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Dihydrotanshinone I induced apoptosis and autophagy through caspase dependent pathway in colon cancer

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ABSTRACT

Background: Dihydrotanshinone I (DHTS) was previously reported to exhibit the most potent anti-cancer activity among several tanshinones in colon cancer cells. Its cytotoxic action was reactive oxygen species (ROS) dependent but p53 independent.
Purpose: To further study the anti-cancer activity of DHTS and its molecular mechanisms of action in colon cancer both *in vitro* and *in vivo*.
Methods: Caspase activity was detected by fluorescence assay. Apoptosis was detected by flow cytometry and TUNEL assay. Protein levels were analyzed by western blotting. Knockdown of target gene was achieved by siRNA transfection. Formation of LC3B puncta and activation of caspase-3 were detected by confocal fluorescence microscope. *In vivo* anti-colon cancer activity of DHTS was observed in xenograft tumors in NOD/SCID mice.
Results: Anti-colon cancer activity of DHTS by inducing apoptosis and autophagy was observed both *in vitro* and *in vivo*. Mitochondria mediated caspase dependent pathway was essential in DHTS-induced cytotoxicity. The apoptosis induced by DHTS was suppressed by knockdown of apoptosis inducing factor (AIF), inhibition of caspase-3/9 but was increased after knockdown of caspase-2. Meantime, knockdown of caspase-2, pretreatment with Z-VAD-fmk or NAC (N-Acety-L-Cysteine) efficiently inhibited the autophagy induced by DHTS. A crosstalk between cytochrome c and AIF was also reported.
Conclusion: DHTS-induced caspase and ROS dependent apoptosis and autophagy were mediated by mitochondria in colon cancer. DHTS could be a promising leading compound for the development of anti-tumor agent or be developed as an adjuvant drug for colon cancer therapy.

Introduction

The search for effective regimens with minimal adverse effects for the treatment of colon cancer remains the top priority of cancer research. So far, a number of traditional Chinese medicinal preparations and their components have been reported to exhibit promising anti-cancer activities, they are potential candidates for anti-cancer drug development. Meanwhile, the mechanisms of beneficial preventive and therapeutic effects achieved by traditional and complementary medicine are currently being deciphered in molecular medicine. Our preliminary *in vitro* studies showed that several tanshinones had potent anti-cancer activity in various colon and liver cancer cells and dihydrotanshinone I (DHTS) was the most potent compound (Wang et al., 2013). The anti-cancer activity of DHTS was reactive oxygen species (ROS) but not p53 dependent. However, the underlying mechanisms of cytotoxic action of DHTS are still not well understood.

Programmed cell death, which has been recognized since the 1960s, is any type of cell death during which the cell uses specialized intracellular machinery to kill itself. Two important types of programmed cell death are apoptosis and autophagy. Indeed, current anticancer treatments, including many chemotherapeutic agents as well as ionizing radiation therapy, actually activate apoptosis to utilize the apoptotic machinery to kill cancer cells. However, autophagy can both stimulate and prevent cancer depending on the context. On one hand, cancer cells may utilize autophagy to survive with altered metabolism in the hostile tumor microenvironment, suggesting the potential of autophagy inhibition in cancer therapy. On the other

Abbreviations: DHTS, dihydrotanshinone I; ROS, reactive oxygen species; DAPI, 4′, 6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase dUTP Nick-end Labeling; O.C.T, optimal cutting temperature.

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hand, high levels of autophagy might directly lead to autophagic cell death in cancers.

Apoptosis can be mediated by extrinsic and/or intrinsic pathways, and caspase activation was observed in both pathways (Ouyang et al., 2012). Caspases are synthesized as inactive preforms and cleave to aspartate residues upon activation. Caspases can be divided into two distinct groups, the initiator caspases including caspase-8 and -9 as well as the executioner caspases, such as caspase-3 and -7. Initiator caspases are present in the cell as inactive monomers and their activation is promoted by dimerization, which happens when initiator caspases are recruited to large molecular weight protein complexes that act as signaling platforms (Fan et al., 2005; Lamkanfi and Kanneganti, 2010; Tait and Green, 2010; Woltering, 2010). Caspase-2 has been reported as an initiator caspase, its role is very special in apoptosis since caspase-2 gene produces several alternative splicing isoforms (Bouchier-Hayes and Green, 2012). The inclusion of exon 9 leads to an in-frame stop codon in caspase-2 short isoform (casp-2S) mRNA, thus producing a truncated protein that inhibits cell death. Whereas the exclusion of exon 9 results in caspase-2 long isoform (casp-2 L) mRNA, which produces protein product inducing cell death (Brynychova et al., 2013; Han et al., 2013; Iwanaga et al., 2005; Puccini et al., 2013). Caspases cleave a number of different substrates in the cytoplasm or nucleus, leading to many morphologic features of apoptotic cell death. Activation of caspases can be initiated from different entry points, such as the plasma membrane upon ligation of death receptor (receptor mediated pathway) and the mitochondria (mitochondria-mediated pathway), etc. The mitochondrial pathway is initiated by the release of apoptotic factors such as cytochrome c, apoptosis inducing factor (AIF) and endonuclease G from the mitochondrial intermembrane space by mitochondrial outer membrane permeabilization (MOMP), which is a complex process that involves numerous molecular players including Bcl-2 family (Brenner and Grimm, 2006; Kuwana and Newmeyer, 2003). The release of cytochrome c into the cytoplasm subsequently triggers caspase-3/7 activation through formation of the cytochrome c/Apaf-1/caspase-9 apoptosome complex.

In this study, the anti-cancer activity of DHTS and its molecular mechanisms of action in colon cancer were investigated. DHTS was reported to induce apoptosis and autophagy in colon cancer both *in vitro* and *in vivo*. Caspase activation accompanied by the crosstalk between AIF and cytochrome c played the dominant role in DHTS-induced cytotoxicity.

Materials and methods

Materials

HPLC grade authentic standard of DHTS was purchased from Chengdu Congon Bio-tech Co., Ltd. (Sichuan, China). Z-VAD-FMK (pan caspase inhibitor), Z-IETD-FMK (caspase-8 inhibitor), Ac-DMQD-CHO (caspase-3 inhibitor IV) and Z-LEHD-FMK (caspase-9 inhibitor) were from Calbiochem (Darmstadt, Germany). Ac-DEVD-AMC (caspase-3/7 substrate), Ac-IETD-AMC (caspase-8 substrate) and Ac-LEDH-AMC (caspase-9 substrate) were from EMD Millipore (Darmstadt, Germany). NucBuster TM Protein Extraction Kit was from EMD Biosciences (Darmstadt, Germany). Mitochondria extraction kit for cells was from Millipore (Billerica, MA). Primary antibody of GAPDH was purchased from CHEMICON. Primary antibody of AIF was from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies were from Cell Signaling. FlexiTube siRNA for human AIFM1 (Gene accession: NM_001130846), HS-Cap2-10 (Gene accession: NM_001224) and All Stars Neg. SiRNA AF 555 were purchased from QIAGEN Science (Germantown, Germany). Jetprime Transfection reagent was from Polyplus transfection SA (St. Louis, MO, Illkirch FRANCE). Antibodies of Alexa-Fluor 488 goat anti-rabbit, Alexa-Fluor 488 goat anti-mouse, Alexa-Fluor 555 donkey anti-mouse were obtained from Molecular Probes (Eugene, OR). Unless otherwise specified, all chemicals used in this study were purchased from Sigma (St. Louis, MO).

Cell culture

Human colon cancer HCT116 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD) and was routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; GIBCO/BRL, NY), 100 mg/l penicillin G and 100 U/ml streptomycin sulfate at 37 °C in 5% CO_2.

RNA interference

The expression of AIF or caspase-2 was efficiently lowered using predesigned target-specific siRNA purchased from Qiagen Science. FlexiTube siRNA was transfected into cells using Jetprime™ (Polyplus-transfection Inc.) following the protocol provided. All Stars Neg. siRNA AF 555 served as sham control siRNA.

Apoptosis analysis

Cells were seeded and cultured overnight in 24-well plate. DHTS were then added into the medium except the control and vehicle control groups. Cells with 0.1% (*v*/*v*) DMSO served as vehicle control. Apoptosis was detected after 24 h treatment by flow cytometry. To verify the role of AIF and caspase-2 in the apoptotic activity of DHTS, apoptosis in cells with AIF or caspase-2 knockdown was determined. The role of caspases in apoptotic activity of DHTS was also defined. In this regard, cells were pretreated with Z-VAD-fmk, Z-IETD-FMK or Z-LEHD-FMK (40 μM) for 1 h before DHTS incubation.

Activity assay of caspases

Cells were treated with various concentrations of DHTS (3.13–20 μM) for 48 h. For the activity assay, Ac-DEVD-AMC (1 μg/μl), Ac-IETD-AMC (1 μg/μl) or Ac-LEDH-AMC (1 μg/μl) and cell lysate were added into Protease Assay Buffer in 96-well plate. Reaction mixtures with lysis buffer were used as negative controls. Cells treated with DMSO (0.1%) were treated as vehicle control. The reaction mixtures were incubated for 1 h at 37 °C. The AMC liberated from the substrates was measured using spectrofluorometer of Victor 2 plate reader (Perkin Elmer, Massachusetts, USA) with an excitation wavelength of 380 nm and an emission wavelength of 430 nm.

Detection of AIF, cytochrome c, Bax, Bcl-xl, LC3B-I/II and p62

Expression of AIF, cytochrome c, Bax, Bcl-xl, LC3B-I/II and p62 was detected by western blotting using routine method. In brief, samples from cell culture or tissue were harvested and lysed in protein lysis buffer at 4 °C for 30 min. After lysis, samples were centrifuged at 16,000 × g at 4 °C for 20 min. The protein in the supernatant was collected and measured with Pierce® BCA Protein Assay Kit according to the manufacturer's protocol. Proteins were subjected to SDS–PAGE (8–12%) and detected by western blotting. To detect the role of ROS in caspase-3 cleavage and LC3B-II, cells were pretreated with NAC (2 mM) for 1 h. To detect the autophagic flux, cells were cotreated with bafilomycin A1 (BAF) (100 nM), a blocker of autophagic degradation. Equal amount of proteins were resolved by SDS–PAGE followed by a standard immunoblotting procedure and developed using an ECL development kit (GE healthcare, UK limited).

Detection for the translocation of cytochrome c, AIF, Bax, Bcl-xl and RAPR cleavage

Cell fractions including mitochondrial protein, cytoplasmic protein and nuclear protein were extracted according to protocols provided by respective kits. For isolation of whole-cell protein, cells were