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rBTI induces apoptosis in human solid tumor cell lines by loss in mitochondrial transmembrane potential and caspase activation

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ABSTRACT

The molecular mechanisms and the possible effects of a recombinant buckwheat trypsin inhibitor (rBTI) on the induction of apoptosis in the human solid tumor cells (EC9706, HepG2 and HeLa) were investigated. An MTT assay showed that rBTI could specifically inhibit the growth of solid tumor cells in a dose- and time-dependent manner. Analysis by flow cytometry indicated that the apoptosis of several tumor cells increased after treatment with rBTI in range of 6.25–50 μg/ml. DNA electrophoresis analysis showed the 'DNA ladder', typical of apoptosis. rBTI-induced apoptosis was shown to involve Bax and Bak up-regulation, Bcl-2 and Bcl-xl down-regulation, release of cytochrome *c* from the mitochondria to the cytosol, activation of caspase-3 and -9 and disruption of the mitochondrial transmembrane potential ($\Delta\psi_m$). The z-DEVD-fmk caspase-3 inhibitor significantly inhibited rBTI-induced apoptosis. We concluded that rBTI can induce the apoptosis in several types of human solid tumor cells and promotes apoptosis through the mitochondrial apoptotic pathway.

1. Introduction

Apoptosis is a cell-initiated death course that is controlled by changes in gene expression and activation, and is one of the important mechanisms in maintaining the stability of an organism. It is quite significant in embryogenesis, development of organs and maintaining healthy organisms (Lockshin and Zakeri, 2004; Ashkenazi and Dixit, 1998; Green and Reed, 1998). At the same time, it plays a very important role in the development and treatment of cancer. It has been well established that apoptosis is tightly regulated by a set of genes that promote either apoptosis or cell survival. Although a number of stimuli appear to trigger apoptosis, there are two major signaling pathways of apoptosis: the extrinsic pathway, which acts through ligand-mediated activation of death receptors on the cell surface, and the intrinsic pathway, which acts through the mitochondria. Mitochondria play a critical role in the regulation of various apoptotic processes including drug-induced apoptosis. Both the extrinsic and the intrinsic pathways lead to the caspase cascade, activating a series of caspases, which then leads to cell death (Khosravi-Far and Esposti, 2004; Jacotot et al., 1999; Wang, 2001).

Protease inhibitors are widely distributed in nature, found in many kinds of animals, plants and microorganisms. In particular, serine protease inhibitors from plants are well-known defense compounds that also regulate endogenous proteases. These proteins that are expressed in developing seeds are assumed to play an important role in inhibiting trypsin and chymotrypsin of external origin. Two major serine protease inhibitors, Kunitz inhibitors and Bowman–Birk inhibitors (BBIs), have been extensively studied in plants (Ryan, 1990). They play key regulatory roles in many biological processes, including the blood coagulation system, the complement cascade, apoptosis and the hormone processing pathways (Azzouz et al., 2005; Enari et al., 1995; Inoue et al., 2005). In recent years, it has been found that proteinase inhibitors can induce apoptosis of cancer cells *in vitro*; therefore, proteinase inhibitors have been receiving attention as potential anti-cancer agents. It has been reported that the Bowman–Birk family of inhibitors, obtained from soybeans and other legumes, are potentially nutritionally relevant anti-carcinogens, particularly with respect to colon cancer (Gladysheva et al., 2001; Foehr et al., 1999). The manufacture and application of proteinase inhibitors in anti-carcinogens treatments have also made good progress, and have taken an important role in curing malignant tumors. At the present time, research in the area of recombinant proteinase inhibitors as anti-neoplastic medical treatments is still not evident. Very little is known about recombinant proteinase inhibitor anti-carcinogens drug.

Buckwheat (*Fagopyrum Esculentum Moench*) is a crop grown for the floury endosperm of its seeds (achenes), with worldwide cultivation, especially in Asia, Russia, Europe, North America, and Australia. Buckwheat contains a rich supply of amino acids,

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abundant vitamins B_1 and B_2, dietary fiber, proteins, minerals and vitamin P (Skrabanja et al., 2001; Park et al., 1997; Pomeranz, 1983). Due to its unique chemical and bio-activity properties, buckwheat has many uses in food products and medicine. Several protease inhibitors from buckwheat seeds have been reported (Belozersky et al., 1995). Among them, a buckwheat inhibitor (BWI)-1 protein extracted from common buckwheat seeds with a molecular weight of 7.7 kDa is a potato inhibitor I family member (Dunaevsky et al., 1997). It was found that BWI-1 and BWI-2a extracted from buckwheat seeds could inhibit T-acute lymphoblastic leukemia cells (Park and Ohba, 2004).

However, very little is known about the molecular mechanisms and the toxicity of protease inhibitors from buckwheat. Previously, we made high purity recombinant buckwheat trypsin inhibitor (rBTI) by cloning, expression and one-step affinity purification (Li et al., 2006; Zhang et al., 2007). A homology analysis showed that the amino acid sequence of rBTI obtained in our laboratory is totally identical to that of BWI-1, and its molecular size and inhibitory activity are similar to those of BBIs. The analysis of inhibitory activity showed that the rBTI could strongly inhibit trypsin in specific activity assays. In addition, this primary investigation indicated that rBTI can induce apoptosis in the human leukemia K562 cells (Wang et al., 2007), but was much less toxic to normal human peripheral blood mononuclear cells (PBMCs). However, the mechanism of rBTI-induced apoptosis and its effect on other kinds of cancer cells are poorly understood. To further evaluate the feasibility of rBTI as an inducer of apoptosis and to explore its potential application as an anti-cancer agent, the effects of rBTI on the induction of apoptosis of the human solid tumor cell lines (EC9706, HepG2 and HeLa) were investigated in this study. Additionally, we focused on the molecular mechanisms and the possible pathways involved in rBTI-induced apoptosis of tumor cells.

2. Materials and methods

2.1. Chemicals and reagents

RPMI 1640 was purchased from Gibco Life Technologies (NY, USA). Fetal calf serum (FCS) was purchased from the Institute of Hematology (Hang Zhou, China). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma (St Louis, MO, USA). Annexin V-FITC Apoptosis Detection Kit was obtained from Pharmingen-Becton Dickinson (San Diego, CA, USA). Apoptosis DNA Ladder Detection Kit, cytochrome *c* Releasing Apoptosis Assay Kit, Mitocapture Mitochondrial apoptosis detection kit, Caspases Colorimetric Assay Kit, and z-DEVD-fmk (caspase-3 inhibitor) were purchased from BioVision (Mountain View, CA, USA). An enhanced chemiluminescence (ECL) kit was purchased from Amersham (GE Healthcare, UK). Classical Total RNA Isolation Kit was from Bio Basic Inc. (Ontario, Canada). Antibodies against Bcl-2, Bcl-XL, Bax, Bak, and actin were purchased from Santa Cruz Biotechnology, Inc. (CA, USA).

2.2. Cell lines and culture

The human esophagus cancer cell line EC9706 (was kindly presented by Prof. Ming-rong Wang of Institute of Tumor, Chinese Academy Medical of Sciences, Beijing, China), the human hepatoma cell line HepG2, human cervical carcinoma cell line HeLa, human embryonic kidney cell strain HEK 293 (were kindly presented by Prof. Quan Chen of Institute of Zoology of the Chinese Academy of Sciences, Beijing, China), and human normal liver cell strain HL-7702 (was kindly presented by China Institute for Radiation Protection) were cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated FCS with 8 U/ml gentamycin sulfate, 15 mM HEPES and in a humidified 5% CO_2 atmosphere.

2.3. Cell growth inhibition assay

Recombinant buckwheat trypsin inhibitor (rBTI) was obtained according to our Lab's protocol (Zhang et al., 2007). Human carcinoma cell lines (EC9706, HepG2, and HeLa), human embryonic kidney cells HEK 293, (which are considered to have more normal characteristics than cancer cells), and human normal liver cells HL-7702 were plated in 96-well microtiter plates at a density of 1×10^6 cells/ml in 100 μl of the complete RPMI-1640 medium. After cells were permitted to adhere for different time points, fresh medium, containing various concentrations of rBTI (6.25–100 μg/ml), BTI (nature buckwheat trypsin inhibitor at 12.5–100 μg/ml), or the mutant R, D-rBTI (12.5–100 μg/ml) was applied for indicated times. Cells were incubated at 37 °C in 20 μl of MTT (5 mg/ml) for 4 h. After the medium and MTT were removed, 150 μl of DMSO was added to each well, and then placed on a plate shaker for 5 min at room temperature. For each well, absorbance at 570 nm was measured using a microtiter plate ELISA reader (Bio-Rad model 550). Cell survival rate was calculated as the percentage of MTT inhibition as follows: Percentage of survival = (mean experimental absorbance/mean control absorbance) × 100%.

2.4. Staining of apoptotic cells with DAPI

After treatment with 50 μg/ml rBTI at 37 °C for 24 h, cells were washed with 0.1 mol/L PBS (pH 7.2) and re-suspended in the fixation solution (4% paraformaldehyde) for about 10 min. 100 μl of cell suspension (1×10^6 ml^{-1}) was stained with 5 μl of DAPI (2 μg/ml) for 10 min. Apoptotic cells were evaluated by fluorescence microscopy.

2.5. Flow cytometric analysis of cell apoptosis

The flow cytometric analysis of annexin V-PITC and PI-stained cells was performed using the Apoptosis Detection Kit according to the manufacturer's protocol. After 1×10^6 cells were treated with designated concentrations of rBTI (0 μg/ml (control), 12.5–100 μg/ml), cells were collected and washed twice with PBS. After the cells were centrifuged at 2000 × *g* for 5 min, they were re-suspended in 500 μl of binding buffer, containing 5 μl of fluorescence-conjugated annexin V, and 2.5 μl of PI, and incubated for 30 min in the dark at room temperature. Following this, the cells were analyzed by flow cytometry (Elite ESP, Coulter, USA) using Cell Quest software (Darzynkiewicz et al., 1992).

2.6. DNA fragmentation analysis

DNA fragmentation analysis was performed using an Apoptosis Ladder Detection Kit. After incubation with the designated concentrations of rBTI (0 μg/ml (control), 12.5–100 μg/ml), 1×10^7 cancer cells were harvested by pipetting and rinsing with ice-cold PBS. The cell pellets were re-suspended in 100 μl of lysis buffer, incubated for 10 min at 37 °C, and centrifuged at 5000 × *g* for 10 min. Genomic DNA was extracted via the kit's manufacturer's protocol. The DNA was then electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. The gel was visualized and photographed under ultraviolet light.

2.7. Measurement of mitochondrial transmembrane potential (MMP, $\Delta\psi_m$)

We next investigated the changes in MMP using the Mitocapture Apoptosis Detection kit. The kit utilizes Mitocapture, a cationic dye that fluoresces differently in healthy vs. apoptotic cells by fluorescence microscopy using a band-pass filter or analyzed by flow cytometry using FITC channel for green monomers and PI channel for red aggregates. With normal mitochondrial function, MMP is high and the red fluorescence is predominant. However, when there is mitochondrial injury, MMP is reduced, leading to an increase in green fluorescence. Briefly, after being treated with 50 μg/ml rBTI for the indicated amounts of time, cells were collected and washed twice with PBS. Cells were then incubated with 1 μl/ml of Mitocapture in warm PBS for 15 min at 37 °C, then washed and re-suspended in PBS. Fluorescence was analyzed by fluorescence microscopy using a band-pass filter or by flow cytometry using the FITC channel for green monomers and the PI channel for red aggregates.

2.8. Preparation for cytosolic and mitochondrial fractions

After treatment with 50 μg/ml of rBTI for indicated times, EC9706 cells were prepared for staining using the cytochrome *c* Releasing Apoptosis Assay kit according to the manufacturer's protocol. Briefly, 1.0×10^6 cells were pelleted and washed once with ice-cold PBS. Cells were resuspended in Cytosol extraction buffer mix containing DTT and protease inhibitors, and incubated on ice for 10 min. The lysates were then subjected to centrifugation at 700 × *g* for 10 min at 4 °C and the supernatants were centrifuged again at 10,000 × *g* for 30 min at 4 °C. These supernatants were collected as cytosolic fractions, and the pellets were resuspended in mitochondrial extraction buffer mix containing DTT and protease inhibitors for 10 s and used as mitochondrial fractions.

2.9. Western blot analysis

To prepare the whole-cell extract, cells were washed twice with cold PBS, lysed in cold RIPA extraction buffer (1 × PBS, 0.5% deoxycholic acid sodium salt, 1% Triton X-100, 0.1% SDS, 1 mM PMSF, 1% leupeptin, and 1% aprotinin) for 30 min on ice. The lysates were centrifuged at 12,000 × *g* for 10 min at 4 °C, the supernatants collected and protein concentration determined by Bradford's method. Proteins were separated by 12% SDS–polyacrylamide gel electrophoresis (20 μg lane) and electroblotted onto nitrocellulose membrane. Membranes were incubated in blocking solution consisting of 5% non-fat milk in TBST (10 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween-20) for 1 h, then immunoblotted with the primary antibody rabbit anti-Bcl-2 antibody, Bax antibody, Bcl-XL antibody, Bim antibody, cytochrome *c* antibody, and actin antibody, and subsequently by a secondary anti-rabbit IgG antibody conjugated with horseradish peroxidase. Bands were detected using an ECL