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Extracellular signal-regulated kinase, receptor interacting protein, and reactive oxygen species regulate shikonin-induced autophagy in human hepatocellular carcinoma

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ABSTRACT

Shikonin, a naphthoquinone derived from the Chinese medicinal plant *Lithospermum erythrorhizon*, shows potential to be a cancer chemotherapeutic agent. Our previous data demonstrate that high doses (about 6 μM) of shikonin induce apoptosis in human hepatocellular carcinoma (HCC) cells. Here, we discovered that a low dose of shikonin (2.5 μM) and a short treatment time (12 h) induced autophagy, as evidenced by the upregulation of microtubule-associated protein 1A/1B-light chain 3 (LC3)-II, the formation of acidic autophagic vacuoles (AVOs), and the punctate fluorescence pattern of GFP-LC3 protein. Next, we investigated the mechanism and found reactive oxygen species accumulation after shikonin treatment. The reactive oxygen species scavengers NAC and Tiron completely blocked autophagy. We further found activation of ERK by generation of reactive oxygen species and inhibition of RIP pathway, which are at least partially connected to shikonin-induced autophagy. Moreover, experiments *in vivo* revealed similar results: shikonin caused the accumulation of reactive oxygen species and phospho-ERK and thus induced autophagy in a tumor xenograft model. These findings suggest that shikonin is an inducer of autophagy and may be a promising clinical antitumor drug.

1. Introduction

Human hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related lethality worldwide, with over 662,000 deaths each year (Organization, 2006), and approximately half of them occur in China because of hepatitis B transmission (Sun et al., 2002). Surgical resection or liver transplantation can only be used for some selected patients without extensive disease, poor liver function, or extrahepatic metastases (Cillo et al., 2004; Obed et al., 2008). Percutaneous ethanol injection (Yamamoto et al., 2001), transcatheter arterial chemoembolization (Lee et al., 1997) and radiation therapy (Chen et al., 2006) are usually performed for unresectable tumors, but they are only suitable for small HCC tumors in the early phase. The usual outcome of HCC is very poor because only approximately 10–20% of tumors can be completely removed by surgery (Belghiti et al., 2003). Large, late-stage, metastatic, or unresectable HCC tumors will result in fatality within 3–6 months (Bruix and Sherman, 2011). Sorafenib is the only advanced HCC drug approved by the United States FDA (Kane et al., 2009). Therefore, the development of novel HCC drugs is urgently needed.

Shikonin, a type of naphthoquinone, is extracted from *Lithospermum erythrorhizon*, a Chinese herbal plant also call zicao, which have been broadly used to treat carbuncles, macular eruptions, measles, sore throat, and burns in China more than 2000 years ago (Chen et al., 2002). Shikonin has the ability to induce apoptotic cell death or trigger cell cycle arrest in various types of cancer cell lines such as A375-S2, Hela, and T24 (Wu et al., 2004a; Wu et al., 2004b; Yeh et al., 2007). The anti-tumor mechanisms of shikonin include the Bcl-2 family proteins (Hsu et al., 2004) and NF-κB pathways (Min et al., 2008). In our previous studies, we found shikonin can

Abbreviations: 3-MA, 3-Methyladenine; ALT, Alanine transaminase; AO, Acridine orange; AVO, Acidic vesicular organelles; CMC, Carboxymethyl Cellulose; CML, Chronic myelogenous leukemia; CCTCC, China center for type culture collection; CQ, Chloroquine; DCFDA, Dichlorofluorescein diacetate; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, Dimethyl sulfoxide; ERK, Extracellular signal-regulated kinase; FBS, Fetal bovine serum; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GFP, Green fluorescent protein; HCC, Hepatocellular carcinoma; JNK, c-Jun N-terminal kinase; LC3, Microtubule-associated protein 1A/1B-light chain 3; LD50, Median lethal dose; MAPK, Mitogen-activated protein kinase; MDA, Malondialdehyde; NAC, N-acetyl-L-cysteine; Nec-1, Necrostatin-1; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; PARP, Poly (ADP-ribose) polymerase; PBS, Phosphate buffered saline; PCD, Programmed cell death; RIP, Receptor interacting protein; RIPA buffer, Radioimmunoprecipitation assay buffer; SDS, Sodium dodecyl sulfate; SHK, Shikonin; SPF, Specific pathogen free; TEM, Transmission electron microscopy; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling

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induce CML cell apoptosis via the stress-related c-Jun-N-terminal kinase (JNK) pathway (Mao et al., 2008) and HCC apoptosis though the reactive oxygen species/AKT and reactive oxygen species/RIP/NF-κB pathways (Gong and Li, 2011). These findings suggest that shikonin could be a promising anti-cancer drug. However, its effects need to be understood in more detail, especially on other cell death pathways and the mechanisms of action.

Recently, autophagy has received much attention in the tumor therapy and oncogenesis fields (Gozuacik and Kimchi, 2004). Normally, autophagy is a physiological process that ensures that cells survive and overcome external adverse environments, such as nutrient deprivation, and internal cellular stresses, such as damaged mitochondria or other organelles (Ravikumar et al., 2010). Autophagy, as a survival mechanism, facilitates the maintenance of intracellular homeostasis by degrading long-lived proteins and damaged organelles and plays roles in many human diseases and physiological processes (Mathew and White, 2011). On the other hand, enhanced autophagy may cause autophagic cell death and become a tumor-suppressing pathway (Kimmelman, 2011). Autophagy defects in mice are associated with susceptibility to genomic damage and tumorigenesis (Takamura et al., 2011). Although the dual functions of autophagy in oncogenesis and cancer therapy are not well understood, investigation of the mechanisms of autophagy can promote the development of strategies for cancer prevention and treatment.

2. Material and methods

2.1. Cell culture and reagents

BEL7402 and Huh7 were purchased from CCTCC (Wuhan, China) and cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL) (HyClone). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

Shikonin was purchased from Enzo (Farmingdale, NY). Carboxy-H2DCFDA and MitoTracker Red were purchased from Invitrogen (Carlsbad, *CA*). Acridine orange (AO), 3-methyladenine (3-MA), N-acetyl-L-cysteine (NAC), and Tiron were obtained from Sigma-Aldrich (St. Louis, MO). PD98059 was from Cell Signaling Technology (Beverly, MA). Nec-1 was from Biomol (Plymouth Meeting, PA).

pRK-HA-RIP and its empty vector pRK were kept in our lab. The target sequence of RIP RNAi was CCACTAGTCTGACGGATAA, which were cloned into the pSUPER plasmid (Oligoengine, Seattle, WA). pSUPER-GFP siRNA (Oligoengine) was used as the negative control.

2.2. Autophagy detection

The pEGFP-LC3 plasmid was a gift from Dr. Tamotsu Yoshimori (National Institute of Genetics, Mishima, Japan). For GFP-LC3 detection, cells were transfected with pEGFP-LC3 for 24 h before receiving treatments. Then, cells were observed under a fluorescence microscope (Olympus BX51). Autophagic cells, which contained five or more GFP-LC3 green dots, were counted. For Acridine orange (AO) staining assay, cells were stained with acridine orange (1 μg/mL) at 37 °C for 30 min before observation. Under a fluorescence microscope, red acidic vesicular organelles (AVOs) stained by acridine orange in autophagic cells were visualized. To detect mitophagy, after shikonin-treatment, GFP-LC3 pre-transfected HuH7 cells were incubated with MitoTracker Red (100 nM) for labeling mitochondria at 37 °C for 30 min. and then observed under a confocal fluorescence microscopy.

2.3. Reactive oxygen species measurement

Reactive oxygen species accumulation was measured by flow cytometry (Epics XL, Beckman Coulter). After treatment, cells were incubated with 1 μM DCFDA at 37 °C for 20 min, and then flow cytometry analysis was performed. Data were processed using FlowJo software (Tree Star, OR).

2.4. Western blotting analysis

After treatment, cells were collected and then lysed in 1% SDS (sodium dodecyl sulfate) on ice. Next, cell lysates were heated to 95 °C for 20 min and then centrifuged at 12,000*g* for 10 min. The supernatant was subjected to quantitative analysis for protein concentration by using a Pierce BCA (bicinchoninic acid) Protein Assay Kit (Thermo scientific). Equal amounts of protein (20 μg) from each sample were separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electropheresis) (Amresco) and then transferred to PVDF (polyvinylidene difluoride) membranes (Millipore). After blocking for 1 h with 5% fat-free milk (Bio-Rad) in Tris-buffered saline and 0.1% Tween-20 (TBST) at room temperature, membranes were incubated in TBST containing 2% fat-free milk with specific primary antibodies at 4 °C overnight. The next day, membranes were washed with TBST for 10 min three times and then labeled by HRP-conjugated secondary antibodies in TBST containing 2% fat-free milk for 1 h at room temperature. After washing in TBST again, the protein immunoblots were exposed to film by a chemiluminescence method using HRP substrates (Millipore).

For western blotting *in vivo*, tumor samples were homogenized and sonicated in RIPA buffer (20 mM pH 7.5 Tris–HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride) on ice. Next, the lysates were centrifuged at 12,000*g* for 15 min at 4 °C, and then the supernatant was subjected to quantitative measurement of protein concentration and western blotting. The samples were then treated similarly to those in the *in vitro* western blot analysis.

The antibody against microtubule-associated protein 1 light chain 3 (LC3) was purchased from Sigma-Aldrich. Antibodies against full-length and cleaved parts of caspase-8,-9, and PARP, antibodies for detecting total ERK, phospho-ERK (Thr202/Tyr204), RIP, NF-κB p50, and RelA p65 were purchased from Cell Signaling Technology. The antibody against GAPDH and HRP-conjugated secondary antibodies against mouse and rabbit IgG were from Beyotimes (Nantong, China).

2.5. *in vivo* experiments

The nude mice xenograft model was widely used in determining *in vivo* anti-cancer effects of drugs. All animal experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Wuhan University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Wuhan University (Permit Number 2011032). All mice were housed in the SFP environment with a 12 h day/night cycle at room temperature (21 ± 2 °C). All cages had an independent ventilating system, supplied unlimited food and water, and fed no more than 5 mice per cage.

Male BALB/c SPF nude mice (4–6 weeks old, median 5 week old) were obtained from the Hunan SJA Laboratory Animal Co., Ltd. (Changsha, Hunan, China). 5×10^6 Huh7 cells suspended in 0.2 mL PBS were injected into the right axilla of each mouse. When the tumor volume reached 200–300 mm^3, 27 mice were randomly distributed into 3 groups ($n=9$). Mice were administered a gavage of 2.5 or 5 mg/kg body weight of shikonin suspended in 0.1% (w/v)