The inhibitory effect of a Korean herbal medicine, *Zedoariae rhizoma*, on growth of cultured human hepatic myofibroblast cells

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Abstract

The aim of this study was to assess the effect of ZR on the growth of cultured human hepatic myofibroblast cells (hMF). The zedoary (*Zedoariae Rhizoma*) made from the dried rhizome of *Curcuma zedoaria Roscoe* is an herbal drug used as an aromatic stomachic. The plant is a perennial herb which is natively distributed throughout Korea and is a traditional Korean herbal medicine. *Zedoariae rhizoma* is a bioactive traditional medicine with anti-tumor, anti-atherosclerosis, anti-inflammation, and growth-regulating properties. During the course of liver fibrogenesis, hMF, mostly derived from hepatic stellate cells, proliferate and synthesize excessive amounts of extracellular matrix components. To evaluate the antiproliferative effect of a traditional herbal medicine, *Zedoariae rhizoma* water extracts (ZR) was examined on the growth inhibition of hMF since proliferation of hMF is known to be central for the development of fibrosis during liver injury, and factors that may limit their growth are potential antifibrotic agents. The aim of this study was to test the effects of ZR on the proliferation in cultured hMF. hMF were obtained by outgrowth from human liver explants. ZR markedly reduced serum driven cell proliferation, as assessed by nuclear autoradiography experiments and measurement of actual cell growth. Growth inhibition was totally reversed after removal of the ZR. ZR potently inhibited hMF growth (IC$_{50}$=8.5 µg/ml), in a pertussis toxin-insensitive manner. Analysis of the mechanisms involved in growth inhibition revealed that ZR rapidly increased prostaglandin E2 production and in turn cAMP, which inhibited hMF proliferation, did not affect cAMP levels. Production of cAMP by ZR was abolished by NS-398, a selective inhibitor of cyclooxygenase (COX)-2. Also, ZR potently induced COX-2 protein expression. Blocking COX-2 by NS-398 blunted the antiproliferative effect of ZR. We conclude that ZR inhibits proliferation of hMF, probably via an intracellular mechanism, through early...
COX-2-dependent release of prostaglandin E2 and cAMP, and delayed COX-2 induction. Our results indicated a novel role for ZR as a growth inhibitory mediator and pointed out its potential involvement in the negative regulation of liver fibrogenesis. The results that ZR exhibits potent antiproliferative and antifibrogenic effects toward hMF, indicated that ZR might have therapeutic implications in chronic liver disease.

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**Keywords:** Zedoariae rhizome; Cellular proliferation; Human hepatic myofibroblasts (hMF); Antifibrotic agents; Growth inhibition; Prostaglandin E2; COX-2; Antiproliferative effect; Liver fibrogenesis

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**Introduction**

Hepatic fibrosis, the main hallmark of chronic liver disease, is characterized by the accumulation of smooth muscle α-actin-positive mesenchymal cells, or myofibroblast (MF) cells, within the expanding fibrous septa or in the perisinusoidal spaces (Schmitt-Graff et al., 1991). Liver fibrosis is characterized by increased deposition and altered composition of extracellular matrix during chronic injury. Hepatic stellate cells (also called Ito cells, perisinusoidal cells, or fat-storing cells) are the main source of MF cells and play a central role in liver fibrogenesis (Iredale, 2001; Gressner, 1991). These cells display a quiescent phenotype in normal liver and acquire myofibroblastic features following acute or chronic liver injury (Iredale, 2001; Gressner, 1991; George et al., 2004). In addition, studies in experimental models of liver fibrosis indicate that portal fibroblasts might also contribute to hepatic MF cells (Tang et al., 1994). In the course of fibrogenesis, hepatic MF cells proliferate (Iredale, 2001; Gressner, 1991) and synthesize most extracellular matrix components (Takahara et al., 1992) that accumulate in fibrotic liver (Li and Friedman, 1999), such as interstitial and basement membrane (type IV) collagen. This fibrogenic response is characterized by intense proliferation and accumulation of myofibroblasts that actively synthesize extracellular matrix and proinflammatory cytokines, as demonstrated in experimental models and culture studies (Olaso and Friedman, 1998). Evidence for heterogeneity in the liver myofibroblast population has been provided recently, and it has been described in rat that two populations of myofibroblasts with fibrogenic potential, hepatic stellate cells and hepatic myofibroblasts, accumulate during chronic liver injury (Knittel et al., 1999a,b). Given their greatly enhanced mitogenic properties, identification of agents that may regulate the growth of these cells has been the topic of several recent studies. It was reported that the growth inhibitory properties of endothelin-1 (ET-1), tumor necrosis factor-α (TNF-α) and C-type natriuretic peptides (Mallat et al., 1996, 1998) are involved in human hepatic myofibroblasts (hMF). Recently, it was also shown that proliferation of hMF is tightly controlled by cyclooxygenases (Mallat et al., 1996, 1998; Gallois et al., 1998; Davaille et al., 2000), the rate-limiting enzymes in the conversion of arachidonic acid into prostaglandins and thromboxane. Indeed, ET-1 and TNF-α markedly inhibit proliferation of hMF through a pathway that involves induction of cyclooxygenase-2 (COX-2) and production of prostaglandin E2 (PGE2) and prostaglandin I2 (Mallat et al., 1996, 1998; Gallois et al., 1998).

There are detailed descriptions of the clinical experiences and prescriptions of liver fibrosis in traditional Korean medicine. Zedoariae rhizoma or Curcuma zedaria R (Zingiberaceae), a traditional Korean medicine originating in China, is medicinal plant and used for treatment of symptoms such as weakness caused by fatigue and weakness after illness (Jiangsu New Medical College, 1977).
The plant has been being at present used as a Korean (Korean name, Bongchul) and Japanese herbal medicine (Japanese name, Gajutsu), which is listed in the Korean Pharmacopoeia II as aromatic stomachic, emmenagogus, or for the treatment of ‘Ohyul extravasated blood’ syndrome caused by blood stagnation. Furthermore, *Zedoariae rhizoma* water extract (ZR) also have been used as an important fragrance and spice in Asian countries. As chemical constituents of this plant, many sesquiterpenes, such as furanogermanone (Toda, 2003), germacrone (Morikawa et al., 2002), and (+)-germacrone 4,5-epoxide (Mosmann and Coffman, 1993; Murakami et al., 2000; Matsuda et al., 2000; 2001a,b; Yoshikawa et al., 1994; 1996; 1997a,b; 1998; 2000a,b), have been isolated from *Zedoariae rhizoma*, and these sesquiterpenes have been reported to exhibit antihepatotoxic and anti-ulcer effects (Yoshikawa et al., 1994; Toda, 2003). With respect to its bioactive constituents of natural medicines and medicinal foodstuffs (Yoshikawa et al., 1994; 1996; 1997a,b; 2000a,b; Murakami et al., 2000; Matsuda et al., 2000; 2001a,b), it has been reported that the sesquiterpene constituents from ZR exhibited potent vasorelaxant activity. In addition, absolute stereostructures of carabran-type sesquiterpenes, curcumenone, 4S-dihydrocurcumenone, and curcarabranols A and B, were determined on the basis of physicochemical and chemical evidence (Yoshikawa et al., 1998). Furthermore, it was also communicated on hepatoprotective activity of the 80% aqueous acetone extract and several known constituents from ZR and their plausible mechanisms of action (Matsuda et al., 2001a,b).

Many kinds of traditional herbal medicines have immunomodulating activities, e.g., B cell mitogenic activity (Furuya et al., 2001), activation of macrophages (Yoon et al., 2003), enhancement of natural killer (NK) activity (Kaneko et al., 1994) and action on hematopoietic stem cells. Natural herbal medicines such as ZR have been used empirically in the treatment of hepatic cancer and fibrosis, but the mechanism by which they bring about antitumor and antifibrosis effects are unclear. Although ZR is being used to treat human hepatic tumor and fibrosis and known to have antimetastatic effects, however, its antifibrosis activity, as one of the pharmacological and biological actions of ZR have not been thoroughly investigated to date. In a preliminary study, ZR showed a mitogenic activity and antiproliferative properties in many cell types, including hMF (Park et al., unpublished results). Given the pivotal role of hMF cell in hepatic fibrogenesis, there is a tremendous interest in drugs including traditional herbal medicine that might limit the proliferation and/or matrix component synthesis of these cells. This led us to investigate the role of ZR as a possible growth inhibitory factor for these cells. In this study, we report that ZR inhibits proliferation of hMF, probably via an intracellular mechanism involving early COX-2-dependent release of PGE2 and cAMP and delayed COX-2 induction. Our results shed light on a novel role for ZR as a growth inhibitory mediator and point out its potential involvement in the negative regulation of liver fibrogenesis.

**Materials and methods**

**Materials**

NS-398 was from Sigma (St. Louis, MO, USA). Fetal calf serum was from Jeil Biotech Services Co. (Daegu, Korea), and pooled human AB-positive serum were supplied by the National Transfusion Center and from GIBCO BRL (Life Technologies, CA, USA). [methyl-³H]Thymidine
(25 Ci/mmol) and [35S]GTPS were from ICN Pharmaceutical (Beverly, CA, USA). L-[5-3H]proline (19 Ci/mmol) was from Amersham Life Science (Arlington Heights, IL). L-[4,5-3H]Leucine (108 Ci/mM) was from ICN. cAMP radioimmunoassay kit and COX-1 and COX-2 specific antibodies were from Cayman (CA, USA). The protein assay kit was from Bio-Rad (Richmond, CA, USA). All other chemicals were from Sigma (St Louis, MO, USA). CellTiter 96 AQueous One Solution Cell Proliferation Assay was from Promega (CA, USA). Media were obtained from GIBCO BRL (Life Technologies, CA, USA). All tissue-culture plastic ware was from Falcon Plastics (Becton Dickinson, Oxnard, CA), except for 96-well plates, which were from Costar (Cambridge, MA). Penicillin, streptomycin and fungizone were from Sigma. N-ethylmaleimide, β-aminopropionitrile, 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and smooth muscle α-actin antibody (clone 1A4) were from Sigma. Gelatin and ascorbic acid were from Merck (Darmstadt, Germany).

Preparation of extract of Zedoariae Rhizoma

Dried Zedoariae Rhizoma (9.0 kg, cultivated in Yongchun, Korea and purchased from Kyungju Market Co., Kyungju) was identified by one of the authors, SD Park. A voucher of the plant is on file in our laboratory (2002.09. ZR-2-D3). The plant was finely minced and extracted. ZR was prepared as a hot water extract from medical plant (20 g). Spray-dried ZR was manufactured by a herb medicine hospital as the test drug. Extraction was carried out by boiling the mixture of herbs in 10 parts water at 95–100 °C for 1 h, and the extract was spray-dried in a hot air stream. The Zedoariae rhizoma was dissolved in distilled water for administration to mice. As for in vitro use, ZR was dissolved in distilled water, and then centrifuging the mixture at 7500 rpm for 30 min. After filtration, the aqueous extract was lyophilized and stored at –20 °C. The ZR was dissolved in pyrogen-free isotonic saline (Sigma Chemical, MO, USA) and filtered through a 0.2 μm filter (Microgen, Laguna Hills, CA, USA) before use.

Cell isolation and culture

hMF were obtained by outgrowth of explants prepared from surgical specimens of normal liver, as described previously (Preaux et al., 1997). This procedure was performed in accordance with ethical regulations imposed by the Korean Ministry of Health and Welfare legislation, as previously described (Shim et al., 2004). Adult liver tissue was obtained either from hepatic tissue that had been surgically excised by debridement of a traumatized liver or a hepatocytomized liver specimen for the treatment of massive liver fracture. Fetal liver tissues were obtained from abortion (at 26 weeks of gestation) performed to safeguard mothers from harm associated with a pregnancy with the provisions in law of Maternal and Child Health Care of the Republic of Korea. The study was approved by the Institutional Ethical Committee. For the primary culture, human MF were obtained from the livers of organ donors and fetal livers at 26 weeks of gestation. Adult livers and fetal livers were donated from the Liver Research Institute (Prof. T-H Chung), Kyungpook National University Hospital, Daegu, Korea. The liver tissue was placed immediately in cold 10% fetal bovine serum/Dulbeco’s modified Eagle’s medium (10% FBS/DMEM) supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml), and then washed 2–3 times with the same medium. The liver tissues were cut with scissors into small pieces and transferred to a 50 ml falcon tube. The larger fragments
were allowed to settle and the supernatant was recovered. The cell suspension was filtered through a gauze and washed with DMEM three times by centrifugation at 50 x g for 3 min. Freshly isolated hMF were seeded in 10 ml of 10% FBS/DMEM supplemented with penicillin (100 units/ml) and streptomycin (100 µg/ml) at a density 10^8 cell per plastic dish (diameter 100 mm) at 37 °C under 5% CO₂ in air. The cells were harvested at the indicated times for all experiments and stored at –70 °C in 10% DMSO/45% FCS/45% DMEM.

Cells were used between the 3rd and 7th passage, and all experiments were performed on hMF made quiescent by a 3-day incubation in serum-free Waymouth medium, unless otherwise indicated. The myofibroblastic nature of the cells was routinely evaluated by electron microscopy and positivity for smooth muscle α-actin by immunohistochemistry, as described previously (Preaux et al., 1997). The cultures were also found to express two markers of rat hepatic myofibroblasts, fibulin-2 and interleukin-6, and not the protease P100, a marker for rat hepatic stellate cells (Knittet al., 1999a).

[^35]S[GTPS binding assay

Confluent hMF were made quiescent by incubation in Waymouth medium without serum for 48 h. Cells were then washed with ice-cold phosphate-buffered saline (PBS) medium, scraped in buffer A (20 mM Tris, pH 7.4, 500 µM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml leupeptin, 1 µg/ml aprotinin, 0.5 µg/ml pepstatin), and homogenized with a Polytron homogenizer. The homogenate was spun 5 min at 5,500 x g, and the pellet was discarded. The supernatant was centrifuged for 40 min at 43,000 x g, and the resulting pellet was resuspended in buffer A and frozen at 80 °C until use. Membranes (10 µg of protein/assay) were incubated for 45 min at 30 °C in buffer B (20 mM Tris, pH 7.4, 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 100 µM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 0.5 µg/ml pepstatin) containing 3 µM GDP unless otherwise indicated, 50 pM[^35]S[GTPS (0.5 µCi/assay), and varying concentrations of ligands. The samples were then rapidly filtered on GF/B glass microfiber filters (Whatman) presoaked in buffer C (20 mM Tris, pH 7.4, 10 mM MgCl₂, 100 mM NaCl, and 1 mM β-mercaptoethanol). The filters were washed three times with 2 ml of buffer C and counted. Specific binding was calculated as the difference in bound radioactivity in the absence or presence of 10 µM unlabeled GTPS and did not exceed 20% of the total binding.

Pertussis toxin (PTX) treatment of hMF

Confluent hMF were made quiescent by incubation in Waymouth medium without serum for 48 h. Cells were then treated for 24 h with either 3 or 100 ng/ml PTX or vehicle, and DNA synthesis was measured as described below. Alternatively, membranes were prepared as described above and used in GTPS binding assays.

Cyclic AMP assay

Cells were seeded in 24-well plates, grown to confluence, and serum-deprived for 48 h in Waymouth medium. Following preincubation with 0.6 mM isobutylmethylxanthine for 15 min, hMF were stimulated with PBS containing effectors. When indicated, cells were preincubated for 60 min with either the COX-2 inhibitor NS-398 (5 µM) or vehicle (Me2SO). Samples were processed as described
previously (Mallat et al., 1996), and cyclic AMP was assayed by a commercial radioimmunoassay (Cayman, USA).

**Prostaglandin release**

Confluent monolayers in 96-well plates were serum-deprived for 48 h, washed in PBS, and further incubated in Waymouth medium. Following pretreatment with 5 μM NS-398 or vehicle, cells were stimulated with the indicated effectors over various times. Release of PGE2 was assayed by a specific enzyme immunoassay as described previously (Pradelles et al., 1985).

**DNA synthesis assay**

DNA synthesis was measured in triplicate wells by incorporation of [³H]thymidine, as described previously (Tao et al., 1999). Confluent hMF were made quiescent by incubation in Waymouth medium without serum for 48 h. Cells were then stimulated for 24 h with human serum following pretreatment for 30 min with NS-398, dexamethasone, or vehicle. [³H]Thymidine (0.5 μCi/well) was added during the last 8 h of incubation.

**Cell proliferation assay**

Cell proliferation was assessed by two tests:

*Mitochondrial deduction of tetrazolium salt MTT to formazan (Gonzalez and Tarloff, 2001)*

This conversion has been shown to be proportional to the number of living cells in the culture (Gonzalez and Tarloff, 2001). hMF were seeded in sextuplicate in 96-well plates at a density of 2,000 cells/well in 200 ml DMEM 5/5. Twenty-four hours after seeding (day 0), the growth-inhibitory effect of ZR was studied over a 7-day incubation period in Waymouth’s medium containing 10% human serum, with or without ZR. Medium and additive(s) were renewed on day 3 and day 5. In the second part of the experiment, the reversibility of the effects of ZR was studied. The drug was withdrawn on day 8, and hMF were further incubated for 7 days. At each time point, MTT reduction was assessed by adding 1 mg/mL MTT to the cultures for 1 hour and measuring the optical density of the product at 540 nm in a Multiscan microplate reader (Micro Device, USA). Net inhibition was expressed as follows:

\[
\% \text{ net inhibition} = (1 - \frac{B - A}{C - A}) \times 100
\]

where A represents optical density on day 0, and B and C are the optical densities observed at the end of the incubation period in the presence of ZR and control medium, respectively.

*Actual cell growth*

Actual cell proliferation was assessed as described previously (Tao et al., 1999). Briefly, cells were seeded in 96-well plates at low density (5000/well) in DMEM containing 5% human serum and 5% fetal calf serum (5:5), allowed to attach overnight, and made quiescent by a 48-h incubation in serum-free medium. Cells were then incubated for 3 days with 5% human serum in the absence or presence of 10 μg/ml ZR that was renewed daily. The medium was changed for PBS, and CellTiter 96 AQueous One Solution reagent was added to each well, and absorbance was recorded at 490 nm.
Nuclear autoradiography experiments

For nuclear autoradiography, human hMF were grown to confluence in six-well plates (35-mm diameter) and made quiescent by a 3-day incubation in serum-free medium. Cells were then incubated for 30 hours in 1 mL Waymouth’s medium containing 10% human serum, with or without ZR. [\(^3\)H]Thymidine (2 µCi/ml) was added 8 hours before the end of the experiments. Cultures conditioned media underwent electrophoresis phosphate-buffered saline, fixed in 4% paraformaldehyde for 10 minutes, and air-dried. They were covered with NTB2 Kodak emulsion and exposed for 3 days. Labeling was developed with Kodak Dektol developer and fixed in Kodak Unifix. The number of nuclei with overlapping silver grains was counted in random fields. Data from 10 to 20 high-magnification fields per culture were averaged. Triplicate cell cultures were used for each experimental point. In control cultures performed in these experimental conditions, we have previously shown that 40% to 50% of the nuclei are labeled (Mallat et al., 1994).

![MTT assay graph](image)

Fig. 1. Reversible antiproliferative effect of ZR on hMF. A) MTT assay. Cells (2,000/well) were incubated for the indicated periods in medium containing 10% human serum, in the absence or in the presence of ZR. On day 8, ZR-treated cells were switched back to control medium. Cell growth was assessed at each time point by a colorimetric test, as described in Materials and methods. Results are the mean ± SEM of five experiments and are expressed as optical density. ZR growth-inhibitory effect was significant by ANOVA (\(P < 0.01\)). (B) Representative morphological observation of hMF. Cells (5 × 10^6 cells/ml) treated with 0 (a), 5 µg/ml (b) and 10 µg/ml (c) of ZR for 24 hrs were observed at high magnification (× 400).
Results are expressed as mean ± S.E. of n experiments. Results were analyzed by repeated measures analysis of variance or two-tailed Student’s t test, as appropriate, with p < 0.05 considered significant.

Statistics

Results are expressed as mean ± S.E. of n experiments. Results were analyzed by repeated measures analysis of variance or two-tailed Student’s t test, as appropriate, with p < 0.05 considered significant.

Results

Effects of ZR on growth of serum-stimulated hMF

Fig. 1A shows the effects of various concentrations of ZR on serum-stimulated cell growth, as assessed by the reduction of MTT. Cell proliferation was dose-dependently decreased by ZR over the time course of incubation (P < 0.01 by ANOVA). After 8 days of treatment, net growth inhibition was 32% at a concentration of 5 μg/ml and 76% at 10 μg/ml. These results were confirmed by measurement of actual cell growth, the corresponding figures being 20.6% and 47.8% after 4 days of treatment with ZR (Table 1). In addition, assessment of DNA synthesis by nuclear autoradiography showed that ZR reduced the number of replicative hMF (Table 2). The number of labeled nuclei was decreased by 15% and 32% at a ZR concentration of 5 μg/ml, and by 76% and 53% at 100 μg/ml. The antiproliferative effect was not related to a toxic effect of the drug, because cells showed normal morphology upon phase-

Table 1
Effect of ZR on serum-stimulated proliferation of hMF

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell number on day 4 (× 10^3 per well)</th>
<th>Net growth inhibition (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>257 ± 87</td>
<td></td>
</tr>
<tr>
<td>ZR (5 μg/ml)</td>
<td>204 ± 67</td>
<td>20.6 ± 4.6</td>
</tr>
<tr>
<td>ZR (10 μg/ml)</td>
<td>134 ± 34</td>
<td>47.8 ± 3.5</td>
</tr>
</tbody>
</table>

hMF (2 × 10^5/35-mm well) were incubated in medium containing 10% human serum, in the absence (control) or in the presence of ZR, as described in Materials and methods. Cells were counted on day 4. Mean cell number on day 0 was 26 × 10^3 ± 1 × 10^3 per well. Results are the mean ± SEM of five separate experiments.

* Calculated as described in Materials and methods.

Table 2
Effect of ZR on hMF DNA synthesis as assessed by nuclear autoradiography

<table>
<thead>
<tr>
<th>ZR (μg/ml)</th>
<th>Nuclei with overlapping silver grains (n/field)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Control (0)</td>
<td>52 ± 1</td>
</tr>
<tr>
<td>ZR (5 μg/ml)</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>ZR (10 μg/ml)</td>
<td>33 ± 3</td>
</tr>
</tbody>
</table>

Confluent quiescent hMF were stimulated for 24 hours with 10% human serum, in the absence or in the presence of various concentrations of ZR. [3H]Thymidine was present over the last 12 hours of the experiment. Cells were subsequently processed for nuclear autoradiography analysis, as described in Materials and methods. Nuclei with overlapping silver grains were counted. Triplicate cell cultures were used for each experiment, and data from 10 to 20 random high-magnification fields per culture were averaged. Results are expressed as mean ± SEM.
contrast microscopy (Fig. 1B). In addition, when cultures were exposed to ZR for 7 days, and further switched back to control medium, cells rapidly resumed a normal growth, as assessed by MTT reduction (Fig. 1A).

![Graph A](image1)

**A)** Cell Growth Assay (A490) (fold over basal levels)

![Graph B](image2)

**B)** Concentration of ZR (µg/ml)

![Graph C](image3)

**C)** [3H]–Thymidine incorporation (% of control)

Fig. 2. ZR inhibits the proliferation of hMF. A, Effects of ZR on DNA synthesis of hMF. Confluent cells were made quiescent by incubation in serum-free medium over 2 days. Cells were stimulated for 24 h with varying concentrations of ZR, in the presence of 2% human serum. [3H]Thymidine incorporation into DNA was measured as described under “Experimental Procedures.” Results represent the mean ± S.E. of six experiments and are expressed as percent of control. *p < 0.05 compared with control for concentrations over 1.0 µg/ml. B, Effect of ZR on hMF growth. Cell growth was assayed at day 0 and day 2 as described under “Experimental Procedures,” without or with 2% human serum in the absence or presence of 10 µg/ml ZR or vehicle, which was added every day. Results are the mean ± S.E. of three experiments. p < 0.05 for ZR versus control. C, Inhibition of DNA synthesis by ZR is pertussis toxin-insensitive. Confluent quiescent cells were pretreated for 24 h with 3 ng/ml pertussis toxin or vehicle. DNA synthesis was then measured as described in A. Results represent the mean ± S.E. of three experiments. The inhibitory effect of ZR was not significantly different in hMF treated with toxin (PTX) or vehicle. The inset shows the effects of 3 and 100 ng/ml pertussis toxin on DNA synthesis measured with 2% human serum.
ZR inhibits proliferation of hMF in a pertussis toxin-insensitive manner

We investigated the effects of ZR on the proliferation of hMF. ZR had no mitogenic effects on hMF (not shown) but dose-dependently reduced serum-stimulated DNA synthesis (Fig. 2A). A maximal 45% inhibition was observed at 10 μg/ml ZR, with an IC₅₀ of 8.5 μg/ml. Direct assessment
of cell growth confirmed the antiproliferative effect of ZR, with a 35% inhibition of cell growth being observed after 2 days (Fig. 2B). The growth inhibitory effects of ZR were insensitive to pertussis toxin (Fig. 2C), although pertussis toxin treatment was effective, since it reduced the mitogenic effect of serum. Moreover, pertussis toxin was maximally effective since DNA synthesis in response to serum was reduced to the same extent at 3 and 100 ng/ml of the toxin (Fig. 2C, inset).

**COX-2 mediates the growth inhibitory effects of ZR in hMF**

The next series of experiments were performed to analyze the signaling events that mediate inhibition of hMF proliferation by ZR. We first examined the effects of ZR on PGE2 and cAMP levels, two growth inhibitory mediators for hMF (Mallat et al., 1996, 1998; Gallois et al., 1998). Following addition of ZR, cAMP production rose within 5 min, maximally increased to 17-fold by 10 min, declined after 15 min, and returned to basal levels after 90 min (Fig. 3A). Stimulation of cAMP production by ZR was totally blocked by the selective COX-2 inhibitor NS-398 (Fig. 3A) and unaffected by PTX treatment (Fig. 3B). These data suggested that ZR raises cAMP following PGE2 production by COX-2. Accordingly, ZR caused a rapid 5.2-fold activation of PGE2 production, which correlated with the time course of cAMP production (Fig. 3C), and was blocked

![Graph A](image)

**A)**

**Fig. 4.** ZR induces COX-2 protein expression and activity in hMF. A, ZR induces COX-2 protein expression but has no significant effect on COX-1. Whole cell extracts obtained from quiescent hMF treated by 10 μg/ml ZR for various times were analyzed by Western blot of COX-2 or COX-1 proteins. Results show a typical experiment repeated three times. B, ZR stimulates PGE2 release. Confluent quiescent cells were pretreated for 60 min with 5 μM NS-398 or vehicle (Me2SO) and were further stimulated with 10 μg/ml ZR over various periods. PGE2 release was assayed by enzyme immunoassay as described under “Experimental Procedures.” Results show a typical experiment repeated twice.
by NS-398 (Fig. 3C, inset). It should be noted that NS-398 decreased basal PGE2 production, although COX-2 is constitutively expressed in hMF (see Fig. 3A), in agreement with the previous evidence showing that COX-2 accounts for basal PGE2 production in these cells (Gallois et al., 1998). Therefore, these results demonstrate that ZR stimulates an early COX-2-dependent production of PGE2, leading to cAMP synthesis. Since COX-2 is also an inducible enzyme, and given its central role in the inhibition of hMF proliferation (Mallat et al., 1996; Gallois et al., 1998), we investigated the effect of ZR on COX-2 protein expression. As shown in Fig. 4A, ZR caused a strong induction of COX-2, which was maximal after 3 h and remained elevated for at least 8 h. In contrast, ZR did not significantly affect COX-1 expression. Induction of COX-2 was associated with a 2.5-fold increase in PGE2 production, an effect totally blocked by the COX-2 inhibitor NS-398 (Fig. 3B). Finally, we used NS-398 and dexamethasone, which selectively inhibit COX-2 activity and transcription (Gallois et al., 1998), respectively, in order to determine the role of COX-2 in the growth inhibitory effect of ZR. As shown in Fig. 5, both agents blunted the antiproliferative effect of ZR. Taken together, these results suggest that ZR inhibits the growth of hMF by a pathway involving both early PGE2 and cAMP productions, as well as delayed COX-2 induction.

Discussion

Proliferation of myofibroblasts is central for the development of liver fibrosis during chronic liver diseases. The present study shows that the growth of hMF is inhibited by ZR and constitutes the first evidence for an antiproliferative effect of this extracts. These data suggest that ZR may play a key role as a negative regulator of liver fibrogenesis.

In this work, we studied the effects of ZR on the growth of hMF cells. We showed that ZR decreases proliferation of hMF, as shown by the colorimetric determination of hMF growth. These results are confirmed by measurement of actual cell growth and by autoradiography experiments.
Two lines of evidence argue against a toxic effect of ZR: 1) There were no morphological alteration of cells at microscopic examination, and 2) the inhibitory effect was totally reversed after ZR removal. Our results are in keeping with the growth-inhibitory effects of ZR observed in human fibroblasts (data not shown). The signaling pathway involved in the antiproliferative of ZR toward hMF has not been investigated in this study. Cell growth inhibition was significantly enhanced by addition of increasing concentrations of ZR. Recently, several reports presented that natural dietary plants may play an antioxidative role in the prevention of aging and carcinogenesis and may offer effective protection from lipid peroxidative damage in vitro and in vivo (Galli and Costa, 1995; Hamelmann et al., 1999). Therefore, much attention has been focused on natural antioxidants, in particular it was reported the ZR may exert an anti-aging action and anticancer activity [data not shown], although little is yet known about the pharmacological effects or active ingredients. When the growth inhibitory effect of a water soluble extract from the ZR was investigated using hMF cells, the herbal medicine directly inhibited the proliferation of hMF cells, and induced cell death in hMF cells, in a dose-dependent manner. Therefore, a major point of the present study is that ZR exhibits potent antiproliferative effects in hMF, which contrasts with its mitogenic properties in many other cells. Whether this growth inhibitory effect of ZR is linked to an intracellular effect or to its plasma membrane receptors remains to determine, although both effects are not mutually exclusive. In hMF, several lines of evidence argue for an intracellular mechanism for ZR action. First, growth inhibitory effect of ZR is observed at microgram concentrations.

Growth inhibitory properties of ZR on hMF rely on a novel signaling pathway that involves both early and delayed production of PGE2. Rapid production of PGE2 is ensured by constitutive COX-2, as shown by the dramatic reduction of basal PGE2 production by the selective COX-2 inhibitor NS-398. These data suggest that, although COX-1 is constitutively expressed in hMF, the major part of PGE2 levels found in basal conditions derives from constitutive COX-2. The rapid increase in PGE2 production in turn leads to elevation of cAMP, a growth inhibitory mediator that blocks early events involved in hMF proliferation (Mallat et al., 1996). Moreover, in these cells, PGE2 and cAMP show antiproliferative properties (Davaille et al., 2000). Thus, it is interesting to study whether ZR could provoke growth arrest. In hMF, ZR also ensures delayed PGE2 secretion, following COX-2 induction. These data constitute the first description of a regulation of COX-2 by ZR. Blocking COX-2 by either dexamethasone or NS-398 abrogates the antiproliferative effect of ZR, indicating that COX-2 mediates the growth inhibitory effect of ZR. Recent studies indicated that activation of COX-2 is a key event in the inhibition of hMF proliferation (Davaille et al., 2000). It was shown that the mitogenic effects of PDGF-BB and thrombin result from a balance between a promitogenic and a COX-2-dependent growth inhibitory pathway (Mallat et al., 1998). Therefore, the present data further support a central role of COX-2 in hMF growth inhibition. Early COX-2-dependent cAMP will block early steps involved in hMF proliferation, such as extracellular signal-regulated kinase and c-Jun NH2-terminal kinase activations (Mallat et al., 1996). The consequences of COX-2 induction by ZR remain to be determined but may involve regulation of more distal events, such as cell cycle components.

The establishment of an effective treatment for hepatic fibrosis in Korea is urgently required, because the death rate for liver fibrosis or cirrhosis in this country is still high. Mixed natural herbal medicines have been used clinically to treat hepatic fibrosis patients, or they have been used as adjuvant therapy, because they are associated with minimal side effects and can also decrease the side effects of chemotherapy and/or radiotherapy (Inuma et al., 2003). Oka et al. (1995) reported that the mixed herbal
medicines, Sho-saiko-to, prevented the development of hepatocellular carcinoma in patients with cirrhosis. Furthermore, it was also reported that this medicine could inhibit the proliferation of cancer cell lines by the induction of apoptosis and arrest at the G0/G1 phase (Yano et al., 1994). Furthermore, Shimizu et al. (1999) have reported that Sho-saiko-to reduces the deposition of type I collagen and the number of α-smooth muscle actin positive-stellate cells in the liver and inhibited, not only lipid peroxidation in cultured rat hepatocytes and stellate cells. Among the active components of Sho-saiko-to, baicalin and baicalein were found to be mainly responsible for the antioxidative activity. It was also shown that the traditional Chinese medicinal herb, *Salvia miltiorrhiza* (Dan-Shen in Chinese), reversed the fibrosis caused by carbon tetrachloride treatment in a rat experimental model of hepatic fibrosis (Wasser et al., 1998). Rats treated with the herb had reduced levels of transforming growth factor-beta1, procollagens I and III and tissue inhibitor of metalloproteinase-1 transcripts and an increased level of matrix metalloproteinase-13 transcript. These findings suggest that certain kinds of natural herbal medicines such as Sho-saiko-to and *Salvia miltiorrhiza* function as potent antifibrosuppressants, and may be effective in the treatment of patients with liver fibrosis or cancer, or may prevent the development of liver fibrosis or clinical cancer.

ZR has been used in traditional Chinese medicine for treating liver, lung and rectal tumors. Thus, as a another experiments carried out, the effects of aqueous extracts of the herb on benzo[a]pyrene 7,8-dihydrodiol. (BaP 7,8-DHD) and benzo[a]pyrene 7,8-dihydrodiol-9,10-epoxide (BPDE)-induced mutagenesis using *Salmonella typhimurium* TA100 as the bacterial tester strain and rat liver 9000 × g supernatant (S9) as the metabolic activation system were reported previously (Wong et al., 1992). We also determined the effects of ZR on BaP 7,8-DHD and BPDE binding to calf thymus DNA. Organosoluble metabolites of BaP 7,8-DHD and water-soluble conjugates of BaP 7,8-DHD and BPDE were analyzed by high-performance liquid chromatography and alumina column liquid chromatography (data not shown). Mutagenesis assays revealed that the herb produced a significant concentration-dependent inhibition of histidine-independent (His+) revertants induced by BaP 7,8-DHD and BPDE. ZR also inhibited BPDE-induced mutagenesis in a concentration-dependent manner in the absence of S9. ZR significantly inhibited BaP 7,8-DHD and BPDE binding to DNA. ZR also inhibited the formation of organosoluble metabolites of BaP 7,8-DHD and decreased the formation of water-soluble conjugates of BaP 7,8-DHD and BPDE. However, the fraction of the total radioactivity in the watersoluble conjugates present as sulfate and glutathione was increased by ZR. Glucuronide fraction was decreased. The results of this study suggested that the Chinese medicinal herb possess antimutagenic properties and further suggest that they act as blocking agents through a scavenging mechanism.

In summary, the present study brings out two major findings, a novel role for ZR as a growth inhibitory natural extracts, via COX-2 activation. At present, our data rather favor the hypothesis of an intracellular antiproliferative action of ZR. Accumulation of hepatic myofibroblasts is central in the development of liver fibrosis, as shown in experimental models of liver injury and in human chronic liver diseases (Borkham-Kamphorst et al., 2004). Therefore, our results point to a potential mechanism by which the herbal medicine ZR acts a potential medicine for treatment of liver fibrosis.

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References


