Shikonin Induces Apoptotic Cell Death via Regulation of p53 and Nrf2 in AGS Human Stomach Carcinoma Cells

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Abstract
Shikonin, which derives from Lithospermum erythrorhizon, has been traditionally used against a variety of diseases, including cancer, in Eastern Asia. Here we determined that shikonin inhibits proliferation of gastric cancer cells by inducing apoptosis. Shikonin's biological activity was validated by observing cell viability, caspase 3 activity, reactive oxygen species (ROS) generation, and apoptotic marker expressions in AGS stomach cancer cells. The concentration range of shikonin was 35-250 nM with the incubation time of 6 h. Protein levels of Nrf2 and p53 were evaluated by western blotting and confirmed by real-time PCR.

Our results revealed that shikonin induced the generation of ROS as well as caspase 3-dependent apoptosis. c-Jun-N-terminal kinases (JNK) activity was significantly elevated in shikonin-treated cells, thereby linking JNK to apoptosis. Furthermore, our results revealed that shikonin induced p53 expression but repressed Nrf2 expression. Moreover, our results suggested that there may be a co-regulation between p53 and Nrf2, in which transfection with siNrf2 induced the p53 expression. We demonstrated for the first time that shikonin activated cell apoptosis in AGS cells via caspase 3- and JNK-dependent pathways, as well as through the p53-Nrf2 mediated signal pathway. Our study validates in partly the contribution of shikonin as a new therapeutic approaches/agent for cancer chemotherapy.

Key Words: Shikonin, p53, Nrf2, ROS, JNK, Apoptosis

INTRODUCTION
Similar to radiotherapy treatments, many anti-cancer drugs are commonly associated with adverse drug effects (ADEs) and often have serious side effects. Furthermore, the high rates of recurrence after resection necessitate the use of alternative anti-cancer agents with fewer and less serious ADEs. Phytochemicals has been highlighted by recent studies as natural anti-cancer agents with little toxicity by acting against the propagation of cancer cells (Filomeni et al., 2005a).

Shikonin (Fig. 1A), a naphthoquinone isolated from the traditional herbal medicinal plant Lithospermum erythrorhizon, has significant pharmacological activities including anti-tumor, antimicrobial, anti-inflammatory, and wound healing activities (Lee and Lin, 1988; Chen et al., 2002). Traditional medicine composed of naphthoquinone has been proven to be effective in breast cancer (Liu et al., 2005; Leung and Fong, 2007) as well as been used for patients with liver and lung cancer (Liu et al., 2005). The results of recent studies also suggested that shikonin has a significant anti-proliferative action against various types of cancer cell lines. Shikonin has also been shown to have the potential to regulate the expression of p53 in association with proapoptotic proteins (Hsu et al., 2004; Wu et al., 2004; Mao et al., 2008).

Apoptosis is a type of programmed cell death characterized by physiological and morphological changes in the affected cells, and many chemotherapies target the cellular mechanisms of apoptosis and other cell death pathways. Apoptosis is known to be triggered by various internal and external stimuli, one of them being reactive oxygen species (ROS). ROS create a condition of oxidative stress that can induce a cellular response (Filomeni et al., 2005b, Matsuzawa and Ichijo,

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The results of recent studies indicate a possible association between Nrf2 and apoptotic regulation by p53 as well as the possibility of cross-talk between the Nrf2- and p53-induced responses (Faraonio et al., 2006).

Our past study has already shown that shikonin elicits antiproliferative activity on AGS cells via induction of G2/M cell cycle arrest and regulation of the Egr1-p21 signaling pathway (Kim et al., 2014a). In this study, we investigated whether there is an association between the antiproliferative activity of shikonin on stomach cancer cells and induction of p53 and Nrf2 expressions. In order to examine the role of shikonin in the induction of apoptotic cell death, we have isolated shikonin from Lithospermum erythrorhizon (unpublished data). Our results showed that shikonin treatment induced p53 expression and accumulation of excessive ROS, while simultaneously leading to the inhibition of Nrf2 expression. These findings suggest that shikonin may be able to enhance the effectiveness of chemotherapy.

MATERIALS AND METHODS

Cell culture, reagents, and antibodies

The human gastric adenocarcinoma cell line AGS (p53 wild type) and Kato 3 (p53 null type) (Korean Cell Line Bank, Seoul, Korea) were cultured in RPMI 1640 medium (WelGENE Inc., Daegu, Korea) supplemented with 10% fetal calf serum,
and 0.1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO₂. Antibodies against procaspases 3, cleaved caspase 3, cleaved PARP were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against β-actin, Nrf2, p53, phospho-JNK, JNK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FLAG and β-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). The anti-mouse or anti-rabbit secondary antibodies were purchased Cell Signaling Technology. Shikonin, Sp600125 and Ac-DEVD-CHO were obtained from Sigma-Aldrich.

**MTT assays to measure cytotoxicity**

Cell viability was determined by the MTT assay according to established protocols. The cells were dispensed in 96-well flat bottom microtiter plates (SPL, Pocheon, Gyeonggi, Korea) at a density of 5×10^3 cells per well, treated with various concentrations of shikonin for 6 h, following which, MTT solution (2 mg/mL) was added to each well and the cells incubated for a further 4 hr. After removal of the MTT solution 100 µL of DMSO was added to each well and absorbance was measured at 540 nm using a microplate spectrophotometer (Bio-Rad, Hercules, CA, USA).

**Caspase assays**

Caspase activation was evaluated using Caspase-Glo 3 kits (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Briefly, AGS cells were plated in 96-well clear-bottom plates (Lonza, Basel, Switzerland). The cells were treated with shikonin. After 6 h, assay reagent (100 µl) was added to each well. The plate was incubated in the dark for 30-60 min, and luminescence was measured using a SpectraMAX 250 Optima plate reader (Molecular Device Co., Sunnyvale, CA, USA).

**Measurement of intracellular ROS and oxidative damage**

The fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA, Sigma-Aldrich) was used to monitor the intracellular accumulation of reactive oxygen species (ROS). After treatment, the cells were incubated in RPMI-medium containing 6 µM of the probe at 37°C for 30 min, washed, and then analyzed by flow cytometry (BD, Franklin Lakes, NJ, USA). Malondialdehyde (MDA) levels were measured using a commercial assay kit (Cayman chemical, Ann Arbor, MI, USA) according to the manufacturer’s instructions. Proteins were determined by the method of Bradford (Kim et al., 2011b).

**Plasmid and siRNA transient transfection**

The ARE-luciferase reporter plasmid was generated using the pGL3-promoter vector (Promega). ARE sequence was cloned between the kpnI and SacI restriction sites upstream of the SV40 promoter (Akhdar et al., 2009). Down-regulation of Nrf2 was obtained by transient transfection with a small interference RNA (siRNA) against Nrf2 (Sigma-Aldrich).

**Luciferase assay**

Cells were then transfected with 100 ng/well ARE luciferase reporter construct together with 2.5 ng/well pRL-SV40 renilla luciferase construct (Promega) using 2 µL/well Lipofectamine™ 2000 (Invitrogen, Grand Island, NY, USA) and allowed to incubate for 24 h, following which the cells were treated with shikonin for 6 h. The cells were then harvested in passive lysis buffer and analyzed using a dual-luciferase reporter assay system on Zenyth multilabel plate reader (Anthos Lab, Heerhugowaard, North Holland, the Netherlands), following the manufacturer’s instructions. Relative light units of the p21 luciferase construct were normalized to those of the Renilla luciferase construct to control for transfection efficiency. Experiments were performed in triplicate.

**Western blotting**

Western blot analysis was carried out as previously described (Kim et al., 2012, Ko et al., 2014b). Cell pellet was re-suspended in RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS and protease inhibitors). Proteins were transferred onto a nitrocellulose membrane after SDS-PAGE. The membranes were blocked with 5% skim milk in TBST buffer (in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.02% Tween 20) for 1 h. Then, primary antibody incubation was performed overnight and followed by incubation with secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) for 1 h. Detection was done with the Enhanced Chemiluminoscence reagent (Santa Cruz Biotechnology).

**Immunofluorescence staining**

Immunostaining for the indicated proteins was performed as previously described (Shim et al., 2011, Ko et al., 2013). AGS cells were cultured on coverslips, fixed with 4% paraformaldehyde and permeabilized. Cells were stained with anti-rabbit polyclonal antibody (1:400) and with Alexa Fluor conjugated anti-rabbit antibody (1:500, Invitrogen) for 2 h at room temperature. The coverslips were mounted onto glass slides using mounting media containing 4’6-diamidino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA, USA), and examined under a Zeiss fluorescence microscope (Carl Zeiss, Jena, Germany).

**Reverse transcription PCR and Quantitative reverse transcription-PCR**

Total RNA was isolated and cDNA synthesis and RT-PCR analyses were carried out as described previously (Kim et al., 2011a). Real-time PCR reactions were performed in duplicate in the Light Cycler 480 PCR (Roche, Indianapolis, IN, USA) system following the manufacturer’s instructions, and relative quantification was carried out with 2^(-ΔΔCT) using the abundance of glyceraldehydes 3-phosphate dehydrogenase (GAPDH) as the endogenous house-keeping control.

**Statistical analysis**

Each experiment was duplicated at least three times. Data are plotted as the mean ± SD. The statistical significance of the assay was evaluated using SPSS software (SPSS, Chicago, IL, USA). Significance was set at p<0.05.

**RESULTS**

**Shikonin treatment induces cell death in gastric cancer cells**

We initially investigated the dose-dependent effects of shikonin treatment (0-250 nM for 6 h) on cell viability of stomach cancer cells. Our results of the MTT assay showed that induced cell death in AGS cells in a dose-dependent manner,
especially at or higher than 35 nM of shikonin treatment, in which the decreased cell viability was essentially not observed below that concentration. Interestingly, p53 null type cells showed strong resistance to shikonin, where Kato 3 cells did not progress to cell death even after being incubated in 250 nM of shikonin for 6 h (Fig. 1B). Also, we investigated whether expression levels of p53 and p53-dependent gene, phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), were changed by shikonin treatment. Shikonin-treated cells exhibited dose-dependent increase in the RNA expression levels of p53 and NOXA (Fig. 1C). As Bcl-2 family members occupy pivotal positions in the induction of apoptotic signal pathway, treating the cells with 100 ng/mL of shikonin induced Bax RNA level, but reduced that of Bcl2 (Fig. 1D). These results may possibly indicate the involvement of p53activity and apoptosis induction in the cytotoxic effects of shikonin.

**Shikonin-elicited apoptosis is caspase 3 dependent**

As shown in Fig. 1, shikonin treatment resulted in the induction of cell death as well as modulation of apoptotic genes in AGS cells. To further scrutinize whether shikonin induces the apoptotic signal pathway, we evaluated the levels of caspase 3 and poly (ADP-ribose) polymerase (PARP) proteins, which serve as biomarkers of apoptosis. Fig. 2 indicates that AGS cells underwent caspase-dependent apoptosis in a dose-dependent manner when incubated with shikonin for 6 h. The results from western blot analysis clearly showed that cleaved forms of PARP were effectively elevated and procaspase 3 reduced at 125-250 nM shikonin treatment (Fig. 2A). Also, exposure to shikonin increased caspase 3 activity (Fig. 2B). To further confirm these findings, we explored the roles of caspase 3 in shikonin-induced apoptosis by using cells pretreated with caspase 3 inhibitor Ac-DEVD-CHO (50 μM). These pretreated cells showed inhibited activities of proteolyzed forms of caspase 3 and PARP, indicating that the caspase 3 inhibitor inhibited shikonin induced apoptosis in AGS cells (Fig. 2C).

**Shikonin treatment resulted in the elevation of ROS level in cells**

As the generation of ROS plays a pivotal role in apoptotic cell death, we investigated whether shikonin induced ROS levels in AGS cells. As shown in Fig. 3A, a dose-dependent ROS accumulation was observed in AGS cells within 6 h of exposure to 62.5, 125 and 250 nM of shikonin. To test whether the shikonin treatment actually induced oxidative stress-related damage, we measured the levels of MDA, a lipid peroxidation product that is commonly used as a presumptive measure in ROS-mediated injury. As expected, MDA levels were also increased by shikonin in a dose-dependent manner (Fig. 3B). Taken altogether, our results suggested that ROS generation was markedly induced by treatment with shikonin. Other study suggested that AGS cells are equipped with an efficient antioxidant defense system (Filomeni et al., 2005a), where gastrointestinal-derived cell lines may exploit the well-known GPx antioxidant enzymes, among the five members being gastrointestinal-derived GPx (GI-GPx) (Chu et al., 2004). Thus, we determined the expression of GI-GPx by real-time PCR method. As seen in Fig. 3C, the mRNA level of GI-GPx was dramatically declined up to 40% of its control level upon shikonin treatment in the AGS cell line.

**Shikonin modulates Nrf2 and p53 activities**

It can be presumed from Fig. 1A that AGS (p53 wild) cell viabilities were more sensitivity to shikonin exposure than that of Kato 3 (p53 null) cells. Since p53 is known to involve in the suppression of Nrf2-mediated activity (Fararionio et al., 2006), we investigated whether incubation in shikonin-treated media would induce any changes in the expressions of p53 and Nrf2 in the AGS cells. Clearly, shikonin induced an increase in the expression levels of p53 in a dose-dependent manner, where 250 nM shikonin-treated cells exhibited approximately three fold higher expression level than that of the non-treated cells (Fig. 4A). In contrast, Nrf2 expression showed a significant decrease in shikonin-treated cells. To confirm these data, the levels of these two proteins were investigated by Western blot analysis, which also revealed correlated data in cells treated with 125-250 nM shikonin in AGS cells. On the contrary, when Kato 3 cells were subjected to the same dosage of shikonin treatment, we found that there was no significant change in the expression of Nrf2 (Fig. 4B).
Shikonin reduces translocation of Nrf2 into the nucleus and activates the Nrf2/ARE signaling in AGS cells

The Nrf2/ARE signal pathway is considered to play an important role in cellular protection, but it has also been suggested to function in acquiring drug resistance in various cancer cells. Since Fig. 4A, 4B suggested that shikonin reduced Nrf2 expression level, we examined how the Nrf2/ARE signal transduction was affected in AGS cells treated with shikonin.
by performing the immunofluorescence and luciferase assays. As shown in Fig. 4C, Nrf2, which was localized in the cytosol of untreated cells, was slightly translocated into the nucleus at low concentrations of shikonin (62.5 nM). However, at high dosage of shikonin (250 nM), there was no Nrf2 that was localized in the nucleus. In contrast, in Kato 3 cells treated with high dosage of shikonin, Nrf2 was evidently translocated into the nucleus. Shikonin activation of the Nrf2/ARE signal pathway was then examined in an ARE-luciferase reporter assay.

Fig. 6. Role of Nrf2 in the cell response against shikonin treatment. (A-C) Cells were either untreated or transfected with a non-targeting siRNA (scramble) or a siRNA directed against Nrf2 (siNrf2). The cells were cultured in the absence or presence of shikonin for 6 h. (A) The indicated proteins were analyzed by western blotting. β-actin was used loading control. (B) JNK is the activating kinases of p53 and Nrf2. Cells were in the absence or presence of 10 µM Sp600125 after treatment with various concentration of shikonin for 6 h, and then the indicated proteins were analyzed by western blotting. β-actin was used loading control. (C) AGS cells were transfected with the pGL3-ARE reporter vector and then treated with various concentration of shikonin in the presence or absence of Sp600125 for 6 h. Firefly luciferase activity was normalized to Renilla activity. *p<0.05 versus vehicle. The data are expressed as mean ± SD for triplicates.
Interestingly, we observed little to no change in the 4ARE-promoter activity after 6 h treatment with shikonin (Fig. 4D). Thus, our results revealed that Nrf2/ARE signaling pathway may be inhibited by shikonin treatment, especially at high concentration of 250 nM.

**JNK is associated with shikonin induced apoptosis of AGS cells**

Shikonin has been shown to induce chronic myelogenous leukemia (CML) cell apoptosis through JNK activation (Mao et al., 2008). JNK belongs to the MAPK family and act by responding to various stress stimuli. In addition, phosphorylation of JNK activates its downstream molecules, among them being p53 (Seger and Krebs, 1995). To determine the role of JNK in shikonin-induced apoptosis, we analyzed the protein activity of phosphorylated JNK. It can be suggested from Fig. 5A that the shikonin treatment results in activation of JNK in a dose-dependent manner. Pretreatment of the AGS cells with SP600125, a JNK’s chemical inhibitor, resulted in significant attenuation of the cell’s signaling response to shikonin treatment observed in cells without SP600125 pretreatment. As shown in Fig. 5B, the proteolysed forms of PARP and caspase-3 upon shikonin treatment were no longer present in cells pretreated with SP600125. Furthermore, we evaluated whether this inhibition could affect the signal transduction leading to Nrf2-mediated cell responses. We found that cells treated with shikonin showed reduced Nrf2 activity level. Interestingly, contrary to the non-pretreated cells, the SP600125 pre-treated cells exhibited a dose-dependent increase in the Nrf2 level (Fig. 5B). Furthermore, we also observed a concomitant increase in the 4ARE-promoter activity in cells pretreated with JNK inhibitor (Fig. 5C).

**Nrf2 is a sensitive modulator in shikonin-induced cell death**

We have already seen in Fig. 4 that shikonin inhibited Nrf2 and ARE-promoter activities via p53-dependent pathway. We thus investigated whether Nrf2 was a sensitive modulator of shikonin-induced cell death in AGS cells and if so, whether it functioned by affecting p53 expression. AGS cells were transfected with either control or Nrf2 siRNA for 24 h followed by incubation in shikonin-treated media for 6 h (Fig. 6). As shown in Fig. 6A, 6B, the siNrf2-transfected cells showed significantly higher mRNA and protein levels of p53 than in the cells treated with control. Similarly, we observed higher activation levels of proapoptotic proteins, c-PARP and c-caspase 3, in shikonin treated cells transfected with Nrf2 siRNA compared to control. Since several genes encoding antioxidant enzymes (Gastrointestinal glutathione peroxidase, GI-GPx) contain functional or putative ARE sequence in their 5’-flanking regions, we investigated the effect of shikonin treatment on GI-GPx mRNA levels in AGS cells transfected with a siRNA directed against Nrf2. Although shikonin significantly declined the expression levels of GI-GPx genes in both siNrf2-transfected and non-transfected cells, the GI-GPx mRNA level was decreased to more extent than that in scramble siRNA transfected cells (Fig. 6C).

**DISCUSSION**

Many traditional anti-cancer agents used in the treatment of various cancers are associated with drug-resistance (Jones et al., 1995), which have led researchers to come up with new strategies and approaches to chemotherapy. In this context, many natural herbs and pigments, which have long been used in Asian traditional medicine, are beginning to be revealed of their chemotherapeutic effects by scientists in the field of modern clinical medicine. Shikonin is such a natural compound that has been used in traditional medicine (Guo et al., 1991). There are many reports on shikonin demonstrating its potential anti-cancer effects, including topoisomerase 2-mediated DNA cleavage (Fuji et al., 1992) and inhibition of cancer proliferation via regulation of the cell cycle (Wu et al., 2004). However, the molecular mechanism of shikonin-induced cell death has not been fully elucidated. Here, we provide the first experimental evidence that shikonin induces the apoptosis of gastric adenocarcinoma cells via simultaneous induction of p53 and repression of Nrf2 expression.

Many anti-cancer drugs require in cancer cells the generation of ROS, which contributes to drug-induced apoptosis via various cellular responses (Simon et al., 2000). ROS is also known to play important roles in the cell signaling and homeostasis (Devasagayam et al., 2004). In this study, we found that shikonin is capable of inducing the accumulation of ROS that is may act as a stimuli to signal cell death. We have also observed the involvement of caspase and JNK inactivation of apoptotic pathway by shikonin treatment, in which both the caspase 3 and JNK inhibitor pre-treatment resulted in the inhibition of shikonin-induced cell death in AGS cells. We also found that shikonin is an activator of the p53-mediated signal pathway as well as repressor of the Nrf2-related pathway, both of which were associated with the generation of ROS and shikonin-induced apoptotic cell death.

p53 is a crucial transcriptional mediator that regulates a variety of biological processes including survival, proliferation, apoptosis, and differentiation through downstream targets. Our results showed that p53-dependent apoptosis was induced by shikonin treatment in AGS gastric cancer cells. In general, DNA damage-dependent activation of p53 induces a significant accumulation of ROS that is pivotal for p53-dependent signaling transduction of apoptosis (Polyak et al., 1997, Macip et al., 2003). Our data showed that shikonin-treated cells maintained their p53 expression, but not Nrf2 expression. It has been reported that p53 is negatively regulated by proteasomal degradation, such as that by Mdm2. However, in a recent study, Nrf2 was found to contribute to the basal expression of Mdm2. In the case of Nrf2, siRNA-pretreated cells showed a higher expression of p53 than control siRNA-pretreated cells. It is therefore possible that an additional level of p53 regulation exists that functions through the Nrf2 activity, thereby allowing the dampening of Mdm2-based proteasomal degradation of p53 upon shikonin treatment (Kwak et al., 2003). On the other hand, p53 may suppress the transcription of Nrf2 target genes driven by the ARE promoter (Faracionio et al., 2006; Wakabayashi et al., 2010). When p53 is highly up-regulated, such as in cells exposed to etoposide, p53 has the ability to negatively regulate the Nrf2 target gene transcripts. Shikonin was also found to induce p53 activation down-regulate Nrf2 and ARE promoter activity. Also shikonin functions in topoisomerase 2 inhibition, in a manner similar to etoposide. Therefore, shikonin may regulate Nrf2 which in turn p53 signaling, or p53 may regulate Nrf2.

Altogether, the present study demonstrated for the first time that shikonin elicited antiproliferative effects on human gastric
carcinoma cells via caspase- and JNK-dependent apoptotic pathways. Such effects were associated with accumulation of ROS as well as concurrent activation of p53 and inhibition of Nrf2 activities. Interestingly, our results also suggested inter-relationships in p53 and Nrf2, in which p53 activation upon shikonin treatment was lost upon siNrf2-transfection, and vice versa. Taken altogether, we confirmed the anticancer effects of shikonin, the naphthoquinone derivative that has long been used as traditional Chinese medicine for treatment of various diseases, including cancer (Lee and Lin, 1988). In this sense, our study may also contribute to discovering new therapeutic approaches for solving resistance in cancer chemotherapy.

CONFLICT OF INTEREST

We declare that we have no conflicts of interest.

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