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Cytotoxic effect of sea anemone pore-forming toxin on K562 chronic myeloid leukemia cells

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Abstract

Chronic myelogenous leukemia (CML) is one of prevalent cancer worldwide. In spite of various designed drugs, chemoresistance remains the main obstacle in cancer cure. Therefore, developing novel strategy for treatment of CML is an urgent need. Fragaceatoxin C (FraC) is novel protein toxin from a sea anemone called *actinia fragacea* with great impacts against cells by pore formation and disturbing cell membrane integrity. The aim of this study was evaluation of FraC toxin toxicity against K562. The bacteria cells harboring expression vector of FraC were induced by IPTG and purified by Ni^{2+}-NTA sepharose affinity chromatography. Then, purified toxin activity was evaluated using RBC hemolytic test. Eventually, evaluation of FraC cytotoxicity and apoptosis were performed using MTT and flow cytometery assays, respectively. Our results revealed that FraC toxin decreased K562 cells viability in a dose- and time-dependent manner with a whole destroy of cancer cells at 35.00 µg mL^{-1} after 72 hr. Furthermore, flow cytometery analysis indicated that FraC toxin enhanced necrosis along with apoptosis in K562 cells in a dose dependent manner. We speculated that FraC toxin could be considered as a novel candidate for cancer cell researches and treatments provided that it should be turned into a specific agent by engineering and directing to cancer cell membrane.

Introduction

Leukemia is a high-frequency malignant disease accounting for approximately 30.00% of all cancers occurring in children. By and large, leukemia falls into four malignant groups, namely, acute non-lymphoid leukemia, chronic myeloid leukemia, acute lymphocytic leukemia, chronic lymphoma leukemia in terms of morphological, laboratory and lymphoid or cellular myeloid roots.[1,2] Generally, selective action, increase in the function of anti-cancer chemical drugs and low complications of drugs for normal cells of the body are the major challenges in treating cancer.[3,4] Also, another challenge facing cancer treatment is the resistance of cancer cells to commonly used chemical drugs. It is established that the chimeric Bcr-Abl oncogene with uncontrolled/persistent tyrosine kinase activity frequently is associated with growth factor-independent cell proliferation, survival as well as resistance to chemotherapeutic agents-induced apoptosis in CML patients. Therefore, research on discovering new antitumor agents has been become one of the main purposes in medical treatment.[5]

Pore-forming toxins (PFTs) are one of the nature strongest biological weapons produced in a wide range of organisms namely bacteria, insects, marine invertebrates, poisonous reptiles and mammals to defend against predators, competition and other biochemical and physiological mechanisms.[6] They exert their cytotoxic activity via attachment to the cell membrane and formation of the oligomeric pores.[7] The molecular weight of most cytotoxic pore forming agents is ranging from 5.00 to 80.00 kDa.[8] Mammalian cells are resistant to PFTs and cell death depends on cell type and drug dosage. Bacteria and aquatic organisms are the most toxin producing organisms among other pore forming-producing organisms.[9]

According to the recent studies, protein toxins such as *Pseudomonas* exotoxin, *Diphtheria* toxin, and *Clostridium*

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perfringens enterotoxin have potential for application in cancer therapy.[10] In addition to bacterial protein toxins, nowadays, sea anemone pore forming toxins have gained a great attention.[11]

Actinia fragacea or so called strawberry, an inhabitant of the intertidal zone of the northern rocky coast of Spain,[12] contains a toxin named Fragaceatoxin C that bears a hemolytic activity. It was cloned and partially characterized by Gonzalez-Manas group in 2009.[13,14] The cDNA for FraC is available under the accession number FM958450 in GenBank. Structurally, FraC contains 179 amino acids with relative molecular weight of 20 kDa. Moreover, the crystal structure of FraC was resolved in 2010 and deposited in the RCSB PDB under the accession number 3LIM.[15] Due to potential applications and importance of pore forming toxins in cancer treatment, in the present study we aimed to investigate the anti-cancer effect of FraC on K562 cell line.

Materials and Methods

Chemicals and kits. Yeast extract and tryptone were purchased from Scharlau Company (Barcelona, Spain). Isopropyl-b-D-thiogalactopyranoside (IPTG) was from Bio Basic (Toronto, Canada). The RPMI and fetal bovine serum (FBS) were obtained from Gibco (Dublin, Ireland). *Ni^{2+}-NTA* agarose resin (30210) was taken from Qiagen (Venlo, The Netherland). Annexin V-FITC Apoptosis Detection Kit was from Invitrogen (Paisley, UK). Bradford and cell proliferation assay kits were provided by Arsam Fara Zist (Urmia, Iran).

Cells and plasmid. The plasmid *pET28a* containing the FraC protein-coding gene which was already cloned in biochemistry lab of faculty of Veterinary Medicine in Urmia University was used as an expression vector.[8] For FraC expression *E. coli* BL21 was used as a host which was purchased from Pasteur institute of Iran, Tehran, Iran.

Expression and purification of FraC. First a fresh colony was selected from a solid bacterial culture medium and inoculated into 5.00 mL of LB culture medium containing 50.00 µg mL^{-1} kanamycin in a 50.00 mL falcon and incubated at 37.00 ˚C overnight in a shaking incubator at 150 rpm. Next, 1.00 mL of the initial culture medium was transferred to 100 mL of the new LB medium and incubated at 37.00 ˚C in a shaking incubator at 150 rpm until the OD 600 reached 0.60 - 0.80. Eventually, the culture was induced with 1.00 mM IPTG at 28.00 ˚C for 12 hr in a shaking incubator at 160 rpm.

Purification of FraC toxin. The full procedure of FraC purification was thoroughly described in our previous study.[8] Briefly, FraC-expressing bacteria were centrifuged at 1,500 *g* for 20 min at 4.00 ˚C. The supernatant was eliminated and the pellet sediment was suspended in 10.00 mL of lysis buffer (phosphate buffer, pH 7.40 containing 300 mM NaCl and 5.00 mM imidazole) and sonicated on ice. Next, the mixture was centrifuged at 13,000 *g* at 4.00 ˚C and supernatant collected as a crude cytosolic protein for affinity purification. The total protein was loaded on the column, washed by wash buffer, the same as lysis buffer except 25.00 mM imidazole, and then eluted by elution buffer, phosphate buffer containing 300 mM imidazole. Finally the FraC fractions were collected and their protein concentrations were spectrophotometrically determined using Bradford method.[8]

FraC hemolytic activity assays. This was performed according to Bellomio *et al.* study with slight modification.[13] Briefly, human fresh red blood cells (RBCs) were washed three times with PBS and their absorbance at 700 nm was adjusted to around 1. Hemolytic assay was performed by recording the changes in turbidity in a Unico UV2100 spectrophotometer (Unico, Dayton, USA) after 20 min incubation of RBCs with various concentrations of the purified FraC. Next, the absorbance of the toxin-treated samples was measured. Hemolytic activity was expressed as percentage using the following formula:

$$Hemolysis\ (\%) = (A_{max}-A_{abs}) / (A_{max}-A_{min}) \times 100$$

where, A_{max} and the A_{min} represented the absorbance of the intact and completely lysed RBCs, respectively, and A_{abs} was the absorbance of each sample recorded after 20 min incubation with various concentrations of FraC.[13]

Cell culture. Human leukemia K562 cell line was obtained from Pasteur Institute (Tehran, Iran) and maintained in RPMI-1640 medium supplemented with 10.00% FBS and penicillin (100 µg mL^{-1}; Gibco) and streptomycin (100 U mL^{-1}) in a 5.00% CO_2 humidified atmosphere at 37.00 ˚C. Cell numbers and viabilities were assessed using a hemocytometer and the abilities of the cells to exclude trypan blue.

MTT assay. Cytotoxicity was estimated by the MTT reduction assay kit. Viable cells with active mitochondria reduced the yellow tetrazolium salt giving dark blue water-insoluble formazan crystals. Briefly, 1.00×10^4 cells in exponentially phase were seeded per well in 96-well plates in triplicates 12 hr before treatment. Cells were treated by different concentrations of FraC (concentrations of 2.17, 4.35, 8.70, 35.00, and 70.00 µg mL^{-1}) and were incubated at 37.00 ˚C in 5.00% CO_2 for 24, 48 and 72 hr. Then, the cells were treated with 25.00 µL MTT (5.00 mg mL^{-1}) solution for 4 hr. After incubation, 150 µL formazan solubilizing solution was added to each well. The formazan dye crystals were solubilized for 30 min and the color intensity was determined at the wavelength of 570 nm with an ELISA Reader (Awareness Technology Inc., Palm City, USA). Results were expressed as the percentage of MTT reduction assuming that the absorbance of the control cells was 100%.

Flow cytometry analysis of cell death. To quantify the extent of cells apoptosis, double labeling method with FITC-Annexin V and propidium iodide (PI) were performed.

In each well of the 6-well plate, 500 × 10^3 cells were cultured 24 hr before treatment with 2.17, 4.35, 8.70, 35.00 µg mL^{-1} of FraC toxin, and 48 hr later, the cells were collected and cell suspension was transferred to micro-tubes. Cells were centrifuged for 5 min at 1,200 rpm. Supernatant was removed and the cell precipitate was washed with a phosphate buffered saline (PBS) and with a binding buffer. Then, 5.00 µL of annexin V was added to the cell suspension, incubated for 15 min at room temperature in a dark place and washed with PBS. Eventually, the 10.00 µL of PI (50.00 µg mL^{-1}) was added to the samples and stored 10 min in dark place and then evaluated by flow cytometer (FACScan, Franklin Lakes, USA).

Statistical analysis. Statistical comparisons were performed using the GraphPad Prism Software (version 5.0; GraphPad Inc., San Diego, USA). Each experiment was repeated three times in an independent manner (n = 3). To analyze the significance of difference between the results Student's *t*-test was used. All data were expressed as mean ± SD. Significance of $p < 0.05$ was set in all tests.

Results

Evaluation of hemolytic activity of recombinant FraC. To find out whether the purified FraC maintained its conformation and hence its activity during the expression and purification, hemolytic activity of FraC on the RBCs was evaluated. FraC exhibited a potent hemolytic activity in a dose-dependent manner at concentrations ranging 0.0 to 70.00 µg mL^{-1} (Fig. 1). In this regard, roughly 30.00% hemolysis was obtained at the concentration of 2.17 µg mL^{-1} followed by 50.00% and 100% hemolysis at the concentrations of 4.00 and higher than 35.00 µg mL^{-1}, respectively, within 20 min. These results clearly showed that the FraC conformation and its activity were preserved during the expression and purification.

FraC effects on the K562 cell line. To reveal growth inhibitory effects of recombinant FraC, exponentially growing human leukemia K562 cells were cultured for 24, 48 and 72 hr in the presence of different concentration of FraC toxin and the cytotoxicity was estimated using MTT assay. As depicted in Figure 2 cell viability was inhibited by 10.00% to 80.00% after 72 hr of cells exposure to 1.00 to 70.00 µg mL^{-1} toxin in a dose-dependent manner. Estimation of IC50 represented that the 50.00% inhibitory effect of FraC was achieved at 35.00, 17.50 and 8.70 µg mL^{-1} for 24, 48 and 72 hr toxin exposure, respectively.

Analysis of cell death mechanism by flow cytometry. To obtain further evidence about the anti-proliferative effect of FraC, we surveyed the induction of apoptosis or necrosis occurrence following toxin treatment by double staining with PI and annexin V. Our results were presented in Figure 3 showing that 48 hr treatment with 35.00 µg mL^{-1} FraC toxin provoked early and late apoptosis in K562 by almost 15.00% relative to un-treated cells. Also, the rate of necrosis was increased depending on the dose, as well so that nearly 13.00% was attained in 35.00 µg mL^{-1} after 48 hr. The results indicated that necrosis in contribution with apoptosis promoted FraC-induced cell death. The rates of early and late apoptosis, and necrosis rates are specified in Figure 3.

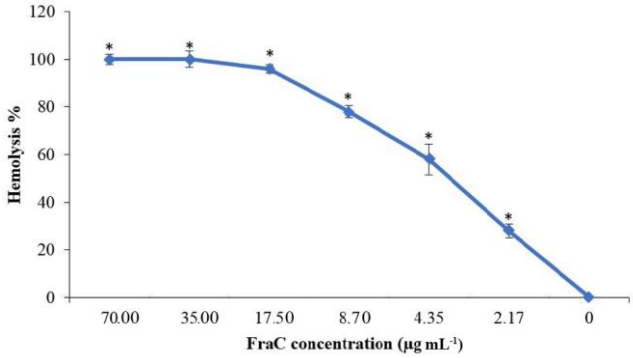

Fig. 1. Hemolytic activity of various concentration of FraC. Representative results are the mean of at least three independent experiments. Asterisk indicates significantly different from control cells ($p < 0.05$).

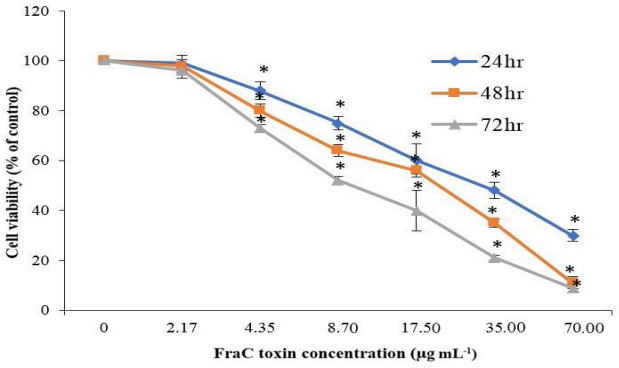

Fig. 2. Growth inhibitory effects of FraC on K562 cells. Relative cell growth was evaluated using MTT assay compared to un-treated cells. The data are the means of three-independent measurements ± SD. Asterisk indicates significantly different from control cells ($p < 0.05$).

Discussion

Natural toxins, in particular pore-forming toxins which produced and secreted by a large number of organisms have been extensively characterized. Several researches have focused on their function and application. Nowadays, the cancer treatment potential of pore forming toxins has captured much attention.[11,16]

The mostly-studied PFTs, so-called BC2 and Eqt II toxins are isolated from sea anemone, the *Bunodosome Caissarum* species and *Actinia equine*, respectively. Both of them which belong to the cytolysins family exhibited a toxic effect on human U87 cells and A172 glioblastoma cell line.[11] Moreover, because of their pore-creating function they are used to increase the effectiveness of anticancer drugs by facilitating their entry into cytosol.

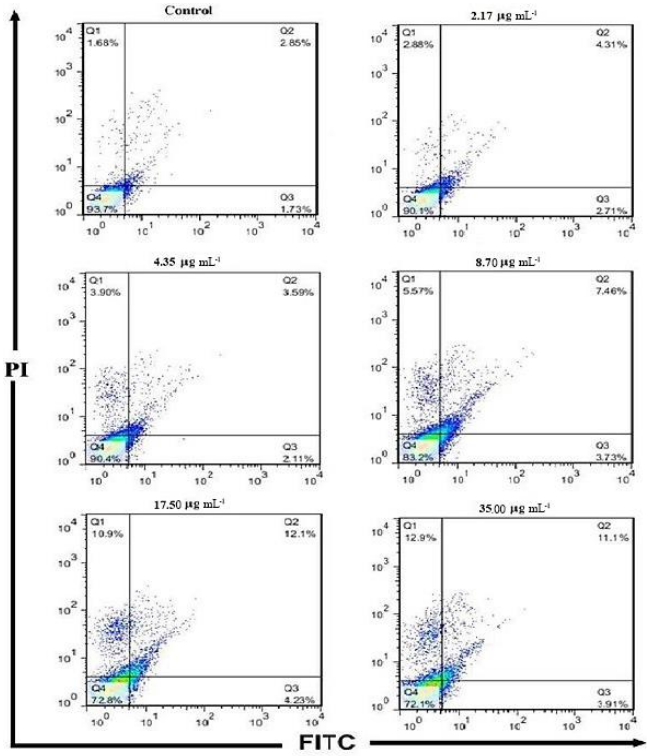

Fig. 3. Effect of various concentration of FraC toxin on the extent of cell death induction in K562 cells. The percentage of apoptotic cells was measured using AnnexinV/PI double staining. Q1, PI positive and Annexin V negative indicate necrotic cells. Q2, PI and Annexin V positive represent cells in late apoptosis. Q3, PI negative and Annexin V positive show cells in early apoptosis. Q4 exhibits PI and Annexin V negative as indicator of viable cells.

In comparison with the cytotoxicity of anticancer drugs, their combination with cytolysin makes their dose 10-300 times less than when they are applied alone.[11]

Since there has been evident that FraC kills cells through creating pore in cell membrane, we attempted to address whether it acted on K562 cells or not. To fulfill this, the present research was conducted to investigate the anti-cancer impact of newly-introduced FraC toxin of sea anemone on K562 cell line. Recombinant optimization and production of FraC was already implemented.[8] In this study initially FraC toxin was purified and its function was examined on RBC to ensure its activity during purification process. As shown in Figure 1, a potent hemolytic activity is evident at the concentrations ranging 0.00 to 70.00 µg mL^{-1}, so that, all of cells have been destroyed above 35.00 µg mL^{-1}. These results clearly implied that the FraC conformation and its activity were preserved during the expression and purification process.

In order to investigate the anti-cancer effects of FraC, K562 cell line was employed to examine the growth inhibitory and cell death mechanism. It was indicated that at the concentrations of 2.17, 4.35, 8.70, 17.50, 35.00, 70.00 µg mL^{-1}, FraC reduced the growth of cancer cells in a dose and time-dependent manner, so that, significant cytotoxic effect was observed at 35.00 and 70.00 µg mL^{-1} after 72 hr. Moreover, to further analyze the FraC effects on K562, the apoptosis and necrosis rates were surveyed using flow cytometry. The data indicated that the cell death mechanism was mediated most likely through necrosis rather than apoptosis. This finding could be attributed to the pore-forming capability of toxin which in turn disturbed cell membrane integrity.

The cytotoxicity effects of *stichodactyla haddoni, Heteractis magnifica,* and *Paracondylactis sinensis* were investigated on L929 fibroblastic cell line in mouse and P388 leukemia cell. In these studies, decrease of cell viability was observed in a toxin concentration-dependent manner.[11] Fractions III, IV of *Anemonia viridis* have been investigated on PC3, PLC/PRF/5 and A375 cancer cell lines and evaluations indicated that 100 µg mL^{-1} of *A. viridis* IV caused 100% toxicity.[23] In the current study FraC exerted cytotoxic activity on K562 cell line at the concentration of as minimum as 3.00 µg mL^{-1} which led to complete cell death at 70.00 µg mL^{-1}. Furthermore, other studies revealed that *Heteracti smagnifica* toxin increased apoptosis in MCF-7 and T47D breast cancer cells by activating the mitochondrial pathway and activating caspase cascades 3, 8, and 9.

Several reports indicated that cancer cells contained high levels of lipid membrane, cholesterol level, glycosphingnolipid and SM. Additionally, pore-forming cytolysins of sea anemone such as BC2 and Eqt II toxins require a high level of SM and cholesterol to bind to the membrane.[18] These proteins may selectively act against cancer cells.[19-21] BC2 toxin at the concentration of 1.00 µg mL^{-1} and an Eqt II toxin at 10.00 µg mL^{-1} concentration induce cell death with a low dose of chemotherapy drugs through quasi-necrosis in glioblastoma cells which is one of the most common primary tumors of the brain. Also, cytolysin of sea anemone indicated a potent toxic effect with vincristine drug (antimicrobial drug).[11] In U87 cells, cytotoxicity was increased by 300 times more than vincristine. The low dose of chemotherapy drugs when combined with PFTs, leads to more effective cytotoxicity. According to the recent reports, when incubated with St-1 toxin (0.20 - 1.00 mg mL^{-1}), Cos-7 cell permeability was increased with fluorescent PI (668 DL) stain.[22]

Using pore forming toxins for the selective death of the target cells is a new approach to treat cancer.[8,16,17] In our investigation for the first time we reported that FraC cytotoxicity was caused by both necrosis and apoptosis in the course of time and concentration. For converting non-specific pore-forming toxin such as FraC to selective and specific toxin, protein engineering and conjugation of a tumor-specific antigen was suggested. In addition to pore-forming and cytotoxic activity of FraC, it could be used as a channel for carrying agents across impermeable cell

membrane. This was promising because the low concentration of chemotherapy drugs could reduce the effects of chemotherapy complications.

Totally, these findings disclosed the cytotoxic effects of FraC on K562 cancer cell line. Even though, FraC exerted cell death in an unspecific way, however, converting it to a tumor-specific agent by protein engineering might be a promising and novel strategy in cancer treatment.

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Conflict of interest

The authors declare no conflict of interest.

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