., Tsukiyama T., Tokiwa T., *In Vitro Cell. Dev. Biol.*, **39**, 337—339 (2003).
13) Yamazaki T., Shimosaka S., Sakurai M., Mastumura T., Tsukiyama T., Tokiwa T., *J. Electrophoresis*, **48**, 55—58 (2004).
14) Yamazaki T., Tsukiyama T., Tokiwa T., *In Vitro Cell. Dev. Biol.*, **41**, 80—82 (2005).
15) Yamazaki T., Matsumura T., Tsukiyama T., Tokiwa T., *Tiss. Cult. Res. Commun.*, **25**, 137—145 (2006).
16) Yamazaki T., Shimosaka S., Sasaki H., Matsumura T., Tsukiyama T., Tokiwa T., *Toxicology in Vitro*, **21**, 1530—1537 (2007).
17) Chomczynski P., Sacch N., *Anal. Biochem.*, **162**, 156—159 (1987).
18) Ozaki I., Mizuta T., Zhao G., Yotsumoto H., Hara T., Kajihara S., Hisatomi A., Sakai T., Yamamoto K., *Cancer Res.*, **60**, 6519—6525 (2000).
19) Fini M., Cook J. R., Moha R., Brinckerhoff C. E., "Matrix Metallopro-

teinase," ed. by Parks W. C., Mecham W. C., Academic Press, San Diego, 1998, pp. 299—356.
20) Wilson C. L., Matrisian L. M., *Int. J. Biochem. Cell Biol.*, **28**, 123—136 (1996).
21) Nakamura T., Yoshimoto K., Nakayama-Nakamura T., Yoshimoto K., Nakayama Y., Tomita Y., Ichihara A., *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 7229—7233 (1983).
22) Albini A., Benelli R., Noonan D. M., Brigati C., *Int. J. Dev. Biol.*, **48**, 563—571 (2004).
23) Parkkila S., Rajaniemi H., Parkkila A. K., Kivela J., Waheed A., Pastorekova S., Pastorek J., Sly W. S., *Proc. Natl. Acad. Sci. U.S.A.*, **97**, 2220—2224 (1999).
24) Croft D. R., Sahai E., Mavria G., Li S., Tsai J., Lee W., Marshall C. J., Olson M. F., *Cancer Res.*, **64**, 8994—9001 (2004).
25) Xue F., Zhang J. J., Qiu F., Zhang M., Chen X. S., Li Q., Han L. Z., Xi Z.-F., Xia Q., *Chin. Med. J.*, **120**, 2304—2307 (2007).
26) Kohno M., Pouyssegur J., *Ann. Med.*, **38**, 200—211 (2006).
27) Ito H., Dexbury N., Benoit E., Clancy T. E., Zinner M. J., Ashley S. W., Whang E. E., *Cancer Res.*, **64**, 7439—7446 (2004).