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

Yu-Ying Li, Zheng Zhang, Zhan-Hua Wang, Hong-Wei Wang, Li Zhang, Lei Zhu

Key Laboratory for Chemical Biology and Molecular Engineering of Ministry of Education, Institute of Biotechnology, Shanxi University, 36 Wucheng Road, Taiyuan, 030006, China

Blood Center of the Second Hospital in Shanxi Medical University, Taiyuan, 030001, China

A R T I C L E   I N F O

Article history:
Received 1 February 2009
Received in revised form 20 May 2009
Accepted 21 May 2009
Available online 28 May 2009

Keywords:
rBTI
Apoptosis
Mitochondrial transmembrane potential
Cytochrome c
Caspase

A B S T R A C T

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 (ΔΨm). 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.

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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 protease inhibitors can induce apoptosis of cancer cells in vitro; therefore, protease 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 protease 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,
abundant vitamins B₁ and B₂, dietary fiber, proteins, minerals and vitamin P (Skrbanja 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 (BW1)-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 BW1-1 and BW1-2a 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 BW1-1, and its molecular size and inhibitor 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, the 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). MTI (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma (St Louis, MO, USA). Annexin V-PTC 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 (kindly provided 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 provided by Prof. Quan Chen of Institute of Zoology of the Chinese Academy of Sciences, Beijing, China), the human hepatoma cell line HepG2, human cervical carcinoma cell line HeLa, human embryonic kidney cell strain HEK 293 (which were kindly provided 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 provided 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% CO₂ atmosphere.

2.3. Cell growth inhibition assay

Recombinant buckwheat tryptsin 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 × 10⁴ 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), BTT (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 microplate 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-hyde) for about 10 min. 100 μl of cell suspension (1 × 10⁶ 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-PTC and PI-stained cells was performed using the Apoptosis Detection Kit according to the manufacturer's protocol. After 1 × 10⁶ 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 × 10⁶ 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, ΔΨₘ)

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 × 10⁶ 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.
2.10. Reverse transcription-PCR

Total RNA in the cells after being treated with 50 μg/ml rBTI for 24 h was extracted using a Classical Total RNA Isolation Kit according to the manufacturer’s instructions. The reverse transcription reaction was performed using oligo (dT) and AMV-RT reverse transcription enzyme (5 U/ml). The newly synthesized cDNA was amplified by PCR. The following primer pairs were used: for Bcl-2 primers, 5′-GGAGGATTGTGGCCTTCTTTGAG-3′ (forward); 5′-TATGCACCCAGAGTGATGCAGGC-3′ (reverse); for Bcl-xL primers, 5′-GGAGCTGGTGGTTGACTTTCT-3′ (forward); 5′-CCGGAAGAGTTCATTCACTAC-3′ (reverse); for Bak primers, 5′-TGAAAAATGGCTTCGGGGCAAGGC-3′ (forward), 5′-TCATGATTTGAAGAATCTTCGTACC-3′ (reverse); for GAPDH, as an internal control, 5′-GTCAACGGATTTGGTCGTATT-3′ (forward) and 5′-AGTCTTCTGGGTGGCAGTGAT-3′ (reverse). Amplification cycles were: 94 °C for 5 min, then 30 cycles at 94 °C for 30 s, different temperature for 30 s (GAPDH for 45 °C; Bax and Bak for 49 °C; Bcl-2 for 56 °C, Bcl-xL for 61 °C), 72 °C for 60 s, followed by 72 °C for 5 min. The amplified products were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining. The gel was photographed under ultraviolet light.

2.11. Determination of caspase activity

Caspases activities were determined by colorimetric assays using caspase-3, caspase-8, and caspase-9 activation kits according to the manufacturer’s protocol. After treated with designated concentrations of rBTI (0–50 μg/ml), cell lysates were prepared by incubating 2 × 10^6 cells/ml in cell lysis buffer for 10 min on ice. Lysates were centrifuged at 10,000 × g for 1 min. The supernatants (cytosolic extracts) were collected and protein concentration was determined by the Bradford’s method using BSA as a standard. 100–200 μg protein (cellular extracts) was diluted in 50 μl cell lysis buffer for each assay. Cellular extracts were then incubated in 96-well microtiter plates with 5 μl of the 4 mM p-nitroanilide (pNA) substrates, DEVD-pNA (caspase-3 activity), IETD-pNA (caspase-8 activity), or LEHD-pNA (caspase-9 activity), for 2 h at 37 °C. Caspase activity was measured by cleavage of the above substrates to free pNA. Free pNA (cleaved substrates) was measured by absorbance at 405 nm in a microtiter plate reader. Relative caspase-3, caspase-8, or caspase-9 activity was calculated as a ratio the absorbance of treated cells to untreated cells.

2.12. Statistical analysis of data

Data were presented as mean ± SEM. Statistical analysis was carried out by ANOVA followed by a Dunnett t-test, *P < 0.05 was considered statistically significant.

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![Fig. 1. Effects of rBTI treatment on the growth of EC9706, HepG2, and HeLa cells (A–C). Cells were incubated for different time periods. Concentration of rBTI is 25 and 50 μg/ml. (D) Effects of rBTI treatment on EC9706, HepG2, HeLa, HEK 293, and HL-7702 cells. Cells were incubated with various concentrations of rBTI (0–50 μg/ml) for 36 h. (E) Effects of BTI, rBTI and R, D-rBTI treatment on EC9706 cells. Cells were incubated with various concentrations of BTI, rBTI or R, D-rBTI (0–50 μg/ml) for 36 h. Values were given as mean ± SEM of five independent experiments. *P < 0.05 compared with untreated cells.](image-url)
3. Results

3.1. Inhibitory effect of rBTI on human solid tumor cells

To investigate the effects of rBTI on the viability and apoptosis of solid tumor cells, we assayed dose responsiveness or time dependencies as determined by MTT. After cells were incubated separately with the target protein at 37 °C, the absorbance was measured using a microtiter plate reader at 570 nm. The effects on human esophagus cancer cells are shown in Fig. 1A and D, where the cells were treated with either 25 μg/ml or 50 μg/ml rBTI for 6 h, 12 h, 24 h, 36 h, or 48 h or different concentrations of rBTI (0–50 μg/ml) for 36 h. The results showed that rBTI could obviously inhibit the growth of EC9706 cells in a dose-dependent manner at all concentration levels, and that rBTI at 25 and 50 μg/ml could inhibit the growth of EC9706 cells in a time-dependent manner within 6–48 h of exposure. EC9706 cell growth was inhibited by 52.15 ± 3.7% at the 50 μg/ml level in all samples. The effects of rBTI on HepG2 human liver carcinoma cells (Fig. 1B and D), and HeLa human cervical adenocarcinoma cells (Fig. 1C and D) are shown. Cells were treated with either 25 μg/ml or 50 μg/ml rBTI for 6–60 h or various concentrations of rBTI (0–50 μg/ml) for 36 h. rBTI inhibited the survival of HepG2 and HeLa cells in a dose- and time-dependent manner. After cells treatment with 50 μg/ml rBTI, HepG2 cell growth was inhibited by 48.97 ± 4.6%, and HeLa cell growth was inhibited by 43.66 ± 3.0%.

As shown in Fig. 1D, the inhibition rates against growth in EC9706, HepG2, and HeLa cells at the low concentration level of 12.5 μg/ml rBTI, were 25.9 ± 2.4%, 16.9 ± 2.7%, and 12.3 ± 2.5%, respectively. As the treatment dose increased, the inhibitory effect on EC9706 was higher than on any of the other cancer cell line. To test cytotoxicity of rBTI in normal cells, we added rBTI to human embryonic kidney cells HEK293 and human normal liver cell line HL-7702. In contrast, the rate of growth inhibition of human HEK293 cells and human normal liver cells HL-7702 were not more than 11.2 ± 2.4% and 10.6 ± 1.8%, respectively, even at 50 μg/ml rBTI. Thus, rBTI seemed to have lower toxicity against normal cells than against cancer cells. Furthermore, comparison of the effects of rBTI on EC9706 cells with those of the positive control (BTI isolated from buckwheat seeds extract natural trypsin inhibitor) showed that rBTI and BTI inhibited the growth of EC9706 cells equivalently. The negative control (R, D-rBTI, with a mutated active site) showed only minimal growth inhibition (Fig. 1E). In EC9706 cells, when the concentration was 50 μg/ml, the growth inhibition of rBTI was 49.3 ± 5.8%; BTI was 50.1 ± 3.9%, while mutant (R, D-rBTI) was only 11.0 ± 3.0%. These results revealed that rBTI is highly toxic to EC9706 cancer cells. Together, these data indicated that rBTI inhibited the survival of three solid tumor cell lines, the strongest inhibition being in EC9706 cells.

3.2. rBTI induces apoptosis in human solid tumor cells

To define whether rBTI-mediated growth inhibition of several cancer cell lines (EC9706, HepG2 and HeLa) is associated with apoptosis, we used DAPI to investigate the changes in the cells’ nuclei. As clearly shown in Fig. 2A–I, normal cells (control cells without treatment) showed homogeneous staining of their nuclei, the percentages of apoptotic cells were not more than 6.5%. In contrast, when cells were treated with rBTI (50 μg/ml for 24 h), apoptotic cells showed irregular staining of their nuclei because of chromatin condensation and nuclear fragmentation. The apoptosis percentages were 66%, 32.9%, and 55.6%, respectively. Meanwhile, we investigated human normal liver cells HL-7702 with treatment rBTI.

In contrast, no features of apoptosis and necrosis were observed in HL-7702 cells (Fig. 2G–I), the percentages of apoptotic cells were not more than 11.3%.

Simultaneous staining with annexin V-PI/TC and PI distinguished between healthy, early apoptotic, late apoptotic and dead cells.
After 24 h of treatment with or without different concentrations of rBTI, apoptosis in human EC9706, HepG2, HeLa, and HL-7702 cells was analyzed by flow cytometry (Fig. 3A–D). Fig. 3A shows the percentages of apoptotic cells that were undergoing early apoptosis and late apoptosis (includes dead cells). EC9706 cells were 6.2% when the cells were not treated with rBTI (control group), but 12.5%, 26.9%, 30.1%, 66.0%, and 80.8%, when cells were treated with 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, or 100 µg/ml rBTI, respectively. It is important to note that when the concentration of rBTI reached 100 µg/ml, most of the EC9706 cells were apop-

Fig. 3. Flow cytometric analysis of EC9706, HepG2, HeLa, and HL-7702 cells treated with rBTI (6.25–100 µg/ml) for 24 h (A–D). The percentage of cells undergoing apoptosis was determined by three independent experiments. Cells (1 x 10^6) were treated with or without rBTI, washed with 50 mM cold PBS (pH 7.6), suspended in 100 µl of binding buffer, containing 5 µl of fluorescence-conjugated annexin V and 2.5 µl of PI and then incubated for 15 min at 37 °C. The induction of apoptosis by rBTI in cells was analyzed using flow cytometry.
Fig. 3. (Continued).

These results demonstrate that rBTI can induce the apoptosis of EC9706 cells in a dose-dependent manner. Fig. 3B shows the percentages of apoptotic cells that were undergoing early apoptosis and late apoptosis (includes dead cells). Untreated (control group), HepG2 cells were 5.6% in early and late apoptosis, respectively. When the cells were treated with 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml, 50 μg/ml, or 100 μg/ml rBTI, the apoptosis percentages were 14.2%, 27.8%, 34.0%, 27.8%, 14.2%, respectively.
Fig. 4. The fragmented DNA was extracted and analyzed on a 1.5% agarose gel in apoptotic EC9706, HepG2, and HeLa cells treated with rBTI. (A) Lane 1: untreated cells; Lanes 2–4: the cells were treated with 12.5 μg/ml, 25 μg/ml, or 50 μg/ml rBTI for 24 h, respectively; Mr: 100 bp DNA Ladder Marker. (B) Lane 1: untreated cells. Lanes 2 and 3: the cells were treated with 25 μg/ml or 50 μg/ml rBTI for 24 h, respectively. (C) Lanes 1–3: the cells were treated with 50 μg/ml, 25 μg/ml, or 12.5 μg/ml rBTI for 24 h, respectively; Lane 4: untreated cells. The results were representative of three independent experiments with similar results.

55.6%, and 62.5%, respectively. It is important to note that when the concentration of rBTI reached 100 μg/ml, most of the HepG2 cells were apoptotic or dead; the remaining living cells were only 36.5%. These results demonstrate that rBTI can induce the apoptosis of HepG2 cells in a dose-dependent manner. The same analysis was performed on HeLa cells. Fig. 3C shows that the percentages of apoptotic (early and late apoptosis) cells were 6.3% when the HeLa cells were not treated with rBTI (control group), but were 13.1%, 24.6%, 28.8%, 32.9%, and 35.2%, when the cells were treated with 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml, 50 μg/ml, or 100 μg/ml rBTI, respectively. Of note is that most of HeLa cells were in the early or late stages of apoptosis, and relatively few cells were dead. In conclusion, EC9706 cells had a greater apoptotic response than HepG2 or HeLa cells under the same concentration and duration of rBTI treatment. In contrast, the percentages of apoptotic (early and late apoptosis) were not more than 16.3% in HL-7702 cells from 6.25 to 100 μg/ml rBTI (Fig. 3D), suggesting that rBTI is much less toxic to normal cells. This result is in agreement with the data obtained by the MTT assay, and suggests that rBTI treatment of EC9706, HepG2, and HeLa cells showed a remarkable induction of apoptosis.

Fig. 5. Effect of rBTI on the changes in mitochondrial membrane potential. Cells were treated with 50 μg/ml rBTI for the indicated amounts of time. Mitocapture was added to the cells, and incubated at 37 °C in CO2 incubator for 15–20 min. Cells were analyzed immediately by flow cytometry. x-Axis is the fluorescence intensity, y-axis number of cells. Cells with normal mitochondria predominantly display the left-red fluorescence intensity, and apoptotic cells (the right of the green fluorescence intensity). Graphs were shown as representative results from three independent experiments.
3.3. DNA electrophoresis shows that rBTI induced a DNA ladder characteristic of apoptosis

DNA was cut into nucleosomes using DNase during metaphase. 200 base pairs or the integral DNA fragments were obtained. The DNA of apoptotic cells presented as a regular ladder in 1.5% agarose gel, which is typical of cells undergoing apoptosis (Herrmann et al., 1994; Nagata, 2000). For further confirmation of the effects of rBTI on the apoptosis of solid tumor cells, cells were treated with various concentrations of rBTI (12.5–50 μg/ml) for 24 h, and DNA fragmentation was investigated. Compared to untreated cells (control), rBTI treatment led to the appearance of ladder-like strips in the three solid tumor cell lines (Fig. 4A–C). EC9706 cells, HepG2 cells, and HeLa cells showed significantly increased DNA fragmentation after treatment with 50 μg/ml rBTI for 24 h. Together, these data indicated that rBTI-induced apoptosis in EC9706, HepG2, and HeLa cells.

3.4. Mitochondrial transmembrane depolarization during rBTI-induced apoptosis

Mitochondria play a central role in regulating cell death and survival. Mitochondrial permeability changes during apoptosis are an important event because they lead to collapse of the mitochondrial transmembrane electrochemical gradient, then cytochrome c is released into the cytoplasm (Wei et al., 2001; Kroemer and Reed, 2000). To determine the involvement of the mitochondrial mediated pathway in the rBTI-induced apoptosis of tumor cells, we investigated the changes in MMP (Δψm) using the MitoCapture Apoptosis Detection kit. After rBTI treatment of EC9706 cells, the mitochondrial membrane potential dye, analyzed by flow cytometry showed that resulted in a rapid dissipation of Δψm in a time-dependent manner (Fig. 5). Detection under a fluorescence microscope (abbreviated) showed the same changes in Δψm. A collapse of the Δψm was detected as indicated by loss of red fluorescence as early as 2 h after 50 μg/ml of rBTI treatment. This change reached maximum 12 h after rBTI treatment.

3.5. rBTI induces release of cytochrome c from the mitochondria to the cytosol

Cytochrome c release from mitochondria is a critical step in the apoptotic cascade as this activates downstream caspases. Cytochrome c is located in the space between the inner and outer mitochondrial membranes. An apoptotic stimulus, such as a change in MMP, triggers the release of cytochrome c from the mitochondria into the cytosol where it binds to Apaf-1. The cytochrome c/Apaf-1 complex activates caspase-9, which then activates caspase-3 and other downstream caspases (Liu et al., 1996; Li et al., 1997). To examine the release of cytochrome c in rBTI treated EC9706 cells, used Western blotting of both the cytosolic and mitochondrial fractions. EC9706 cells were treated with 50 μg/ml rBTI for the indicated amounts of time, and then the mitochondria and cytoplasm of cells were extracted and analyzed via SDS–PAGE electrophoresis, and Western blotting. As shown in Fig. 6, the first increase in cytochrome c levels occurred in the cytosolic fraction and was observed 3 h after rBTI treatment, followed by a significant increase in cytochrome c levels in the cytosol in a time-dependent manner, along with a concurrent decrease in mitochondrial cytochrome c, suggesting the involvement of mitochondria in rBTI-induced apoptosis.

3.6. Effect of rBTI on the Bcl-2 family

In order to determine whether Bcl-2 family proteins were involved in the apoptotic process induced by rBTI, Bcl-2 family anti-apoptotic gene, including Bcl-2 and Bcl-xl and pro-apoptotic gene, including Bax and Bak, were detected by RT-PCR after 50 μg/ml rBTI treatment for 24 h. As shown in Fig. 7A, Bcl-2 and Bcl-xl mRNA were decreased, Bax and Bak mRNA were increased, while GAPDH mRNA (as an internal control) was not changed. Next, we investigated apoptosis-related protein expression in EC9706 cells by Western blot. As shown in Fig. 7B, treatment with rBTI increased the expression level of Bax and Bak proteins and inhibited the expression level of Bcl-2 and Bcl-xl proteins.

3.7. rBTI induces activation of caspase-9 and caspase-3

The caspase family of proteins, which is comprised of aspartate-specific cystein proteases, also plays a critical role in regulating apoptosis. The key components of the biochemical pathways of caspase activation have recently been elucidated (Stennicke and Salvesen, 1998). In particular, caspase-3 is one of the key executers of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins. To confirm whether caspases are activated after cytochrome c release we measured the changes in caspase-9, -8, and -3 activity in EC9706 cells after rBTI treatment. As shown in Fig. 8A, a dose-dependent increase in the activity of caspase-9 and caspase-3 was observed in rBTI treated cells. There was a significant increase in caspase-9 and -3 activities after 24 h, which increased with the increasing concentration of rBTI. There was an initial 18.9% increase in caspase-3 activity after treatment with 6.25 μg/ml rBTI and an 80.3% increase after treatment with 50 μg/ml rBTI. Caspase-9 showed an initial 17.3% increase after...
treatment with 6.25 μg/ml rBTI and a 71.8% increase in activity after treatment with 50 μg/ml rBTI. However, the activity of caspase-8 was only slightly increased. To further define the role of caspase in rBTI-induced apoptosis, we used a general and potent inhibitor of caspase-3, z-DEVD-fmk. Cells were pretreated with 10 μM of z-DEVD-fmk 2 h before rBTI treatment. Apoptosis was detected 24 h after treatment. As shown in Fig. 8B, z-DEVD-fmk pretreatment significantly inhibited activity of caspase-3, attenuated rBTI-induced apoptosis. These findings suggest that rBTI-induced apoptosis in EC9706 cells was correlated with the activation of caspase-3.

4. Discussion

The death rate of cancer is only inferior to that cardiovascular disease. Although great progress has been made in the treatment of some cancers, the mortality of the majority of patients with solid tumors has not declined in the past 20 years; this is in part due to the comparatively low numbers of anti-tumor drugs. There has been much research to find new and effective drugs for the treatment of various tumors. Recent interest has been focused on protease inhibitors due to their unique role as pro-apoptotic proteins, and they are now being developed into targeted anti-tumor polymeric agents (Malkowicz et al., 2003).

Buckwheat is commonly used in food products and medicine due to its unique chemical and bio-active components. In a previous study, we showed that a recombinant buckwheat trypsin inhibitor (rBTI) induced apoptosis in K562 leukemic cells. It is still not used in clinic cancer treatment because its mechanism and effect on other kinds of cancers are poorly understood. In this paper, we found that rBTI had a significant inhibitory effect on the growth of three solid tumor cell lines. EC9706 cells exhibited a greater growth inhibitory response as compared with HepG2 and HeLa cells. Additionally, rBTI was less cytotoxic to human embryonic kidney cell HEK293 and human normal liver cell HL-7702 than to the cancer cell lines. An inactive mutant of rBTI (R, D-rBTI) could not induce the apoptosis of EC9706 cells when given at the same concentration as normal rBTI (12.5–50 μg/ml). The results also demonstrate that rBTI-induced apoptosis in the solid tumor cells (EC9706, HepG2 and HeLa) in a dose- and time-dependent manner.

The appearance of nuclear condensation and apoptotic bodies is universal characteristics of cells undergoing apoptosis. Fluorescence microscopy of cells stained with DAPI showed that the rBTI-treated EC9706, HepG2, HeLa cells had chromatin condensation and nuclear fragmentation. Quantification of apoptosis via annexin V and PI staining demonstrated that EC9706 cells were highly sensitive to rBTI. When the concentration of rBTI reached 50 μg/ml, the apoptosis of EC9706 cells was almost 50%; at 100 μg/ml, most of the EC9706 cells were dead. Together, these data indicate that rBTI possesses anticancer properties against several types of human solid tumor cells. This may have potential applications in the prevention and treatment of certain tumors.

Apoptosis, one of the most fundamental biological processes in eukaryotes is a well-defined cell-death process, in which individual cells die by activating intrinsic ‘suicide’ mechanisms (Huang and Strasser, 2000). Apoptosis, which is caused by a variety of insults, is thought to have a key role in killing cancer cells. A large number of studies confirm that almost all apoptosis stimulating factors can cause structural damage and mitochondrial dysfunction (Marchetti et al., 1996). Mitochondrial transmembrane potential (MMP, ΔΨm) and mitochondrial permeability changes during apoptosis play an important role in the process (Newmeyer and Ferguson-Miller, 2003). The mitochondria-mediated intrinsic apoptotic pathway is controlled by the members of the Bcl-2 family. The Bcl-2 family consists of pro-apoptotic and anti-apoptotic members (Nakazawa et al., 2003). During apoptosis, Bcl-2 family pro-apoptotic proteins, including Bim, Bax, Bak and Bid, can translocate to the outer membrane of mitochondria, promote the release of pro-apoptotic factors and induce apoptosis. Bcl-2 family anti-apoptotic proteins, including Bcl-2 and Bcl-XL, located in mitochondria, inhibit the release of pro-apoptotic factors and prevent apoptosis. When interacting with activated pro-apoptotic proteins, the anti-apoptotic proteins lose inhibiting ability of pro-apoptotic factors’ release, and again promote apoptosis. Alteration in the levels of anti- and pro-apoptotic Bcl-2 family proteins influences apoptosis (Yang et al., 2006). It causes the loss of mitochondrial membrane potential (ΔΨm), release of cytochrome c from the mitochondria intermembrane space toward the cytosol and proteolytic activation of caspase-9 and caspase-3 (Li et al., 1997; Bossy-Wetzel and Green, 1999).

In this experiment, we investigated the changes in MMP in order to confirm the involvement of the mitochondrial signaling pathway during rBTI-induced apoptosis. Our results demonstrate that treatment with rBTI dramatically induced loss of ΔΨm in EC9706 cells. We also examined the release of cytochrome c from the mitochondria to the cytosol, a prerequisite step for activation of caspase-9 in the mitochondrial apoptosis pathway. Indeed, the levels of cytochrome c in the cytosolic fractions were significantly increased by rBTI treatment in a time-dependent manner, and occurred concurrently with a decrease in mitochondrial cytochrome c. rBTI increased the level of the pro-apoptotic proteins Bax and Bak, decreased the level of the anti-apoptotic proteins Bcl-2 and Bcl-XL. The result indicated that rBTI-induced apoptosis in EC9706 cells by modulating Bcl-2 family proteins activity. Taken together, these data suggest the involvement of mitochondria in rBTI-induced apoptosis.

Activation of the family of caspases is known as a crucial mechanism for the induction of apoptosis. Caspases participate in a
cascade that is triggered in response to pro-apoptotic signals and culminates in the cleavage of a set of proteins, ultimately resulting in disassembly of the cell (Cohen, 1997; Salvesen and Dixit, 1997). Caspase-3 is one of the essential proteases in apoptosis, and is activated by caspase-9. Caspase-9 is considered the initiator caspase involved in the mitochondria-initiated intrinsic apoptotic pathway. Caspase-8 is considered the mediator signal transduction downstream of death receptors located on the plasma membrane. A colorimetric activity assay confirmed that rBTI-induced apoptosis could cause the activation of caspase-3 and caspase-9 in dose-dependent manner in tumor cells, while caspase-8 was slightly activated. More specifically, blockage of apoptosis by the caspase-3 specific inhibitor z-DEVD-fmk significantly inhibited rBTI-induced apoptosis indicates that rBTI-induced apoptosis is mediated by the initiator caspase involved in the mitochondria-initiated intrinsic apoptotic pathway.

In summary, the potential anticancer activity of rBTI against human solid tumor cells (EC9706, HepG2 and HeLa) was investigated. rBTI exhibited a strong inhibitory effect on the growth of EC9706, HepG2, HeLa cells in vitro, the greatest effect on EC9706 cells. The anticancer activity of rBTI could be attributed, in part, to its induction of apoptosis in cancer cells by involving Bax and Bak up-regulation, Bcl-2 and Bcl-xl down-regulation, causing mitochondrial release of cytochrome c into the cytosol, loss in ΔΨm and caspase-3 activation. The rBTI anti-tumor effects reported here are valuable, and further investigation may contribute to making rBTI a valuable anti-cancer drug.

Conflict of interest statement

None.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 30671084), the Science and Technology Department of Taiyuan (2008) and Committee of Science and Technology of Shanxi Province, China.

References
