Induction of Apoptosis by Buckwheat Trypsin Inhibitor in Chronic Myeloid Leukemia K562 Cells

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Buckwheat is an ancient and specialty grain in China. Due to its unique chemical and bio-activity components, buckwheat has been found to have many uses in food products and medicine. However, very little is known about the toxicity of protease inhibitors from buckwheat. Here, the possible effects of a recombinant buckwheat trypsin inhibitor (rBTI) on the induction of apoptosis of the human K562 cell line were investigated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays and flow cytometric analysis. MTT assay showed that rBTI could specifically inhibit the growth of K562 cells in a dose-dependent manner, but there were minimal effects on normal human peripheral blood mononuclear cells (PBMCs). Furthermore, comparison the effects of rBTI on K562 cells with those of negative control (BSA and the complex of BSA and rBTI) revealed that rBTI was highly toxic to K562 cells, and BSA hardly had any inhibition on proliferation in K562 cells. The analysis of flow cytometric indicated that the apoptosis of K562 cells were 31.0%, 32.8%, 35.3% and 52.1% after treated by rBTI in range of 12.5—100 μg/ml, respectively. The results suggested that rBTI can induce apoptosis of K562 cells and that it might be a potential protein drug of the trypsin inhibitor family.

Key words  trypsin inhibitor; buckwheat; K562 cell; apoptosis

Leukemia is a virulent blood disease, which has serious health implications in humans.1,2) Leukemia, as with many other cancers, tends to spread throughout the body without any symptoms. At present, chemical treatment is the main method for treatment of leukemia, but the side effects are a significant drawback.3) Although natural products have long been a fertile source of cures for cancer, there has been a desperate and continuous need for development of new anticancer drugs aimed at killing cancer cells. It is, therefore, imperative to find a new and effective medical treatment that can prevent and cure cancer.4—7) It is known that proteinase inhibitors have multiple functions, including the regulation of endogenous proteases during germination and protection of plants from insects and microorganisms.5—7) 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.8) 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.9) The manufacture and application of proteinase inhibitors in anti-neoplastic treatments have also made good progress, and have taken an important role in curing malignant tumors. Furthermore, the developments in molecular biology and genetic engineering have shortened the time scales needed for basic research, applied research and development of medical treatments, and the boundary between research and development is becoming more ambiguous. At the present time, research in the area of recombinant proteinase inhibitors as anti-neoplastic medical treatments is still not evident. Previously, a high-purity rBTI was obtained by cloning, expression and purification in our Lab., and the analysis of inhibitory activity showed that the recombinant buckwheat protease inhibitors could strongly inhibit trypsin in specific activity assays. In addition, the primary investigation indicated that rBTI can induce apoptosis of IM-9 cells, and almost no toxicity to normal human peripheral blood mononuclear cells (PBMCs) was shown.

To further evaluate the feasibility of buckwheat trypsin inhibitors to induce apoptosis and explore its potential application, in this paper we study the induction of apoptosis by rBTI in human K562 cells using different methods. These results are invaluable in terms of revealing the inhibitory mechanisms and the possible apoptosis-induction pathways that can occur in tumor cells.

MATERIALS AND METHODS

Chemicals RPMI 1640 was purchased from Gibco Life Technologies (NY, U.S.A.). Fetal calf serum (FCS) was purchased from the Institute of Hematology (Hang Zhou, PR, China). MTT was purchased from Sigma (St. Louis, MO, U.S.A.). Annexin V-FITC Apoptosis Detection Kit was obtained from Pharmingen-Becton Dickinson (San Diego, CA, U.S.A.). Apoptosis DNA Ladder Detection Kit was from Nanning KeyGEN Biotech. Co., Ltd. (China). All other chemicals used were of analytical grade.

Preparation of Target Protein cDNA sequence encoding buckwheat trypsin inhibitor (BTI) obtained in our Lab. (GenBank databases under accession no. AY335158) was digested with BamHI and HindIII from pGEM®-T Easy vector. The fragment of BTI was inserted into the pQE-31 expression vector to form the recombinant plasmid pQE-31-BTI. And then the E. coli M15 [pREP4] harbouring expression vector pQE-31-BTI was grown in 1 L of LB medium supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin at 37 °C. When the OD600 reached 0.6—0.7, the culture was treated with isopropyl-β-D-thiogalactopyranoside (IPTG) to the final concentration of 0.5 mM at 37 °C for 4 h.
target protein. The induced cells were harvested by centrifugation at 6000 \( g \) for 30 min at 4 \( ^\circ \) C, followed by ultrasonication for 1 h on ice. The cell debris was removed through centrifugation at 15000 \( g \) for 30 min at 4 \( ^\circ \) C. The supernatant was then loaded on to a Ni\(^{2+}\)-NTA affinity column, pre-equilibrated with buffer I (20 mm Tris–HCl buffer containing 10 mm imidazole and 500 mm NaCl, pH 7.4). The column was thoroughly washed with the same buffer until the optical density at 280 nm was returned to the baseline. The fusion protein of the bound hexahistidine tag at the N-terminus was eluted with buffer II (20 mm Tris–HCl buffer containing 300 mm imidazole and 500 mm NaCl, pH 7.4). The eluent was collected and dialyzed overnight against 20 mm phosphate buffer (pH 7.4) at 4 \( ^\circ \) C. During the expression and purification, rBTI was monitored by SDS-PAGE analysis and Coomassie Blue staining.

**Cell Culture and Treatment Conditions** Human chronic myeloid leukemia K562 cells were cultured in RPMI 1640 medium supplement with 8 U/ml gentamycin sulfate, 15 mm HEPES and 10% FCS in a humidified 5% \( \text{CO}_2 \) atmosphere. PBMCs obtained from volunteers were grown in the above-mentioned culture medium. Cells (1 \times 10^6) were plated in 96-well microtiter plates and in 24-well microtiter plates, and incubated in medium containing various concentrations of rBTI for the indicated times. Following this, apoptosis was examined using different methods.

**MTT Assay** The MTT test is a colorimetric assay that measures the percentage of cell survival. The K562 cells (1 \times 10^6) and normal human PBMCs (2 \times 10^6) were treated with rBTI (12.5—100 \( \mu \)g/ml) and were separately transferred to quadruplicate wells of 96-well microtiter plates at the indicated times. After incubating at 37 \( ^\circ \) C for 24 h, 20 \( \mu \)l MTT was added for 4 h; following this, 80 \( \mu \)l DMSO was added. The color intensity was measured using a microtiter plate reader (Bio-Rad model 550) at 570 nm. In another MTT assay, K562 cells were incubated separately with bovine serum albumin (BSA), rBTI and a mixture of BSA and rBTI (all protein concentrations were 12.5—100 \( \mu \)g/ml). After the same processes, as mentioned above, were performed, the color intensity was measured using a microtiter plate reader.

**DNA Electrophoresis Analysis** After incubation with the designated concentrations of rBTI, 1 \times 10^6 K562 cells were pelleted. The genomic DNA was extracted from the cells that were treated with rBTI using an Apoptosis Ladder Detection Kit. The DNA was then electrophoresed in 1% agarose gel and visualized by ethidium bromide staining. The gel was photographed under ultraviolet light.

**Flow Cytometry Analysis of Apoptosis** Flow cytometric analysis of annexin V-PITC and PI-stained cells was performed using the Apoptosis Detection Kit. A total of 1 \times 10^6 cells were washed in 50 mm cold phosphate buffer at pH 7.6. After the cells were centrifuged at 2000 \( g \) for 5 min, they were re-suspended in 100 \( \mu \)l of binding buffer. The mixture consisted of 5 \( \mu \)l of fluorescence-conjugated annexin V, and 2 \( \mu \)l of PI was added to the cells for 15 min at room temperature. Following this, the cells were analyzed for annexin V binding within 1 h using flow cytometry. 10

**Morphological Observation of Nuclei** After treatment with rBTI at 37 \( ^\circ \) C for 24 h, K562 cells were washed with cold phosphate buffer (pH 7.6) and re-suspended in the fixation liquid (4% paraformaldehyde) for about 10 min. The suspension was centrifuged gently, and the pellet was washed twice with phosphate buffer (pH 7.6). The cell suspensions were dropped onto the slides, and stained with 0.1 \( \mu \)g/ml Hoechst 33258 for 5 min. Nuclei morphology was observed by fluorescence microscopy.

**Statistics** Data were analyzed using ANOVAs and \( t \)-tests. A \( p \)-value of 0.05 or less was determined to be significant.

## RESULTS

**SDS-PAGE Analysis of Target Protein** rBTI was expressed in *E. coli* M15 [pREP4] under IPTG induction. As presented in Fig. 1A, Commassie Blue staining SDS-PAGE showed that, upon IPTG induction, rBTI was expressed at a high level, according for approximately 25% of the total cellular proteins. After cells had been disrupted by sonication, almost all the rBTI existed in the soluble supernatant, and no target protein was found in the insoluble fraction. The rBTI could effectively bind to Ni\(^{2+}\)-affinity resin and was almost completely retained in the column. In the flow-through fraction, no rBTI was detected with Commassie Blue staining SDS-PAGE. The protein was effectively eluted at 300 mm imidazole. From a 1-l flask culture, about 60 mg target protein was obtained, with a purity of >90%. Consistent with theoretical mass, the apparent molecular mass of His-tagged rBTI was about 9.3 kDa by analysis on tris-tricine SDS-PAGE (Fig. 1B) and western blotting (Fig. 1C). The purified protein was used for apoptosis of the tumor cells.

**Effects of rBTI on Cell Proliferation** The MTT assay was used to examine the effects of rBTI on K562 cells and their proliferation. After K562 cells and normal human PBMCs were incubated separately with the target protein at 37 \( ^\circ \) C for 24 h, the color intensity was measured using a microtiter plate reader at 570 nm. As shown in Fig. 2a, rBTI inhibited the survival of K562 cells in a dose-dependent manner, and the inhibitory effects showed statistically significance at the range of 12.5—100 \( \mu \)g/ml (\( p < 0.01 \)), but there were minimal effects on the rate of inhibition of normal human PBMCs. The 50% inhibitory concentration (IC\(_{50}\)) was 100 \( \mu \)g/ml. Furthermore, comparison the effects of rBTI on

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**Fig. 1. Electrophoresis Analysis of Target Protein**

(A) Sample subjected to 15% SDS-PAGE was stained with Comassie Blue. Lane 1 was total protein of cell harbouring pQE-31-BTI before IPTG induction. Lane 2 was supernatant after sonication with IPTG induction. Lane 3 was deposit after sonication with IPTG induction. Lane 4 was flow-through fraction of Ni\(^{2+}\) column. Lane 5, 6 were eluents at 50 mm and 100 mm imidazole, respectively. Lane 7 was purified target protein washed with 300 mm imidazole. (B) Tris-Tricine SDS-PAGE. Lane M showed molecular weight markers. Lane 8 was purified target protein with an approximate molecular mass of 9.3 kDa. (C) Western blotting analysis of target protein.
K562 cells with those of negative control (BSA and the com-
plex of BSA and rBTI, Fig. 2b) revealed that rBTI is highly
toxic to K562 cells, but the proliferation of K562 cells al-
most had not been inhibited in negative control.

DNA Electrophoresis DNA was cut into nucleosomes
using ribozymes during the metaphase 200 base pairs or the
integral DNA fragments were obtained. DNA presented a
regular ladder in 1.5% agarose gel, which is a typical charac-
teristic of cells undergoing apoptosis.11) Our experiment
showed that the DNA of the K562 cells presented ladder-like
strips (Fig. 3). Moreover, the ladder-like DNA is clearer with
an increase in concentration of rBTI from 12.5 to 100 μg/ml.

Flow Cytometric Analysis The cellular toxicity of rBTI
to K562 cells was analyzed by flow cytometry. Apoptotic
cells were observed after 24 h treatment with rBTI and with-
out treatment. The effect of the concentration of rBTI on the
induction of apoptosis in K562 cells was also examined (Fig.
4). From Fig. 4, it was indicated that the apoptosis of cells
was 2.5% when the cells were treated without rBTI, but the
apoptosis were 31.0%, 32.8%, 35.3% and 52.1%, respec-
tively, when the cells were treated with different concentrat-
ions of rBTI (12.5, 25, 50, 100 μg/ml). In addition, when the
concentration of rBTI reached 200 μg/ml, most of the K562
cells were dead. The results showed that rBTI can induce
apoptosis of K562 cells in a dose-dependent manner at the
low does, when the concentration over 100 μg/ml, the K562
cells gradually dead.

Morphological Observation of Nuclei Comparison the
effects of rBTI on K562 cells with that of phosphate buffer,
PH 7.6 alone (control), as showed in Fig. 5, rBTI-treated

Fig. 2. Effects of rBTI Treatment on K562 Cells and PBMCs
(a) Cells were incubated with various concentrations of rBTI for 24 h. The effects of
BSA, rBTI and the mixture of BSA and rBTI treatment on K56 cells. (b) K562 cells
treated with BSA; K562 cells treated with rBTI; K562 cells treated with mixture
of BSA and rBTI. Values are presented as means±S.D. of four determinations.
*p<0.05, **p<0.01.

Fig. 3. Internucleosomal DNA Fragmentation in K562 Cells Treated with
or without rBTI
K562 cells (1×10⁶) were treated for 24 h; the DNA was extracted using an Apoptosis
Ladder Detection Kit. Lanes 1 and 6: no treatment; Lane 2: 12.5 μg/ml rBTI added;
Lane 3: 25.0 μg/ml rBTI added; Lane 4: 50.0 μg/ml rBTI added; Lane 5: 100 μg/ml
rBTI added.

Fig. 4. Flow Cytometric Analysis of K562 Cells Treated with rBTI
(12.5—200 μg/ml) for 24 h
Cells (1×10⁶) treated with and without rBTI were washed by 50ms cold phosphate
buffer (pH 7.6) and suspended in 100 μl of binding buffer. The mixture consisted of
5 μl of fluorescence-conjugated annexin V and 2 μl of PI was added to the cells solu-
tion, then incubated for 15 min at 37 °C. The induction of apoptosis by rBTI in cells
was analyzed using flow cytometry.
cells displayed signs of apoptosis, and the nuclei of K562 cells disintegrated (Fig. 5B). The toxicity tests performed on the nuclei of K562 cells was consistent with these results from DNA electrophoresis and flow cytometric analysis.

DISCUSSION

In recent years, the incidence of various tumors and hematopathies has remained high, whereas the number of anti-tumor drugs is still comparatively low. To find new and effective drugs for treatment of various tumors, much research has been carried out. Recent interest has been focused on protease inhibitors due to their unique role as anti-apoptotics and they are now being developed into targeted anti-tumor polymeric agents. In this paper, we found that rBTI had a significant inhibitory effect on the growth rate of K562 cancer cells, but hardly had any effects on normal human PBMCs (Fig. 2a). BSA could not induce apoptosis of K562 cells at the same concentration of rBTI (12.5—100 μg/ml, Fig. 2b). The results also demonstrated that rBTI induced apoptosis in the cancer cells in a dose-dependent manner. The analysis of flow cytometric indicated that the K562 cells were highly sensitive to rBTI; when the concentration of rBTI reached 100 μg/ml, the apoptosis of K562 cells was almost 50%. The growth inhibition correlated significantly with the degree of treatment-induced cell death and induction of apoptosis in the target cells. When the concentration of rBTI reached 200 μg/ml, most of the K562 cells were dead (Fig. 4). This indicated that rBTI is highly toxic to K562 cells. This may have potential applications in the prevention or treatment of certain tumors.

Apoptosis, which is identified as one of the most fundamental biological processes in eukaryotes in which individual cells die by activating intrinsic ‘suicide’ mechanisms, has been thought to have a key role in damaging cancer cells, by causing a variety of insults. Some tumor cells have a specific receptor for trypsin inhibitors on their surface, when binding occurs, tumor-cell invasion and metastasis can be prevented and the pericellular matrix can also be re-stabilized. Here, the identification of a novel function of rBTI in inhibiting the proliferation of K562 cells is shown, the results are highly valuable for further studies on the induction of the apoptosis pathway and on the mechanism of rBTI.

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