Original Article

Shikonin induces apoptosis and necroptosis in pancreatic cancer via regulating the expression of RIP1/RIP3 and synergizes the activity of gemcitabine

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Abstract: Pancreatic cancer is a lethal solid malignancy with poor prognosis. The optimal therapy for patients with advanced pancreatic cancer remains challenged. Thus, development of novel chemotherapy regimens is extremely urgent. Shikonin (SK) is a naphthoquinone derived from the roots of the Chinese medicinal herb Lithospermum erythrorhizon. It has been considered as effective anti-inflammatory, anti-oxidant, and anti-cancer activity agents in various diseases. In the present study, we found that SK inhibited the growth of human pancreatic cancer and enhanced the anti-tumor effect of gemcitabine in vitro and in vivo. Moreover, SK induced apoptosis and necroptosis in different pancreatic cancer cells by regulating RIP1 and RIP3 expression. The expression of RIP3 correlated with their necrotic response. These results suggest that the combination of SK and gemcitabine may be a promising chemotherapy regimen for pancreatic cancer.

Keywords: Shikonin, pancreatic cancer, necroptosis, gemcitabine, RIP1, RIP3

Introduction

Pancreatic cancer is an aggressive malignancy and the fourth leading cause of cancer-related deaths in developed countries [1-3]. Unlike other types of cancer, the survival rate for this disease has not improved substantially in nearly 40 years because of cancer drug resistance, which is a major obstacle limiting the efficacy of cancer chemotherapy [4]. Therefore, targeting the weak point of cancer, in particular in apoptotic/drug-resistant cancers, is critical for the success of cancer chemotherapy. Gemcitabine (GEM) is one of the most important drugs in the treatment of pancreatic cancer. It mostly induces apoptosis, a compact and complex circuit regulated by numerous anti- and pro-apoptotic components, and can be specifically inhibited by the pan-caspase inhibitor zVAD-fmk [5, 6]. Therefore, drug resistance via anti-apoptotic progression appears to be inevitable. Necroptosis, a newly identified form of programmed cell death, is different from apoptosis [7, 8]. It requires the activity of receptor interacting protein kinases and can be specifically inhibited by Nec-1 (necrostatin-1) [9]. Several recent studies have shown that necroptosis can accelerate cancer cell death or enhance the sensitivity of tumor cells to anti-cancer treatment [10, 11]. Therefore, targeting the weakness of cancer through the induction of necroptosis may have significant potential in pancreatic cancer chemotherapy.

Shikonin (SK), isolated from the plant Lithospermum erythrorhizon, has been investigated as a potential anticancer drug for the treatment of various types of cancer [12-14]. As a traditional Chinese medicine, SK is a naturally occurring naphthoquinone derivative. It has been shown to have anti-cancer activity through the inhibition of growth and the induction of apoptosis in several kinds of cancer cells. Furthermore, Han et al. reported that SK could
Shikonin inhibits pancreatic cancer

induce necroptosis in human leukemia cell lines [15]. Recently, Wang et al. found that SK could suppress the growth of human pancreatic tumors and potentiate the antitumor effects of gemcitabine through the suppression of NF-κB and NF-κB-regulated gene products [16]. In addition, Gara et al. also found that SK could induce human prostate cancer cells via the endoplasmic reticulum stress and mitochondrial apoptotic pathway [17]. However, whether it can target weak point of pancreatic cancer through the induction of necroptosis has not been evaluated before. Therefore, we investigate the effect of SK on cell death in pancreatic cancer in vitro and in vivo.

Materials and methods

Cell lines and reagents

The human pancreatic cancer cell lines AsPC-1, SW1990, PANC-1 and Capan-2 were purchased from the American Type Culture Collection (Manassas, VA, USA) and the Patu8988 cell line was purchased from Cell Biology Expert. Cells were maintained in ATCC’s and Cell Biology Expert’s recommended growth media (Gibco, Grand Island, USA) containing 10% fetal bovine serum (Gibco; Invitrogen®, USA) and 1% penicillin-streptomycin (Gibco BRL, USA) at 37°C in a 5% CO₂ and 95% air-humidified incubator. SK was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) with a purity of >98%. Gemcitabine was obtained from Selleck (USA). MTT, Nec-1 (necrostatin-1) and zVAD (zVAD-fmk) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). SK was dissolved in DMSO to a storage concentration of 50mmol/L. An equal amount (0.1% v/v) of DMSO (vehicle) was present in all the treatment groups including the control.

Cell proliferation assay

Cell viability was measured using the MTT assay according to the manufacturer’s instructions. Cells were seeded at a density of 5×10⁴ cells/well in 96-well flat bottom microtiter plates. After 24, 48 and 72 h of incubation at 37°C, cells were treated with different concentrations (0, 0.625, 1.25, 2.5, 5 and 10 μmol/L) of SK in 200 μl of complete media for the indicated time periods. Cells were then incubated with MTT (0.25 mg/ml) for 2-4 h at 37°C. The formazan crystals in the cells were solubilized with a solution containing 50% dimethylformamide and 20% sodium dodecyl sulfate (pH 4.7). Finally, the level of MTT/formazan was determined by measuring absorbance at a wave length of 570 nm with the SPECTRA (shell) Reader (TECAN, Austria).

Cell-cycle analysis and apoptosis assay

AsPC-1 and PANC-1 cells in logarithmic phase were seeded on to six-well plates at a density of 3×10⁵ cells/well and then placed in serum-starved conditions for 6 h. Subsequently, cells were incubated with different concentrations of SK (5 and 10 μmol/L) or under different conditions (add Nec-1 or zVAD) in incomplete medium. After 24 h, cells were harvested by trypsinization and fixed by pre-cooled 70% ethanol. For the flow cytometric procedure, cell-cycle phases and apoptotic effects were detected by propidium iodide (PI, Invitrogen®) staining and the FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen®). Fluorescence-activated cell sorter analysis was performed with the FACScanto flow cytometer (BD®).

Quantitative real-time RT-PCR analysis

After exposure to SK at 5 μmol/L for 24 h, PANC-1 and AsPC-1 cells were harvested and total RNA was extracted by using the Trizol reagent (Invitrogen®) according to the instructions included in the kit. To detect RIP1 and RIP3 gene expression in different cells, five pancreatic cancer cell lines in logarithmic phase were harvested and total RNA was extracted. The mRNA expression was detected by Real-time RT-PCR using the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems®) and SYBR Green Dye (TaKaRa®). Primers specific for RIP1 and RIP3 were used for qRT-PCR and are available upon request. For the RT-PCR reaction, 200 ng of total RNA was subjected to cDNA synthesis and subsequently amplified with 40 PCR cycles (0.5 sec at 95°C; 10 sec at 60°C; 10 sec at 72°C). The relative expression levels were calculated using the 2^-ΔΔCT method. All experiments were repeated at least three times. PANC-1 and AsPC-1 cells in the logarithmic phase were divided into five groups as follows: normal control, 5 μmol/L SK, 5 μmol/L SK+Nec-1, 5 μmol/L SK+zVAD, and 5 μmol/L SK+Nec-1+zVAD. Real-time Reverse Transcription-PCR was used to analyze
Shikonin inhibits pancreatic cancer

Changes in RIP1, RIP3 and β-actin mRNA expression.

Western-blotting

PANC-1 and AsPC-1 cells were treated with SK at 5 µmol/L for 24 h, rinsed twice in phosphate-buffered saline (PBS), lysed for 2 h in RIPA lysis buffer on ice and centrifuged at 12,000 rpm for 10 min at 4°C. The protein concentration was determined by the BCA protein assay (BCA™ Protein Assay Kit, Pierce, USA). Aliquots containing 40 µg of protein were separated by 12% SDS-PAGE and then transferred onto PVDF membranes. The membranes were incubated with the relevant primary antibodies overnight at 4°C (RIP1, 1:500; RIP3, 1:500, actin, 1:1000; Santa Cruz, USA). The membranes were washed with wash buffer (PBS containing 0.1% Tween-20) and then incubated with the appropriate HRP-conjugated secondary antibodies (1:2000) for 1 h at 37°C. The blots were developed using the ECL-detection system (Santa Cruz), quickly dried, and exposed to ECL film. All experiments were repeated at least three times with similar results.

Figure 1. SK inhibits growth and induces cell death in five human pancreatic cancer cell lines (A-E). SK inhibited cell proliferation in a dose- (0.312-10 µmol/L) and time- (24, 48 and 72 h) dependent manner. Cell viability was measured by the MTT assay and expressed as relative to the untreated controls. Results represent the means ± SD from six independent experiments.
Shikonin inhibits pancreatic cancer

Stealth small interference RNA (siRNA) sequences for RIP3 were designed and synthesized by GenePharma to target RIP3 mRNA. The coding strand for RIP3 siRNA was 5’-GAACUGUUUGUUAACGUAATT-3' for sense and 5’-UUACGUUAACAAACAGUUCTT-3' for antisense; an unrelated siRNA sequence was used as a control. In this experiment, cells were incubated for 12 h and transfected at approximately 50% confluence with 80 nm siRNA duplexes using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. All the experiments were performed 72 h after transfection.

Animals and in vivo experiments

Specific pathogen free female nude mice (athymic, Balb/c nu/nu) aged 4-6 weeks (18-20 g) were purchased from Shanghai Laboratory Animal Co Ltd (SLAC, Shanghai, China). Animals were housed in an individual ventilated caging system on a 12 h light/12 h dark cycle at 22°C and were allowed free access to sterilized water and food. All animal-related procedures were approved by the Animal Ethical Committee of Tongji University. This study was conducted under the permission of the Science and Technology Commission of Shanghai Municipality (ID: SYXK 2011-0111) with the permit number 2011-RES1.

PANC-1 cells in logarithmic phase were suspended in 100 ml of 1:1 serum-free DMEM and Matrigel (BD Biosciences). The cell suspension was injected subcutaneously into the right anterior armpit of nude mice; a total volume of 0.5 ml containing 5×10^7 cells was injected into each mouse to establish an animal model of transplanted tumors.

SK was dissolved in vehicle (2% DMSO). The mice were randomly divided into five groups (5 mice in each group) as follows: group 1, vehicle-treated; group 2 and 3, SK-treated (2.5 mg/kg and 5 mg/kg, respectively); group 4, positive control (GEM 125 mg/kg); group 5, combination therapy (GEM 75 mg/kg+SK 2.5 mg/kg). One month after the transplantation, drugs were intraperitoneally injected every 2 days for 2 weeks. The mice were then sacrificed and the tumor tissues were analyzed and collected for immunostaining.

Table 1. Growth-inhibitory activity of SK on pancreatic cancer cells

<table>
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<tr>
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<th>AsPC-1</th>
<th>SW1990</th>
<th>Panc-1</th>
<th>Patu8988</th>
<th>Capan-2</th>
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<tr>
<td>24 h</td>
<td>1.1</td>
<td>3.75</td>
<td>2.38</td>
<td>1.19</td>
<td>1.87</td>
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<td>48 h</td>
<td>&lt;0.3</td>
<td>0.92</td>
<td>1.91</td>
<td>0.92</td>
<td>1.88</td>
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<tr>
<td>72 h</td>
<td>&lt;0.3</td>
<td>0.90</td>
<td>1.65</td>
<td>0.79</td>
<td>1.45</td>
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Small interfering RNA mediated inhibition of RIP3 expression in AsPC-1 cells

SPSS18.0 software was used for statistical analyses. Data were obtained from three independent experiments and expressed as the mean ± standard deviation (SD). Real-time PCR data were analyzed according to the differences in target gene expression by the paired t-test and were 2^{-\Delta\Delta CT} transformed before the analysis. Statistical analyses were performed by one-way ANOVA and the Chi-squared test. A p-value <0.05 was considered statistically significant.

Results

SK inhibits growth and induces cell death of human pancreatic cancer cells

The inhibitory effect of SK on the growth of AsPC-1, SW1990, Panc-1, Capan-2 and Patu8988 cells was detected by MTT assay. SK inhibited the growth of all five cell lines in a time- and dose-dependent manner at 0.312-10 μmol/L (Figure 1; P<0.05). The IC_{50} values (50% inhibitory concentrations) for SK at 24, 48 and 72 h in the five cell lines were listed in Table 1. Among the five cancer cell lines, AsPC-1 was the most sensitive and Panc-1 was the most resistant line. In AsPC-1 cells treated with high doses of SK, cell death occurred gradually over a period of 3-5 h, which was much faster than that in Panc-1 cells. This suggested that different cell death mechanisms might mediate the effect of SK in the AsPC-1 and Panc-1 cell lines. Therefore we selected these two cell lines for further study.

Effect of SK on apoptosis/necrosis and cell cycle phase distribution

To further investigate the effect of SK on the inhibition of cell proliferation, the percentage of apoptosis/necrosis was evaluated by flow cytometry using Annexin V-FITC/PI staining. The
Figure 2. Flow cytometric analysis of SK induced pancreatic cell death and the effect of SK on cell cycle distribution in PANC-1 and AsPC-1 cells. After a 24 h incubation with low or high concentration of SK, the percentage of necrotic and late apoptotic cells increased significantly, especially in AsPC-1 cells (A, B). No obvious block of cell cycle progression or cell cycle phase accumulation was observed in either cell line (C, D). Results represent the means ± SD from three independent experiments and *P<0.05, **P<0.01.
Shikonin inhibits pancreatic cancer

Percentage of cells undergoing apoptosis increased from 2.66 ± 0.44% in the control group to 39.1 ± 1.21% in the 10 μmol/L SK treatment group in AsPC-1 cells, and from 0.2% ± 2.1% to 37.4% ± 1.74% in PANC-1 cells (Figure 2A). Compared with the control group, 5 μmol/L (P<0.05) and 10 μmol/L (P<0.01) of SK significantly induced apoptosis in both cell lines (Figure 2B).

Interestingly, the percentage of cells undergoing necrosis was significantly different between the two cell lines (Figure 2A and 2B). In AsPC-1 cell line, SK treatment at 2.5, 5 and 10 μmol/L for 24 h induced necrosis in 5.5% ± 1.01%, 20.4% ± 0.91%, and 56.1% ± 1.98% of cells, respectively, whereas under the same conditions, PANC-1 cells showed a decline of cell necrosis (P<0.05), with 0.4% ± 0.5%, 1.2% ± 1.78% and 7.5% ± 1.04% of cells undergoing necrosis, respectively. These data confirm that cell death occurs by different mechanisms when treated with SK in these two cell lines.

Cell cycle phase distribution is an important indicator of cell survival, so we evaluated cell cycle progression by flow cytometry. No obvious cell cycle block or accumulation of cells was observed in these two cell lines (Figure 2C and 2D).

**SK induces necroptosis of AsPC-1 cells**

The dose-dependent effect of SK on the induction of necrosis was greater in AsPC-1 than in PANC-1 cells, as shown in Figure 2A. An accurate assessment of SK-induced necroptosis in PANC-1 cells was not possible because less than 10% of cells underwent necrosis in response to high doses of SK. Therefore, we used the AsPC-1 cell line to determine whether necroptosis was one of the mechanisms of SK-induced cell death. For this purpose, AsPC-1 cells were treated with SK, Nec-1 and zVAD. The results showed that cells treated with SK and Nec-1 underwent apoptosis, whereas those treated with SK and zVAD showed a cell death pattern consistent with necrosis (more than 50%) and non-viable apoptosis (Figure 3A). Compared with treatment with SK alone, the combination of Nec-1 and SK significantly induced apoptosis (P<0.01), whereas zVAD and SK significantly induced necrosis (P<0.01) in AsPC-1 cells (Figure 3B).
Shikonin inhibits pancreatic cancer

Because Nec-1 is a necroptosis inhibitor, our results indicate that Nec-1 changed SK induced cell death from necrosis to apoptosis, suggesting that SK induced necroptosis in AsPC-1 cells. Treatment with Nec-1 or zVAD does not reverse the cell death effect, but can change the cell death mechanism. As shown in Figure 3A, cells treated with SK, Nec-1 and zVAD showed similar responses to those treated with SK and zVAD, suggesting that inhibition of both apoptosis and necroptosis results in true necrosis, although this phenomenon needs further investigation.

**Necroptosis induced by SK is independent of RIP1 but directly targeting RIP3 in AsPC-1 cells**

To investigate the mechanism of necroptosis, the mRNA and protein expression of RIP1 and RIP3 was examined by RT-PCR and western

**Figure 4.** SK upregulates the expression of key enzymes involved in necroptosis. A: Expression of RIP1 and RIP3 in five pancreatic cancer cells. B: SK markedly upregulated RIP1 expression in PANC-1 cells whereas it had no significant effect on RIP3 expression. AsPC-1 cells showed the opposite pattern of RIP1 and RIP3 expression in response to SK treatment, with negative RIP1 protein expression in AsPC-1 cells treated with SK. C: Nec-1 or zVAD had no obvious effect on RIP3 expression. D: Silencing of RIP3 expression resulted in a marked decrease in the inhibition of cell proliferation by SK. Results represent the means ± SD from three independent experiments and *P<0.05, **P<0.01.
Shikonin inhibits pancreatic cancer

Assessment of RIP1 and RIP3 expression in five pancreatic cancer cell lines (Figure 4A) showed differences in RIP1 mRNA expression among the different cell lines. SW1990 cells showed the highest levels of RIP1 expression, whereas Patu8988 showed the lowest levels (P<0.05). Assessment of RIP3 mRNA expression showed the highest levels in AsPC-1 cells, whereas the remaining four cell lines showed no differences in RIP3 expression levels (P<0.01). The RIP1 and RIP3 proteins showed a similar pattern of expression with the exception of AsPC-1 cells, which were negative for RIP1 expression despite showing the highest RIP3 expression. Assessment of RIP1 and RIP3 mRNA and protein expression in response to SK in two cell lines showed a marked increase in RIP1 expression and no significant difference in RIP3 expression in PANC-1 cells, whereas AsPC-1 cells showed the opposite pattern. AsPC-1 cells were negative for RIP1 protein expression regardless of SK treatment (Figure 4B). These results indicate that RIP1 and RIP3 expression may be involved in the mechanism underlying the sensitivity of pancreatic cancer cells to SK treatment.

To investigate the mechanism of necroptosis in AsPC-1 cells, RIP1 and RIP3 expression was determined in cells treated with Nec-1 and zVAD. The results showed that Nec-1 or zVAD had no obvious effect on RIP3 expression (Figure 4C). To further examine whether SK induces necroptosis by targeting RIP3, siRNA mediated silencing of RIP3 expression was followed by assessment of cell growth by the MTT assay and apoptotic and necrotic rates by flow cytometry. Silencing of RIP3 expression remarkably reduced the inhibitory effect of SK on cell proliferation in response to SK treatment for

Figure 5. SK suppresses in vivo growth of PANC-1 tumor xenografts and enhances the anti-tumor effect of gemcitabine in vitro and in vivo. In vitro, combination treatment with SK with GEM significantly inhibited the growth of PANC-1 (A) and AsPC-1 (B) cells compared to treatment with a single agent. In vivo, low and high doses of SK caused a 57.3% (P<0.05) and 68% (P<0.05) reduction in tumor volume, respectively, and combined treatment with SK and GEM resulted in a 99.7% reduction in tumor volume (C, D). Results represent the means ± SD from three independent experiments and *P<0.05.
Next, we investigated the antitumor activity of combination treatment with SK and GEM in vivo. The results showed that combination treatment resulted in a 99.7% reduction in tumor volume, which was more effective than SK and GEM alone. Furthermore, the doses used in the combination treatment were lower than those used for SK and GEM alone (Figure 5C and 5D). Taken together, our data indicate that the combination of SK and GEM has strong antitumor activity in vivo and in vitro in pancreatic cancer.

Discussion

In the present study, we found that SK inhibits the growth of human pancreatic cancer cells in vitro and in vivo. Interestingly, the efficacy of SK to different cell lines remarkably differed, and it happened to be consistent with the expression of RIP3 among these five cell lines. Namely, the efficacy of SK and the expression of RIP3 were highest in AsPC-1 cells without RIP1 expression and lowest in PANC-1 cells with RIP1 expression. As we know, RIP3 has recently been shown to be involved in necroptosis [18-20]. RIP1 and RIP3 interact via their RIP homotypic interaction motif (RHIM) domains to form a RIP1/RIP3 complex that mediates classic necroptosis [21]. And it can be inhibited by Necrostatin-1 (Nec-1), which is a specific inhibitor of RIP1 and has no effect on RIP3 [9, 18]. However, we found that Nec-1 switched SK-induced cell death from necrosis to apoptosis in AsPC-1 cells which were negative for RIP1 expression regardless of SK treatment. And it seems to be unreasonable that Nec-1, which has not shown activity against RIP3 in previous studies, inhibited RIP3 in the absence of RIP1 in our study. In fact, the team of YoungSik also found that Nec-1 inhibited necrosis in a RIP1-independent manner in L929 cells [22]. These data suggest that Nec-1 could have other functions besides the specific inhibition of RIP1, although this needs further investigation.

Since RIP3 was shown to be the target of SK induced necroptosis, we used a specific RIP3 siRNA to silence RIP3 expression. Silencing of RIP3 resulted in a 76% reduction in SK-induced cell death, which indicated that SK can induce rapid and marked necroptosis by targeting RIP3 independent of RIP1. On the other hand, in the presence of RIP1, SK induced mostly apoptosis, which is reasonable considering that RIP1 is involved in the formation of complex together with FADD and caspase-8, which
Shikonin inhibits pancreatic cancer

activates a caspase cascade to induce apoptosis [23]. Under such conditions, necroptosis is not the main cell death mechanism, although it is involved through the formation of the RIP1/ RIP3 complex. These findings suggest that whether cells turn to apoptosis or necroptosis is probably determined by RIP1 and RIP3 expression and their interaction. In addition, SK induces mostly necroptosis in a rapid manner when RIP1 is absent, which suggests that SK-induced cell death could be accelerated by silencing RIP1 expression. Overall, SK can induce necroptosis and/or apoptosis by regulating RIP1 and RIP3 expression and cross-talk.

GEM is the standard treatment for patients with advanced and metastatic pancreatic cancer [24]. The anti-tumor effect of GEM, a deoxycytidine analogue, is mediated by the induction of DNA strand termination and apoptosis [25]. However, the development of drug resistance via anti-apoptotic progression appears to be inevitable [26]. To overcome drug resistance, recent studies have focused on identifying chemotherapeutic agents that can efficiently enhance the antitumor activity of GEM [26-28]. In the present study, we found that SK has beneficial effects when used as a chemopreventive agent in combination with GEM for the treatment of pancreatic cancer in vitro and in vivo. SK may enhance the antitumor effect of GEM by targeting the “weak point” of necroptosis, which is often impaired during tumorigenesis and can be engaged by targeted pharmacological approaches in cancer. However, the precise underlying mechanism remains to be elucidated.

In summary, SK induces apoptosis and necrosis in pancreatic cancer by regulating RIP1 and RIP3 expression. In addition, SK enhances the anti-cancer effect of GEM in pancreatic cancer in vivo and in vitro. These data indicate that the combination of SK and GEM may be a promising chemotherapy regimen for the treatment of pancreatic cancer.

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Disclosure of conflict of interest

None.

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Shikonin inhibits pancreatic cancer


