Enhancement of cisplatin-induced colon cancer cells apoptosis by shikonin, a natural inducer of ROS in vitro and in vivo

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1. Introduction

Colorectal cancer is among the most commonly diagnosed cancer and ranks as the third leading cause of cancer related mortality worldwide [1]. Although surgical resection is the mainly curative therapy in early stage, chemotherapy still remains an important strategy for patients diagnosed with advanced stage. Platinum-based chemotherapy such as cisplatin is a widely used treatment in colorectal cancer [2,3]. However, a majority of cancer patients eventually relapse and develop drug resistance despite initial response to cisplatin [4]. Recently, combined chemotherapy has been found to be a superior treatment strategy [5–7]. Hence, searching of effective chemosensitizers that could augment the efficiency of anticancer drugs and simultaneously overcome multi-drug resistance and side effects is urgently needed.

Compared with normal cells, cancer cells have intrinsically higher levels of reactive oxygen species (ROS) [8,9]. Increased reactive oxygen species in cancer cells is crucial in the initiation and progression of cancer. However, excessive ROS production could be toxic and render cancer cells to be more vulnerable to damage by further oxidative stress induced by exogenous agents [10,11]. Oxidative stress has been reported to induce cell apoptosis via a series of downstream pathways, such as endoplasmic reticulum (ER) stress and mitochondrial cascade [12,13]. Therefore, manipulating ROS levels in cancer cells is a way to selectively kill cancer cells, and has been involved in the anti-cancer effects of several therapeutic agents including auranofin, disulfiram, and piperlongumine [10,14,15].

Shikonin, a naturally occurring compound isolated from the Chinese herbal plant Lithospermum erythrorhizon, has been used for thousands of years for the treatment of diverse ailments [16,17]. Recent studies have demonstrated that shikonin has potent
antitumor potential, inhibiting malignant cell growth and inducing cancer cell apoptosis by inducing intracellular oxidative stress [16,18]. In the present study, our results showed that shikonin effectively potentiated cisplatin-induced apoptosis and exhibited synergistic effects on inhibition of colon cancer cells proliferation. Shikonin contributed to the synergistic effect by strengthening the induction of apoptosis and cell cycle arrest. The mechanistic investigation elucidated that shikonin as a ROS inducer could dramatically enhance cisplatin-induced colon cancer cells apoptosis in vitro and in vivo by triggering ROS-mediated mitochondrial dysfunction pathway. In summary, our study demonstrated that shikonin was an effective synergistic agent to cisplatin and might be a candidate of chemosensitizer for cisplatin-based therapy in clinical application.

2. Materials and methods

2.1. Cell culture and reagents

Cisplatin, Shikonin, l-Glutathione reduced (GSH) and N-acetylcyesteine (NAC) were purchased from Sigma (St. Louis, MO). Human colon cancer cell lines HCT116, HT29, SW620 and normal human colon mucosal epithelial cell line NCM460 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The cells were routinely cultured in RPMI 1640 medium (Gibco, Eggenstein, Germany) containing 10% heat-inactivated fetal bovine serum (Gibco, Eggenstein, Germany) in a humidified cell incubator with an atmosphere of 5% CO2 at 37 °C. Antibodies including anti-Cdc2, anti-Cyclin B1, anti-MDM-2, anti-Bcl-2, anti-Bax, anti-caspase-3 p30/17, anti-GAPDH, goat anti-anti-mouse IgG-HRP, donkey anti-rabbit IgG-HRP and donkey anti-goat IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FITC Annexin V apoptosis Detection Kit I and goat IgG-HRP were purchased from Santa Cruz Biotechnology (Franklin Lakes, NJ).

2.2. Cell viability assay

Cells were seeded in a 96-well plate and allowed to grow overnight. Cisplatin was dissolved in DMSO and diluted with 1640 medium to final concentrations of 2.5, 5, 10, 20 and 40 μM. The cells were incubated with cisplatin or with a combination of cisplatin and shikonin (1 μM) for 24 h before the MTT assay.

2.3. Cell cycle analysis

HCT116 cells were plated on 60-mm plates for 12 h, and then treated with compound for 14 h. The DNA was labeled with Propidium Iodide. Cell cycle analysis was performed in an FACS Calibur flow cytometer (BD Biosciences, CA).

2.4. Cell apoptosis analysis

HCT116 cells were plated on 60-mm dishes for 12 h, and then treated with compound for 24 h. Cells were then harvested, washed twice with ice-cold PBS, and stained with Annexin V-FITC and propidium iodide (PI). Mortality of the cells was determined using a FACS Calibur flow cytometer (BD Biosciences, CA).

2.5. Western blot analysis

Cells or tumor tissues were homogenized in protein lysis buffer, and debris was removed by centrifugation at 12,000 g for 10 min at 4 °C. Protein concentration was determined by using the Bradford protein assay kit (Bio-Rad, Hercules, CA). After addition of sample loading buffer, 50 μg protein samples were electrophoresed and then transferred to poly-vinylidene difluoride transfer membranes. The blots were blocked for 2 h at room temperature with fresh 5% nonfat milk in TBST. The PVDF membranes were incubated with specific primary antibody in TBST overnight at 4 °C. Following three washes with TBST, the blots were incubated with secondary antibodies for 1 h, and the immunoreactive bands were visualized by using ECL kit (Bio-Rad, Hercules, CA).

2.6. Measurement of reactive oxygen species generation

Cellular ROS contents were measured by flow cytometry. After treatment, cells were stained with 10 μM DCFH-DA (Beyotime Institute of Biotechnology, Nantong, China) according to the manufacturer’s protocol. The caspase-3/9 activity was normalized by the protein concentration of the corresponding cell lysate and expressed as percentage of treated cells to that of control.

2.7. Determination of caspase-3/9 activity

Caspase-3/9 activity in cell lysates was determined using a Caspase-3/9 activity kit (Beyotime Institute of Biotechnology, Nantong, China) according to the manufacturer’s protocol. The caspase-3/9 activity was normalized by the protein concentration of the corresponding cell lysate and expressed as percentage of treated cells to that of control.

2.8. Evaluation of mitochondrial membrane potential (ΔΨm)

The cell mitochondrial membrane potential (ΔΨm) was examined by fluorescence microscope using TMRE as specific probe. Cells were treated with compound for 14 h and stained with TMRE in a humidified atmosphere of 5% CO2 at 37 °C for 30 min. Images acquired by the Nikon fluorescence microscope (40× amplification, Nikon, Japan).

2.9. In vivo antitumor study

All animal experiments were complied with the Wenzhou Medical University’s Policy on the Care and Use of Laboratory Animals. Protocols for animal studies were approved by the Wenzhou Medical College Animal Policy and Welfare Committee (Approved documents: 2012/APWC/0216). Five-week-old athymic BALB/cA nu/nu female mice were used for in vivo experiments. HCT116 cells were harvested and injected subcutaneously into the right flank (5 × 106 cells in 100 μL of PBS). Mice were treated by intraperitoneal (i.p.) injection of 10 mg/kg cisplatin once per day, or by i.p. injection of 4 mg/kg shikonin once per day, or with a combination of cisplatin and shikonin according to the same schedules. The tumor volumes were determined by measuring length (l) and width (w) and calculating volume (V = 0.5 × l × w^2) at the indicated time points. At the end of treatment, the animals were sacrificed, and the tumors were removed and weighed.

2.10. Immunohistochemistry

The harvested tumor tissues were fixed in 10% formalin at room temperature, processed and embedded in paraffin. Paraffin-embedded tissues were sectioned (5 μm thick). Tissue sections were primarily stained with indicated antibodies. The signal was detected by biotinylated secondary antibodies, and developed in DAB.
2.11. Statistical analysis

2.11.1. All experiments were assayed in triplicate (n = 3)

Data are expressed as means ± SEM. All statistical analyses were performed using GraphPad Pro. Prism 5.0 (GraphPad, SanDiego, CA). Student’s t-test and two-way ANOVA were employed to analyze the differences between sets of data. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Combination of shikonin and cisplatin exhibited synergistic anticancer effects

To develop a treatment strategy that would be useful clinically, we tested the effect of combination therapy of cisplatin and shikonin by MTT assay. As shown in Fig. 1A, at 1 μM, shikonin did not obviously affect the viability of HCT116 cells. However, combination of cisplatin and shikonin displayed higher antiproliferative activities on HCT116, HT29 and SW620 cells than that of single agent (Fig. 1A–C). Moreover, combined treatment showed less cytotoxicity toward NCM460 normal human colon mucosal epithelial cells than that of single agent (Fig. 1A–C). This is consistent with the synergistic effect of shikonin and cisplatin examined in HCT116 cells. In the following study, the mechanisms of synergistic effect of shikonin and cisplatin were examined in HCT116 cells. First, we performed DNA flow cytometric analysis to examine the change in cell cycle distribution. As shown in Fig. 1E and F, treatment of cells with shikonin in combination with cisplatin resulted in a marked increase in the proportion of G2/M phase cells compared with that of shikonin or cisplatin alone, as reflected by the increase in G2/M peaks from 18.4% (shikonin) and 22.1% (cisplatin) to 33.2% (combination). Consistent with the flow cytometry outcomes, the Western Blot analysis revealed that treatment with shikonin obviously enhanced cisplatin-induced reduction of cell cycle-related proteins such as MDM-2, Cyclin B1, and Bax in HCT116 cells (Fig. 1G). These data suggest that shikonin can act as an enhancer to sensitize cisplatin-induced cell growth inhibition against HCT116 cells by induction of G2/M cell cycle arrest.

3.2. Shikonin enhances cisplatin-induced cell apoptosis by initiating mitochondrial dysfunction

To further elucidate the mechanisms of combined treatment induced growth inhibition, we examined the pro-apoptosis effect of shikonin or/and cisplatin on HCT116 cells using Annexin V/Propidium iodide staining assay. As shown in Fig. 2A and B, combined treatment of shikonin and cisplatin resulted in a marked increase in the proportion of apoptotic cells compared with that of shikonin or cisplatin alone. Consistent with the flow cytometry results, mitochondria dysfunction was confirmed by TMRE staining assay. As shown in Fig. 2G, treatment of cells with shikonin in combination with cisplatin resulted in a marked increase in the proportion of apoptotic cells compared with that of shikonin or cisplatin alone, as reflected by the increase in the percentage of Annexin V-positive cells from 18.4% (shikonin) and 22.1% (cisplatin) to 33.2% (combination). Consistent with the flow cytometry outcomes, the Western Blot analysis revealed that treatment with shikonin obviously enhanced cisplatin-induced reduction of cell cycle-related proteins such as MDM-2, Cyclin B1, and Bax in HCT116 cells (Fig. 1G). These data suggest that shikonin can act as an enhancer to sensitize cisplatin-induced cell growth inhibition against HCT116 cells by induction of G2/M cell cycle arrest.

3.3. Shikonin potentiates cisplatin-induced apoptosis by ROS accumulation

Previous studies have reported that ROS generation could trigger cell apoptosis via activating mitochondrial pathway [13,21]. Shikonin has been found to increase ROS generation and induce oxidative stress in several cancer cell lines [16,22]. Therefore, we determined the production of intracellular ROS in HCT116 cells by flow cytometry. As shown in Fig. 3A, treatment of cell with cisplatin (1 μM) and shikonin (10 μM) alone both slightly induced ROS accumulation, but combined treatment with shikonin and cisplatin resulted in significant increases in ROS levels, and total ROS generation increased to the maximum after 1 h. To confirm whether ROS accumulation is a necessary event in the potentiated apoptosis, two ROS scavenger, NAC and GSH were used in our experiment. The MTT results revealed that scavenging of ROS fully attenuated combined treatment-induced cell growth inhibition against HCT116 cells (Fig. 3B). In addition, it was found that co-treatment with GSH completely reversed combined treatment-induced apoptosis in HCT116 cells (Fig. 3C and D). Inhibition of Bcl-2 and the depletion of mitochondrial membrane potential were also reversed (Fig. 3E and F). These results revealed the vital role of ROS in the synergism. The redox system might be the upstream target for shikonin to enhance the apoptosis induced by cisplatin in colon cancer cells.

3.4. Shikonin enhances in vivo anticancer activity of cisplatin

To evaluate the synergetic effect of shikonin and cisplatin in vivo, immuno-deficient nude mice bearing HCT116 tumor xenografts were employed. After 16 days’ administration, treatment of cisplatin alone slightly inhibited tumor growth. However, xenograft colon tumor growth in nude mice was more effectively inhibited by combined treatment with shikonin and cisplatin in vivo. For instance, combined treatment with shikonin and cisplatin significantly inhibited the tumor weight and tumor volume, but not affected body weight of mice (Fig. 4A–C). Consistent with the tumor size outcomes, combined treatment showed stronger ability in inducing cleaved caspase-3 expression in tumor tissues (Fig. 4D). In addition, we found that combined treatment with shikonin and cisplatin significantly increased the level of lipid peroxidation product (MDA), a marker of ROS, in tumor tissues (Fig. 4E). Taken together, these data support the conclusion that shikonin can synergistically enhance cisplatin-induced tumor growth inhibition in vivo by inducing ROS accumulation.

4. Discussion

Cancer is a major public health issue in the world.

Many therapeutic drugs have been explored and developed for clinical trials [23,24]. Cisplatin is one of the most potent anticancer agents. It exerts clinical activities against a wide spectrum of solid neoplasms and has been used for many years [25]. However, many kinds of cancer cells are insensitive to cisplatin-induced apoptosis...
Fig. 1. Combination of Shikonin and Cisplatin Exhibited Synergistic Anticancer Effects. (A–C) Shikonin enhances cisplatin-induced growth inhibition in colon cancer cells. Briefly, cells were treated with shikonin and cisplatin for 24 h, cell viability was determined by MTT assay. Assays were performed in triplicate. (D) Shikonin and cisplatin combined treatment showed less cytotoxicity toward NCM460 normal human colon mucosal epithelial cells. (E) Shikonin enhances cisplatin-induced cell cycle arrest in HCT116 cells. Cells after treatment for 14 h were collected and stained with PI solution after fixation by 70% ethanol. Cell cycle distribution was analyzed by flow cytometric analysis. (F) Representative histograms from flow cytometry analysis, assays were performed in triplicate. (G) Expression of G2/M cell cycle relative proteins MDM-2, Cyclin B1 and Cdc2 were determined by western blot after treatment for 14 h. GAPDH was used as internal control. (H–J) Western blot results from (G) were calculated and represented as the percent of control. (*p < 0.05, **p < 0.01).
due to various mechanisms such as Bcl-2 family antiapoptosis proteins overexpression and marked increase of glutathione synthesis [26,27]. As a result, patients have to accept higher doses of cisplatin and this strategy will expose patients to higher risks of side effects such as nephrotoxicity and neurotoxicity [28]. Therefore, the development of chemosensitization strategies and chemosensitizing agents are of important clinical implications.

In the present study, we found that combination of shikonin and cisplatin exhibited synergistic anticancer effects. Shikonin in combination of cisplatin trigger apoptosis of HCT116 cells in vitro. Besides, HCT116 cells in tumor xenografts also display apoptotic feature in vivo, such as the increased expression of cleaved caspase-3 was observed after combined treatment. It is well known that mitochondria play a major role in apoptosis triggered by many agents [13,29]. The mitochondrial membrane potential (ΔΨm) play a key role in triggering mitochondria-mediated apoptosis [30]. In this study, shikonin and cisplatin in combination synergistically induced mitochondria-mediated apoptosis in HCT116 cells, as evidenced by the activation of caspase-9 and a decrease in the mitochondrial membrane potential. The permeabilization of mitochondria is closely regulated by Bcl-2 family expression, which could be classified into two groups, pro-apoptotic proteins such as Bax and Bad, and anti-apoptotic proteins such as Bcl-2 and Bcl-xL [31]. Bcl-2 family proteins could bind to the membrane of mitochondria to regulate the ΔΨm in response to apoptotic stimulation [32]. In this study, combined treatment with shikonin and cisplatin significantly decreased Bcl-2 expression, but increased Bax expression. The imbalance of Bcl-2 family expression finally
resulted in the mitochondrial dysfunction and induced mitochondria-mediated apoptosis in HCT116 cells. These results indicate that combined treatment-induced mitochondria-mediated apoptosis of HCT116 cells was fulfilled by regulation of Bcl-2 family expression.

Cancer cells with increased oxidative stress are likely to be more vulnerable to damage by further ROS insults induced by exogenous agents [11,33]. Therefore, manipulating ROS levels by redox modulation is a good way to selectively kill cancer cells without causing significant toxicity to normal cells [10]. In the previous studies, shikonin displays broad-spectrum anticancer activity against a series of human cancer cells through ROS-mediated apoptosis [16,18]. And the mechanism studies reveal that inhibition of ROS accumulation can effectively prevent cancer cells from shikonin-induced apoptosis. Therefore, we assessed the synergistic effects of shikonin and cisplatin. The present study to show that shikonin potentiates the cytotoxic effect of cisplatin in colon cancer cells in vitro and in vivo. Shikonin induced a robust increase in cisplatin-mediated apoptosis via ROS accumulation. To further characterize the importance of ROS in combined treatment, two antioxidants, GSH and NAC were employed. As anticipated, addition of GSH and NAC completely attenuated combined treatment-induced cell growth inhibition against HCT116 cells. Furthermore, combined treatment-induced activation of Bcl-2/Bax pathway was almost completely blocked by GSH, indicating that ROS acts as a key upstream signaling molecules involved in combined treatment-induced activation of mitochondrial pathway. Therefore, based on these results, we proposed that shikonin enhanced cisplatin-induced colon cancer cell apoptosis through generation of ROS.

In summary, we showed the ability of shikonin to enhance cisplatin-induced human colon cancer cell killing in vitro and in vivo. The suppression of apoptosis by GSH validates the critical
role of ROS in combined treatment-induced cell death. Our results suggested that combining low dose of shikonin with cisplatin can serve as a potential combination therapy for the treatment of human colorectal cancer. In addition, we also demonstrated that ROS production could be a good strategy for the development of new anti-cancer drugs.

Conflict of interest

The authors disclose no potential conflict of interest.

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References


