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Isorhamnetin from *Oenanthe javanica* Attenuates Fibrosis in Rat Hepatic Stellate Cells via Inhibition of ERK Signaling Pathway

Mi Kyeong Lee, Hyekyung Yang, Na Ry Ha, Sang Hyun Sung, and Young Choong Kim*

College of Pharmacy and Research Institute of Pharmaceutical Science, Seoul National University, Seoul 151-742, Korea

Abstract – Isorhamnetin isolated from *Oenanthe javanica* significantly inhibited proliferation and collagen production in HSC-T6 cells in concentration- and time-dependent manners. Pretreatment of HSC-T6 cells with isorhamnetin significantly inhibited serum-induced ERK phosphorylation, in a similar manner as PD98059, a known MEK inhibitor. These results suggested that isorhamnetin reduced collagen production in HSC-T6 cells, in part, via inhibition of ERK signaling pathway.
Key words – isorhamnetin, HSC-T6 hepatic stellate cells, antifibrotic, ERK signaling pathway

Introduction

Hepatic fibrosis occurs as the consequence of sustained wound healing response of liver to toxic, infectious or metabolic agents and is characterized by excessive accumulation of extracellular matrix (ECM) leading to ultimate liver dysfunction and irreversible cirrhosis (Friedman, 2003). In the pathogenesis of liver fibrosis, hepatic stellate cells (HSCs) are considered to play a key role. In response to liver damage, HSCs undergo a process of activation, developing a myofibroblast-like phenotype associated with increased proliferation, and/or excessive production and deposition of ECM components, which is the major pathological feature of hepatic fibrosis (Li and Friedman, 1999; Tsukada *et al*., 2006). Therefore, suppression of HSC activation has been proposed as a therapeutic target against hepatic fibrosis (Wu and Zern, 2000; Bataller and Brenner, 2001).

We previously reported that the methanolic extract of the aerial parts of *Oenanthe javanica DC* (Umbelliferae) protected primary cultured rat hepatocytes against CCl_4-induced toxicity. Subsequent activity-guided isolation of the extract afforded a flavonoid, isorhamnetin, which was found as a hepatoprotective compound (Fig. 1) (Kim and Kim, 1994).

Isorhamnetin has been known to have anti-tumor, anti-bacterial and anti-viral activities (Phadungkit and Luanratana, 2006; Ustun *et al*., 2006; Teng *et al*., 2006) as well as hepatoprotective activity (Kim and Kim, 1994; Kupeli *et al*., 2006). However, to date, there has been no report on antifibrotic activity of isorhamnetin. Thus, we attempted to reveal the action mechanism of antifibrotic activity of isorhamnetin by employing HSC-T6 rat hepatic stellate cell line as an *in vitro* system.

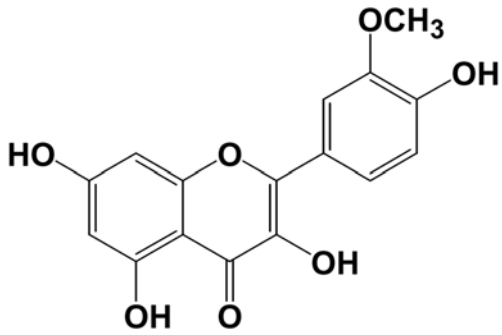

Fig. 1. Structure of isorhamnetin from the aerial parts of *O. javanica.*

Matherials and Methods

Chemicals – Supplement for cell culture and other reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO). Antibodies for phospho-specific ERK and phospho-independent ERK were from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of the highest purity available. Isorhamnetin was isolated from the aerial part of *O. javanica* and its purity was higher than 95.0% (Kim and Kim, 1994).

Culture of HSC-T6 hepatic stellate cells – An immortalized rat hepatic stellate cell line, HSC-T6 (Vogel

*Author for correspondence
Fax: +82-2-888-2933; E-mail: youngkim@snu.ac.kr

et al, 2000), was kindly provided by Prof. S. L. Friedman (Columbia University, New York). HSC-T6 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 95% air-5% CO_2.

Isorhamnetin was dissolved in dimethylsulfoxide (DMSO). Our preliminary study showed that DMSO at a final concentration of 0.1% in media did not affect the cell viability. HSC-T6 cells were treated with vehicle or isorhamnetin to be tested for 48 hr or as indicated. For Western blotting and collagen measurement, HSC-T6 cells were serum-starved for 1 hr before the treatment of isorhamnetin. After pretreatment with isorhamnetin for 1 hr, HSC-T6 cells were then activated by the addition of serum (final concentration in culture, 10%), followed further incubation for 30 min and 48 hr, respectively.

Measurement of inhibitory activity of cell proliferation – Cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HSC-T6 cells were incubated with 0.5 mg/mL of MTT in the last 2 hr of the culture period. Reduction of MTT to formazan was assessed in an ELISA plate reader.

Measurement of collagen content – Collagen content was quantified by Sirius Red-based colorimetric assay (Tullberg-Reinert and Jundt, 1999). Cultured HSC-T6 cells were washed with PBS, followed by fixation with Bouin's fluid for 1 hr. After fixation, the fixation fluid was removed and the culture dishes were washed by immersion in running tap water for 15 min. The culture dishes were air dried and stained by Sirius Red dye reagent for 1 hr with mild shaking on a shaker. Thereafter, the solution was removed and the cultures were washed with 0.01 N HCl to remove non-bound dye. The stained material was dissolved in 0.1 N NaOH and absorbance was measured at 550 nm against 0.1 N NaOH as a blank.

Western blotting – For Western blotting, proteins were extracted using lysis buffer that consists of 1% SDS, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 μg/ml aprotinin and 10 μM phenylmethylsulfonyl fluoride (PMSF). Equal amount of protein, as determined using the bicinchoninic acid (BCA), was loaded on 12% bis-tris electrophoresis gel. After electrophoresis, the gel was transferred to a nitrocellulose membrane and probed with phospho-specific ERK antibodies, and developed using horseradish peroxidase-conjugated secondary antibody and visualized using a chemiluminescence kit (Pierce, Rockford, IL). Equal loading of samples was checked by Ponceau staining and probing with phospho-independent ERK antibody.

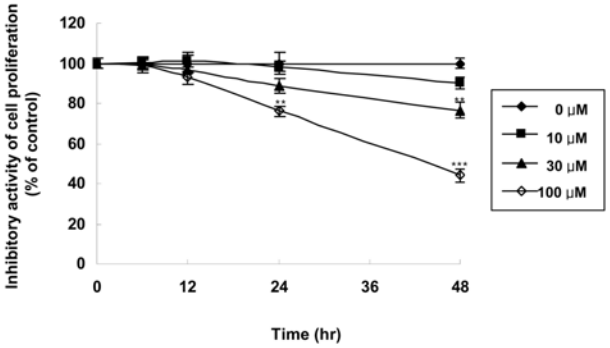

Fig. 2. Effect of isorhamnetin on cell proliferation in HSC-T6 cells. HSC-T6 cells were incubated with isorhamnetin at the concentrations ranging from 10 to 100 μM for indicated times. Cell proliferation was measured by the MTT assay. Inhibitory activity of compounds on cell proliferation (% of control) was calculated as 100 × (Absorbance of compound-treated / Absorbance of control). Results are expressed as the mean ± S.D. of three independent experiments, each performed using triplicate wells. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* vehicle only.

Statistical analysis – The evaluation of statistical significance was determined by the Student's *t*-test with a value of $p < 0.05$ or less considered to be statistically significant.

Results

Effect of isorhamnetin on HSC proliferation – One of the major characteristics of HSC activation, which is known to be a critical role in the hepatic fibrogenesis, is the increased proliferation of the cells (Sato *et al*, 2003). Thus, we determined whether isorhamnetin inhibit the HSC proliferation to assess the antifibrotic activity of isorhanmetin. In our culture system, serum-activated HSC-T6 cells showed high proliferative activity. However, as shown in Fig. 2, when HSC-T6 cells treated with isorhamnetin, proliferation of the cells was significantly decreased in a manner to concentration- and time-dependent. Isorhamnetin significantly decreased HSC proliferation during the period of 24 hr after the treatment at a concentration of 100 μM and further decreased during the period of 48 hr after the treatment at concentrations ranging from 30 to 100 μM. As the cells were treated with isorhamnetin at a concentration of 100 μM for 48 hr, the cell proliferation was decreased up to 50% of the cells which were not treated with isorhamnetin.

Effect of isorhamnetin on the morphology of HSCs – It is known that the culturing of HSCs on plastic plates causes the spontaneous activation of the cells, leading to myofibroblastic phenotype, mimicking the

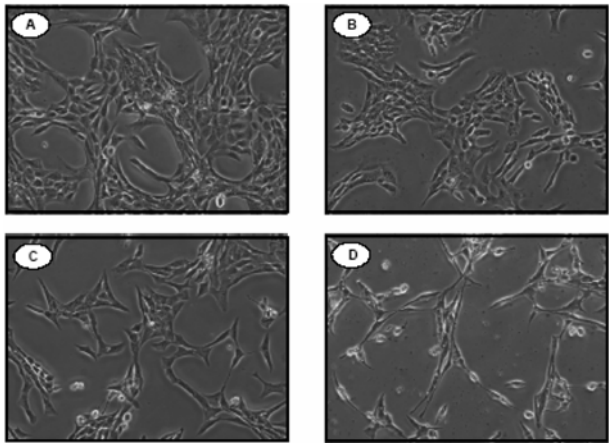

Fig. 3. Effect of isorhamnetin on cell morphology of HSC-T6 cells.
HSC-T6 cells were incubated with vehicle (A) or isorhamnetin at the concentrations of 10 μM (B), 30 μM (C) and 100 μM (D) for 48 hr. Cells were observed with phase contrast microscope (original magnification × 100).

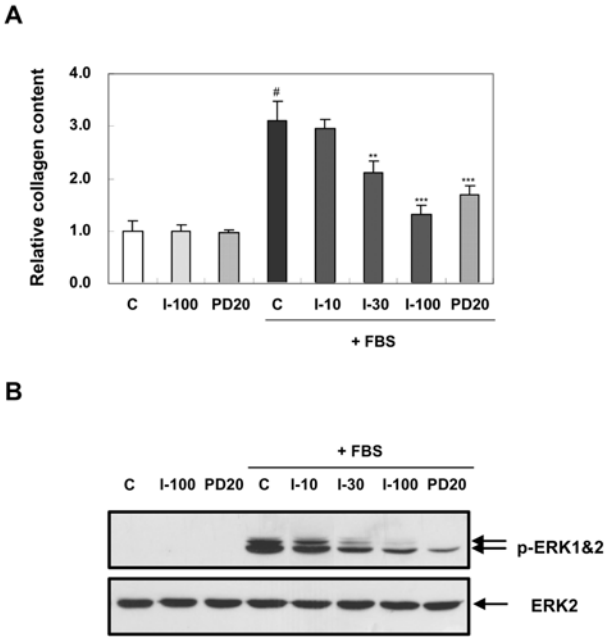

Fig. 4. Effect of isorhamnetin on collagen production and ERK phosphorylation in HSC-T6 cells.
After serum-starvation for 1 hr, HSC-T6 cells were incubated with vehicle, PD98059 (20 μM) or isorhamnetin at the concentrations of 10 μM to 100 μM. After 1 hr incubation, HSC-T6 cells were activated by the addition of serum (final concentration in culture, 10%) and incubated for 30 min for ERK phosphorylation or 48 hr for collagen production. (A) Collagen content was measured by Sirius Red-based colorimetric assay. Results are expressed as the mean ± S.D. of three independent experiments, each performed using triplicate wells. $^{\#}p < 0.001$ *vs* vehicle only and $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* vehicle with FBS. (B) ERK phosphorylation was detected by Western blotting analysis using phosphor-specific ERK1/2 antibody.

process seen in *in vivo* model (Chen and Zhang, 2003). When the HSC-T6 cells were cultured on plastic plates in the absence of isorhamnetin, the cells were grown as flattened and membranous shape, representing myofibroblastic morphology (Fig. 3A). However, if the HSC-T6 cells were treated with isorhamnetin for 48 hr, the morphology was changed to slender cell shape at concentrations ranging from 30 to 100 μM (Figs. 3B-D).

Inhibitory effect of isorhamnetin on collagen production – Excessive production and deposition of ECM such as collagen are other important characteristics of HSC activation, and are responsible for liver dysfunction. Therefore, the effect of isorhamnetin on collagen production in the activated HSC-T6 cells was studied. Activation of HSC-T6 cells by the addition of serum increased the collagen production more than three times when compared to that of control. However, isorhamnetin significantly reduced serum-induced collagen production in concentration-dependent manner over the concentration ranging from 10 μM to 100 μM (Fig. 4A).

Effect of isorhamnetin on ERK signaling – ERK1/2 pathway is known to be associated with HSC activation (Pinzani *et al*., 1998; Zhang *et al*., 2006a). Thus, the effect of isorhamnetin on ERK pathway in activated HSCs was evaluated. In consistent with previous reports, serum dramatically induced ERK phosphorylation as measured by Western blotting using phospho-specific ERK1/2 antibody (Fig. 4B). The serum-induced ERK phosphorylation was inhibited by the pretreatment of HSC-T6 cells with PD98059, a MEK inhibitor. In addition, pretreatment of HSC-T6 cells with PD98059 reduced collagen content increased by serum, which suggested the involvement of ERK signaling in serum-induced collagen production. However, as shown in Fig. 4B, pretreatment of HSC-T6 cells with isorhamnetin exerted concentration-dependent inhibition of ERK phosphorylation induced by serum. Taken together, we suggest that isorhamnetin reduced serum-induced collagen production, in part, via inhibition of ERK signaling.

Discussion

Although liver fibrosis has been regarded as irreversible, recent studies in animal models and patients have suggested that hepatic fibrosis is, at least to some degree, a reversible process (Iredale *et al*., 1998; Issa *et al*., 2004). Elimination of the activated HSCs has been linked to the reversal of liver fibrosis and treatments that induce HSC apoptosis and/or reduce HSC proliferation are currently under investigation as the potential treatment for liver

fibrosis (Bataller and Brenner, 2001).

Suppression of HSC activation generally can be achieved by various pathways, such as inhibition of cell proliferation and/or induction of cell death. Our present study showed that isorhamnetin, a flavonoid isolated from *O. javanica*, inhibited the proliferation of HSC-T6 cells (Fig. 2). Also, the treatment of HSC-T6 cells with isorhamnetin changed the cell morphology from flattened myofibroblastic membranous shape, representing activation state, to slender shape, representing quiescent state (Fig. 3). The treatment of HSC-T6 cells with isorhamnetin was associated neither with increased LDH release in the culture medium nor with modification of the Trypan Blue exclusion test with respect to control cells (data not shown). In addition, morphological changes caused by isorhamnetin were recovered within 24 hr after removing isorhamnetin from the culture medium and the cells kept adherent to the culture dishes (data not shown). These results suggest that the effect of isorhamnetin on HSC-T6 cells is reversible and not caused by direct cytotoxicity.

The relationship between the MAPK cascade and HSC activation has been reported. For instance, increased proliferation by diverse stimuli was inhibited by a MEK inhibitor (Reeves *et al*., 2000; Svegliati-Baroni, 1999). MAPK pathway is also known to play a crucial role in the ECM production in HSCs (Davis *et al*., 1996; Svegliati-Baroni, 1999). In addition, the importance of MAPK signaling in fibrosis was also suggested using *in vivo* model (Marra *et al*., 1999; Svegliati-Baroni, 2003). Our present study showed that antifibrotic activity of isorhamnetin was exerted, in part, by the inhibition of ERK activation induced by serum. However, interestingly, although the inhibition of ERK phosphorylation by the treatment of isorhamnetin at the concentration of 100 μM was weaker than by that of PD98059 at a concentration of 20 μM, the collagen production was more inhibited by the treatment with isorhamnetin (Fig. 4). Thus, we consider that another mechanism might be involved in the antifibrotic activity of isorhamnetin, which needs to be further clarified. In fact, it is believed that besides MAPK signaling, other diverse signaling pathway such as p38, JNK, phosphatidylinositol 3-kinase and protein kinase B (Akt) are also involved in HSC activation (Saxena *et al*., 2004; Zhang *et al*., 2006b; Perez de Obanos *et al*., 2006).

Liver is composed of several different cell types including hepatocytes, HSCs and Kupffer cells and exerts physiological role as well as pathological condition by cross-talking of various cell types. Liver fibrosis can be induced by hepatocellular damage, which causes the release of the cell components like lipid peroxide, metabolites of hepatotoxin and reactive oxygen species, leading to HSC activation (Friedman, 2003). In our previous study, isorhamnetin showed the hepatoprotective activity in primary cultured rat hepatocytes (Kim and Kim, 1994). In addition, isorhamnetin has been reported as an antioxidant (Igarashi and Ohmuma, 1995; Edenharder and Grünhage, 2003). Therefore, we suggest that the antifibrotic activity of isorhamnetin in *in vitro* demonstrated by our present study might be potentiated at *in vivo* model, by the collaboration of diverse activities of isorhamnetin such as hepatoprotective and antioxidative properties. Thus, it will be of interest to investigate further whether isorhamnetin exert antifibrotic effects in *in vivo*, for example, in animal models of liver fibrosis, to explore its therapeutic potentials.

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