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Shikonin Suppresses the Migratory Ability of Hepatocellular Carcinoma Cells

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ABSTRACT: Shikonin is a traditional Oriental medical herb extracted from *Lithospermum erythrorhizon*. Many studies have shown that shikonin possesses anticancer ability against many different cancers, including hepatocellular carcinoma (HCC). Recently, tumor metastasis has been become an important clinical obstacle. However, the effect of shikonin on metastasis by HCC is unknown. The 50% inhibitory concentration (IC_{50}) of shikonin on HCC cells was determined by an MTT assay and the xCELLigence biosensor system. The migratory ability of HCC cells was detected by a transwell migration assay and the xCELLigence biosensor system. Matrix metalloproteinase-2 and -9 (MMP-2 and -9) expression levels were determined by Western blotting, and the activities of MMP-2 and -9 were determined by gelatin zymography. We found that IC_{50} values of HepJ5 and Mahlavu cells to shikonin treatment were around 2 μM. Exposure to a low dose of shikonin (0−0.4 μM) did not influence the survival of HCC cells. Interestingly, exposure to a low dose of shikonin inhibited the migratory ability on HepJ5 and Mahlavu cells. To further dissect the mechanism, we found that treatment with a low dose of shikonin reduced the activities and expression levels of MMP-2 and -9, which were correlated with the decreased cell migratory ability of HCC cells. In addition, we found a decrease of vimnetin expression, but no influence on the expression levels of *N*-cadherin, TWIST, or GRP78. In mechanism dissecting, we found that shikonin treatment may suppress the phosphorylation of AKT and then reduce the NF-κB (NF = nuclear factor) levels, but has no influence on the levels of c-Fos and c-Jun. Furthermore, we also found that shikonin may also reduce the phosphorylation of IκB. We concluded that a low dose of shikonin can suppress the migratory ability of HCC cells through downregulation of expression levels of vimentin and MMP-2 and -9. Our findings suggest that shikonin may be a new compound to prevent the migration of HCC cells.

KEYWORDS: *HCC, migration, metastasis, EMT, vimentin*

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common liver malignancy and causes more than a quarter of a million deaths each year throughout the world.[1,2] HCC is a complex and heterogeneous tumor with frequent intrahepatic spread and extrahepatic invasion, metastasis, and recurrence even after surgical resection, resulting in a poor prognosis.[3] Intrahepatic and extrahepatic metastases take place in more than 50% of patients after excision of HCC according to most published papers, with intrahepatic metastasis more frequently taking place.[4−6] Common sites of extrahepatic metastasis include the lungs, peritoneum, spleen, bones, and lymph nodes.[4−6] HCC invasiveness was correlated with the capacity of tumor cells to invade the portal vein and capsule.[7,8]

Metastasis is a process by which malignant cells move away from the primary tumor through blood or lymphatic vessels and produce tumors at a secondary site. Metastasis takes place as a complex multistep procedure involving adhesion, invasion, and migration of cancer cells.[9] The steps of metastasis require degradation of extracellular matrix (ECM) constituents via proteolytic enzymes.[10] Among these proteolytic enzymes, matrix metalloproteinases (MMPs), including MMP-2 and -9, are the chief ECM-degrading enzymes.[11] It was found that MMP-2 and -9 have the capability to degrade type IV collagen, which is the main constituent of basement membranes. Degradation of basement membranes is regarded as necessary for tumor invasion and metastasis.[11,12] Therefore, MMP

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protein expressions are thought to be favorable targets for anticancer drugs.[13] Consequently, inhibition of MMP activity is an important event for preventing early-stage carcinogenesis, particularly tumor promotion.

Shikonin is a major naphthoquinone compound of the root of *Lithospermum erythrorhizon*, a traditional Oriental medicinal herb. We previously reported the antitumor effects of shikonin on hepatoma SK-Hep-1 cells through the generation of large amounts of intracellular reactive oxygen species (ROS) during the early stage of the apoptotic process, which was subsequently accompanied by disturbance of the mitochondrial transmembrane potential.[14] Recent studies also illustrated that shikonin has significant antihepatoma potential, inducing inhibition of cell growth and proteasomes and inducing cell death and apoptosis in various types of hepatoma cell lines.[15−17] An apoptotic effect induced by shikonin was demonstrated in other hepatoma cells such as Hep G2, Huh7, and BEL7402.[15,17] Generation of ROS resulted in downregulation of AKT, and RIP1/NF-κB (NF = nuclear factor) activity is also involved in shikonin-induced apoptosis.[15] Those studies suggested that shikonin is a potential agent for HCC treatment. However, the role of shikonin in antimetastasis of HCC cells has not yet been well studied.

In this study, we demonstrated that a nontoxic dose of shikonin treatment suppresses the migratory ability of HCC cells. The possible mechanism may be due to the suppression of expression or activity of MMP-9 through the inhibition of the NF-κB signaling pathway and a decrease of the vimentin expression level.

MATERIALS AND METHODS

Chemicals, Reagents, and Cell Cultures. Chemicals (Triton X-100, Tris−HCl, trypan blue EDTA, ribonuclease-A, dimethyl sulfoxide (DMSO), and shikonin (product no. S7576)) were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies targeted to AKT, phospho-AKT, NF-κB phospho-IκB, IκB, MMP-2, MMP-9, *N*-cadherin, GRP78, TWIST, vimentin, actin, tubulin, and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HCC (HepJ5 and Mahlavu) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Grand Island, NY) with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum (Gibco Life Technologies) and 2% penicillin−streptomycin (10 000 U/mL penicillin and 10 mg/mL streptomycin) in a 5% CO_2 humidified incubator at 37 °C as previously described.[18,19]

Cell Viability Assay. Cells were plated at 2×10^4 cells per well in 24-well plates and incubated overnight in a 37 °C incubator containing 5% CO_2. Cells were incubated with different concentrations of shikonin (0−50 μM) to determine the 50% inhibitory dose (IC_{50}) or DMSO as a vehicle control for specific time periods. At selected time intervals, the medium was aspirated. The remaining cells were further incubated with 0.25 mg/mL MTT for 1 h and then extracted with DMSO, and the color change in the extract was measured at 550 nm with a spectrophotometer (GE Healthcare, Piscataway, NJ).

Cell Proliferation Assayed with the xCELLigence Biosensor System. Experiments were carried out in an RTCA DP instrument (Roche Diagnostics, Mannheim, Germany) that was placed in a 5% CO_2 humidified incubator maintained at 37 °C as previously described.[20] Growth curves were constructed using 16-well plates (E-plate 16, Roche Diagnostics). Cells were seeded in an E-plate 16 at 5000 cells per well in 10% fetal calf serum (FCS)-containing medium (four different wells per sample). The plate was then monitored once every 30 s for 4 h and once every 1/2 h thereafter. Data were analyzed using RTCA software 1.2 (supplied with the instrument).

Transwell Migration Assay. The in vitro cell migration was assayed in a BD Falcon cell culture insert (BD Biosciences, San Jose, CA) as previously described.[19−21] Aliquots of cells suspended in serum-free DMEM were seeded into the upper part of each chamber, and the lower compartments were filled with 1 mL of DMEM containing 10% FCS. After incubation for 24 h at 37 °C in 5% CO_2, nonmigrating cells were mechanically removed from the upper surface of the membrane. Cells on the reverse side were stained with 0.1% crystal violet and counted under a microscope at 100× magnification.

Cell Migratory Ability Assayed with the xCELLigence Biosensor System. Experiments were carried out in an RTCA DP instrument (Roche Diagnostics) that was placed in a 5% CO_2 humidified incubator maintained at 37 °C as previously described.[20] Cell migration was assessed using specifically designed 16-well plates (CIM-plate 16, Roche Diagnostics) with 8 μm pores. These plates were similar to conventional transwell plates, with microelectrodes located on the underside of the membrane of the upper chamber. The 10% FCS medium was added to the lower chamber, and 20 000 cells per well were seeded in the upper chamber in serum-free medium (four different wells per sample). The CIM-plate 16 was monitored every 10 s for 40 min and once every hour thereafter. The data were analyzed using RTCA software 1.2.

Assay of MMP-9 and -2 Activities Using Gelatin Zymography. After treatment, the conditioned media were collected and used to assess MMP-9 and -2 activities. The protein concentration was assayed using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA), and samples were mixed with equal volumes of 2× sodium dodecyl sulfate (SDS) sample buffer. Samples were incubated for 5 min at room temperature and electrophoresed on 7.5% SDS−polyacrylamide gel electrophoresis (PAGE) gels containing 2 mg/mL gelatin as a substrate at a constant voltage and 4 °C. After electrophoresis, the gels were rinsed in renaturation buffer (2.5% Triton X-100) on a shaker for 30 min to remove SDS at 4 °C and then incubated overnight at 37 °C in a water bath in activation buffer composed of 50 mM Tris−HCl (pH 7.4) and 5 mM $CaCl_2$. Gels were stained using 0.5% Coomassie blue R-250 for 2 h, followed by appropriate destaining. MMP-9 and -2 activities were detected as a white band on a dark-blue background and quantitated by densitometry.

Silenced Vimentin Expression in HCC. The inhibition of vimentin expression in HCC cells was used shRNA clones purchased from the National RNAi Core Facility (Taiwan, ROC). The target sequence for the human vimentin mRNA (NCBI Reference Sequence code NM_003380) gene was 5′-GCTAACTACCAAGACACTATT-3′ as previously described.[20,21] The nontarget shRNA control vector (SHC002) was purchased Sigma Chemical Co., and the sequence of scrambled shRNA was 5′-CAACAAGATGAAGAGCACCAA-3′. Briefly, 1.5×10^5 cells were washed twice with PBS and mixed with 0.5 μg of plasmid. One pulse was applied for 20 ms under a fixed voltage of 1.4 kV on a pipet-type microporator, Neon (Life Technologies).[22] After 48 h, the cells were harvested for detecting the protein level of vimentin by Western blot analysis and performing the transwell migration assay.

Western Blot Analysis. After treatment, cells were washed with phosphate-buffered saline (PBS), resuspended in a protein extraction buffer for 10 min, and centrifuged at 12000*g* for 10 min at 4 °C to obtain the extracted proteins (supernatant). Protein concentrations were measured with a Bio-Rad protein assay reagent. The extracted cellular proteins were boiled in loading buffer, and an aliquot corresponding to 50−100 μg of protein was separated on a 12% SDS−polyacrylamide gel. After electrophoresis, the proteins were electrotransferred onto a poly(vinylidene fluoride) transfer membrane. After blotting, the membranes were incubated with various primary antibodies overnight and then washed with a PBST solution (0.05% Tween 20 in PBS). Following washing, the secondary antibodies, which were labeled with horseradish peroxidase, were added to the membrane for 1 h and then washed with a PBST solution (0.05% Tween 20 in PBS). The antigen−antibody complexes were detected by enhanced chemiluminescence (GE Healthcare) and detected by VersaDoc 5000 (Bio-Rad Laboratories).[18,19,23,24]

RT-PCR and Quantitative RT-PCR Analysis. Total RNA was extracted from cells using the TRIZOL reagent as recommended by

the manufacturer (Invitrogen). cDNA was amplified from 2 μg of total RNA in a final volume of 20 μL using M-MLV (Moloney murine leukemia virus) reverse transcriptase at 37 °C for 90 min. The primers for real-time polymerase chain reaction (RT-PCR) were as follows: MMP-2 forward, 5′-CTGCGGTTTTCTCGAATCCA-3′; MMP-2 reverse, 5′-GGGTATCCATCGCCATGCT-3′; MMP-9 forward, 5′-CCCTGGAGACCTGAGAACCA-3′; MMP-9 reverse, 5′-CCACC-CGAGTGTAACCATAGC-3′). The quantitative RT-PCR was carried out using ABI SYBR Green Master Mix, and thermal cycling was performed using the ABI StepOne (Applied Biosystems) and the following quantitative PCR protocol: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.[18,19]

Statistical Analysis. All collected data are expressed as the mean ± standard deviation (SD) of at least three separated experiments. The data presented in some figures are from representative experiments, which were quantitatively similar to the data from the replicate experiments. Statistical significance was determined with Student's *t*-tests (two-tailed) comparing two data sets. Asterisks shown in the figures indicate significant differences between the experimental group and the corresponding control condition ($p < 0.05$; see the figure captions).

RESULTS

Determination of the Anticancer Effect of Shikonin on HCC Cells. Several studies have shown that shikonin possesses anticancer effects against different cancers, including HCC.[15,16] First, we determined the anticancer effects of shikonin on HepJ5 and Mahlavu cells. As shown in Figure 1a, shikonin showed an anticancer ability against HepJ5 and Mahlavu cells in a dose-dependent manner. IC_{50} values of HepJ5 and Mahlavu cells with shikonin treatment were around 2 μM. To further confirm the cytotoxic effect of shikonin, we performed a cell proliferation assay using a biosensor system and found that low concentrations of shikonin (<0.4 μM) had no influence on the survival of HepJ5 and Mahlavu cells (Figure 1b). These results indicated that the cytotoxic concentration of shikonin is >0.4 μM.

A Low Dose of Shikonin Suppressed the Cancer Migratory Ability. To understand whether shikonin possesses an antimigratory effect on HCC, we further treated HepJ5 and Mahlavu cells with low concentrations of shikonin (0−0.4 μM), and the migration abilities of HepJ5 and Mahlavu cells were assessed by transwell migration and the xCELLigence biosensor system. As shown in Figure 2a, the number of migrating HepJ5 cells dramatically decreased after they were treated with a nontoxic concentration of shikonin. This experiment was performed using a biosensor system, and we indeed found that low doses of shikonin inhibited the migratory ability of HepJ5 cells (Figure 2b). We further confirmed the anti-migratory effect of low doses of shikonin in Mahlavu cells. As shown in Figure 2c,d, similar results were found in Mahlavu cells. These results suggest that a nontoxic dose of shikonin possesses antimigratory effects.

Effects of Shikonin on MMP-9 and -2 Activities in Hep J5 Cells. To evaluate the inhibition of MMP-9 and -2 activities by shikonin, HepJ5 cells were treated with 0.4 μM shikonin for 3, 6, 12, and 24 h, and conditioned media were collected and analyzed by zymography. As shown in Figure 3a, MMP-9 activity was inhibited 0.89- and 0.88-fold compared to the untreated group at 12 and 24 h in HepJ5 cells. MMP-2 activity was only inhibited 0.94-fold at 24 h in shikonin-treated Hep J5 cells.

Effects of Shikonin on MMP-9 and -2 Expressions by Hep J5 Cells. Because shikonin inhibited MMP-9 and -2 activities by Hep J5 cells, we further evaluated expressions of

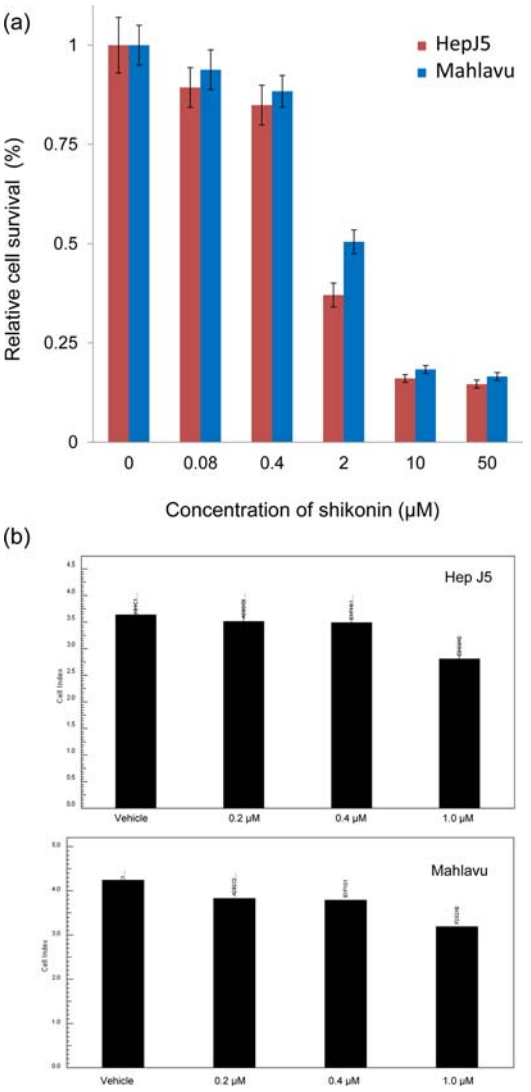

Figure 1. Antiproliferative effect of shikonin on HCC cells. (a) HCC (HepJ5 and Mahalvu) cells were incubated with different concentrations of shikonin (0−50 μM) for 48 h. An MTT assay was performed to determine the rate of cell survival. The *y*-axis indicates the relative cell survival rate (the value of the vehicle group was set to 100%). (b) Concentrations not toxic to HCC cells were determined using the xCELLigence biosensor system. HepJ5 and Mahlavu cells were incubated with shikonin (0−1 μM) on an E-plate 16. The plate was then monitored once every 30 s for 4 h and once every 1/2 h thereafter. Data were analyzed using RTCA software 1.2 (supplied with the instrument). All experiments were independently performed at least three times in triplicate. An asterisk indicates $p < 0.05$.

MMP-9 and -2 in shikonin-treated Hep J5 cells by real-time PCR and Western blotting. As shown in Figure 3b, the transcript levels of MMP-9 were suppressed in a time-dependent manner, but with no influence on the MMP2 transcription levels. MMP-9 expression was inhibited 0.83-fold at 3 h of shikonin treatment, and the inhibition continued to 24 h. Shikonin treatment also slightly inhibited the expression of MMP-2 at various treatment time points.

Effects of Shikonin on Expressions of Migration-Related Proteins in Hep J5 and Mahlavu Cells. To determine whether the decrease in the migratory ability after shikonin treatment was due to the influence of EMT-related marker expressions, we further checked the status of cell

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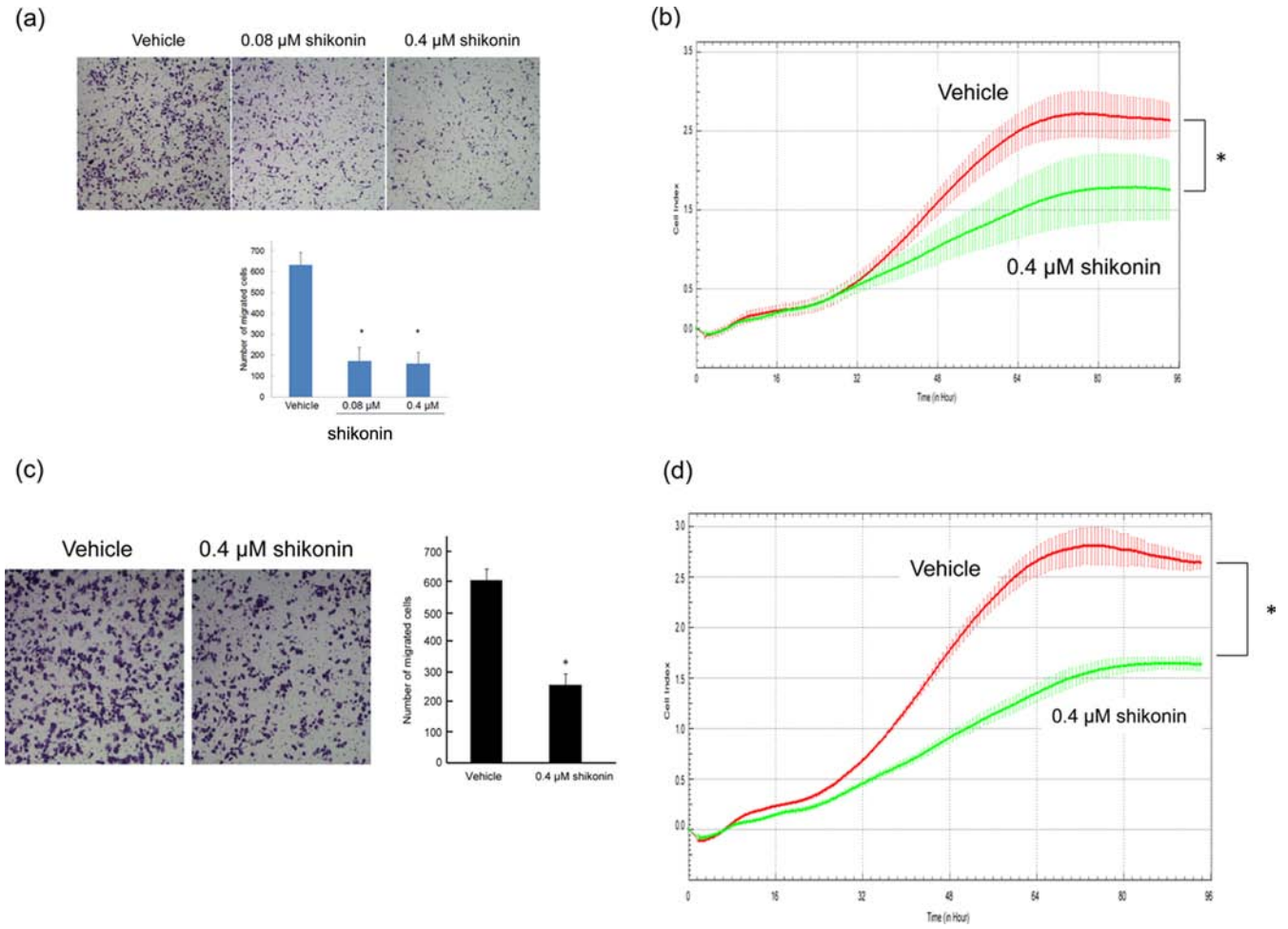

Figure 2. Shikonin suppressed the migratory ability of HCC cells. The migratory ability was determined by a transwell migration assay and the xCELLigence biosensor system. (a) HepJ5 cells were treated with shikonin (0.4 μM) in the inner wells. Migrating cells were calculated after being fixed and stained with crystal violet as described in the Materials and Methods. All experiments were independently performed in triplicate. (b) In the biosensor system, migrating cells were indicated by the cell index. Data were analyzed using RTCA software 1.2 (supplied with the instrument). (c) Mahlavu cells were treated with shikonin (0.4 μM) in the inner wells. Migrating cells were calculated after being fixed and stained with crystal violet as described in Materials and Methods. All experiments were independently performed in triplicate. (d) In the biosensor system, migrating cells were indicated by the cell index. Data were analyzed using RTCA software 1.2 (supplied with the instrument). Experiments were repeated four times. An asterisk indicates $p < 0.05$.

migration-related proteins (vimentin, TWIST, *N*-cadherin, and GRP78) by Western blotting. As shown in Figure 4a, there were no significant differences in the expression levels of TWIST, *N*-cadherin, and GRP78 between shikonin treatment and vehicle control cells. However, the expression level of vimentin was decreased dramatically after shikonin treatment. To further confirm the suppression effect of shikonin in vimentin expression, cells were treated with different amounts of shikonin for 24 h. The levels of vimentin were reduced in a dose-dependent manner (Figure 4b). To further understand whether downregulation of vimentin may contribute to the decrease of migratory ability, we further knocked down the vimentin expression by the shRNA system. As shown in Figure 4c, a decrease of vimentin expression reduced the migratory ability in HepJ5 cells. These results indicate that the decrease in the migratory ability after shikonin treatment might be due to the downregulated vimentin expressions.

Shikonin Treatment Reduced the NF-κB-1 Levels but Not the AP-1 Level. The previous study indicated that transcription factors NF-κB and AP-1 have been reported to control MMP-9 gene expression. We future determined what kind of transcription factors are involved when shikonin reduces the expression levels of MMP-2 and -9. We found that shikonin treatment caused the reduction of NF-κB-1 levels but had no influence on c-Fos and c-Jun (Figure 5a,b). To further dissect the upstream molecules, we checked the status of AKT and phospho-AKT by Western blotting. As shown in Figure 5c, we found that shikonin treatment did not change the protein amount of AKT, but reduced the phosphor-AKT amount. To investigate whether shikonin inhibited the NF-κB signaling pathway, we further checked the phosphorylation state of IκB. As shown in Figure 5d, shikonin treatment did not influence the level of IκB, but decreased the phosphorylation state of IκB. Those results indicate that shikonin reduced the levels of MMP-2 and -9 through suppression of AKT activation and inhibition of the NF-κB signal pathway.

DISCUSSION

Many anticancer drugs have strong cellular cytotoxicity and side effects. Strong anticancer drugs that have no or minimal cytotoxicity and side effects need to be developed. Metastasis and invasion are basic characteristics of malignant cancer cells.

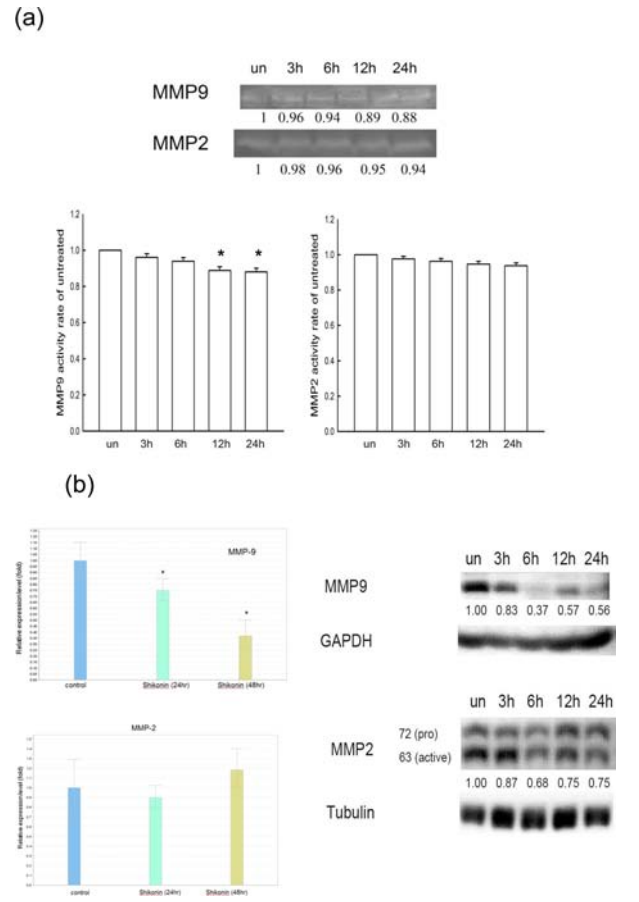

Figure 3. Shikonin treatment suppressed the activities and expression levels of MMP-2 and -9. (a) Activities of MMP-2 and -9 in shikonin-treated HepJ5 cells were determined by zymography as described in the Materials and Methods. (b) Expression levels of MMP-2 and -9 in shikonin-treated HepJ5 cells were determined by real-time PCR and Western blotting. Experiments were repeated four times. An asterisk indicates $p < 0.05$.

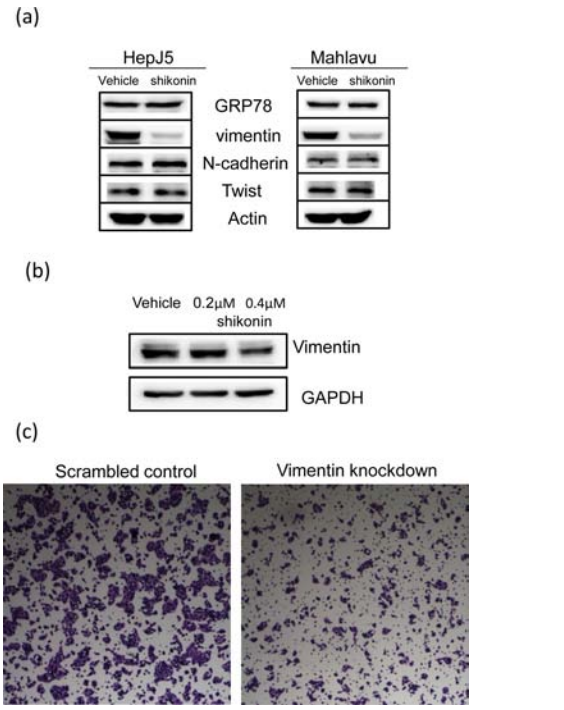

Figure 4. Shikonin treatment suppressed the vimentin expression level, but did not influence the expression levels of *N*-cadherin, TWIST, or GRP78. (a) HCC (HepJ5 and Mahlavu) cells were treated with 0.4 μM shikonin for 24 h. Cells were harvested, and the levels of *N*-cadherin, TWIST, vimentin, and GRP78 were analyzed by Western blotting. (b) HepJ5 cells were treated with 0.2 or 0.4 μM shikonin for 24 h, and the level of vimentin was analyzed by Western blotting. (c) The scrambled control shRNA and vimentin shRNA were transfected into HepJ5 cells by electroporation. After 48 h, the migratory abilities of the scrambled control and vimentin shRNA HepJ5 cells were determined by transwell migration assay. Experiments were independently repeated four times.

Some proteolytic enzymes contribute to these processes, which include degradation of environmental barriers such as the basement membrane and the ECM.[25] The MMP family was shown to play a critical role in the proteolysis of various constituents of the ECM. MMP-2 and -9 of the MMP family are directed to degrade type IV collagen, which is a major component of the basement membrane during cancer invasion and metastasis, and they are expressed by various tumor cells.[26−30] It was demonstrated that the expression of MMP-9 is related to the invasion and progression of cancer.[31] Those studies resulted in research and development of MMP inhibitors to inhibit tumor metastasis. Several novel MMP inhibitors have been developed and are being studied in clinical trials.[32,33]

MMP-2 and -9 are directly involved in human hepatic tumorigenesis and metastasis. This seems to indicate that inhibitors of MMP-2/9, which have dual inhibitory activities on enzyme activities and transcription, may be the best candidates for achieving tumor regression.

Many cancer cells control the expression of MMP-9 by modulating activation of the transcription factor NF-κB through the Ras/Raf/ERK, JNK, and PI-3K/AKT signaling pathways,[34−40] since the promoter region of MMP-9 has NF-κB-binding sites.[11] NF-κB was found to regulate the expressions of some genes, the products of which are involved in tumorigenesis.[41,42] NF-κB can induce activation of MMP-9 and cyclooxygenase 2 (COX-2).[41,42] Thus, several agents able to suppress NF-κB activation have the potential to suppress tumorigenesis and metastasis and have shown therapeutic potential. Min et al. demonstrated that shikonin inhibited tumor invasion via downregulation of NF-κB-mediated MMP-9 expression in human high-metastatic adenoid cystic carcinoma cells.[43] Downregulation of AKT and RIP1/NF-κB activity was found to be involved in shikonin-induced apoptosis in human HCC.[15] Our results demonstrated that shikonin significantly inhibited the activity and expression of MMP-9. Down-regulation of NF-κB-mediated MMP-9 expression may be involved in the metastatic inhibition induced by shikonin in HCC. The interesting obervation is that the expressions of MMP-9 and MMP-2 were lowest at 6 h. The possible explanation is that shikonin treatment may increase the amounts of intracellular ROS during the early stage in human HCC cells.[14] Overproduction of ROS can inhibit or damage many intracellular enzyme expressions. The expressions of MMP-9 and MMP-2 were lowest at 6 h and may be the result of ROS production induced by shikonin.

Herein, we found that shikonin could suppress the migratory ability of HCC cells through suppression of MMP-9 activity at nontoxic doses and a decrease of the expression of vimentin, but had no influence on the expression levels of GRP78,

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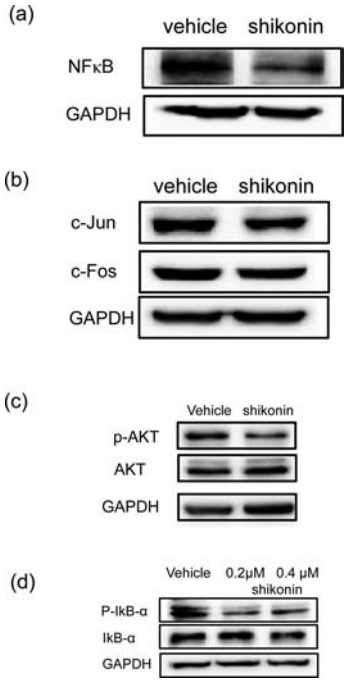

Figure 5. Shikonin treatment influences the expression levels of the NF-κB and AKT pathway but not the AP-1 complex. HepJ5 cells were treated with 0.4 μM shikonin for 24 h. Cells were harvested, and the levels of NF-κB (a), c-Fos and c-Jun (b), and AKT and phospho-AKT (c) were analyzed by Western blotting. (d) Cells were treated with with 0.2 or 0.4 μM shikonin for 24 h, and the level of phospho-IκB and IκB was analyzed by Western blotting.

TWIST, or *N*-cadherin. Our findings indicate that shikonin may be a potential antimetastatic compound in HCC. In addition, we were also interested in seeing whether shikonin also possessed the same effects in different cancer cells. However, we did not see the same effect on prostate cancer cells (data not shown). These results suggest that the suppressive effect of shikonin may be tissue oriented. Currently, we do not know whether a nontoxic dose of shikonin can inhibit the migratory ability of other cancers. This issue is worth further examination.

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Notes

The authors declare no competing financial interest.

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