Original Article

Cytotoxic Effect of Recombinant Fragaceatoxin C on Peripheral Blood Mononuclear Cells

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Abstract

Background & Objective: Actinoporins from sea anemones are potent pore-forming toxins. *Fragaceatoxin C* belongs to this family with a molecular weight of 20 kDa that mainly binds sphingomyelin in membranes and forming 2nm in diameter cation-selective pore. The aim of this study was the examination of recombinant toxin activity against peripheral blood mononuclear cells (PBMCs).

Material & Methods: For recombinant expression of toxin, *E.coli Bl21* was employed. The toxin purified by Ni۲+-NTA Sepharose affinity chromatography. The purity of toxin was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Various dilutions of the toxin were applied on red blood cells, and their hemolytic activity was analyzed by spectrophotometer by recording OD7۰0. To examine the cytotoxic activity of purified toxin, PBMCs were treated with its different concentration. Cytolytic activity of FraC protein on PBMCs was measured using MTT and neutral red uptake assays.

Results: Hemolytic assessment indicated that the toxin had retained its activity after purification. Analysis of PBMCs tests showed that low doses of toxin did not change the viability of cells compared to control cells. The metabolic activity of living PBMCs was only significantly decreased at the higher concentration (800 and 1000 NM) of the toxin. Despite the results of trypan blue tests, obtained data indicated that the Neutral red uptake assay was significantly reduced in PBMCs in a dose-dependent manner.

Conclusion: Despite toxicity against RBCs, FraC is not toxic to the peripheral blood mononuclear cells at lower doses.

Keywords: Actinoporins, Fragaceatoxin C, Peripheral blood mononuclear cells.

Introduction

Cancer therapy yet represents a significant challenge, despite significant advancements in available treatments being made over the past decade (1). Chemotherapy and radiation therapy are effective but have many side effects. Therefore, it is necessary to extend new treatments that are more effective and specific (2). Pore-forming proteins (PFP) are a famous group of toxins present in the poison of sea anemones. Due to their potential to identify and permeabilize cell membranes, these proteins are a new strategy to overcome the problems associated with current cancer (3, 4). Pore-forming toxins (PFT) are secreted as soluble molecules and cause cell-damage by opening pores in lipid membranes, eventually, leading to cell death (5-8). The leading roles of PFTs are defending, attacking, and signaling (9,10). PFTs are generally classified according to structural

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motifs that are employed for insertion within the cell membrane upon pore formation. They are classified into two groups α-PFTs and β-PFTs (10,11). Actinoporins are a class of small and basic α-PFTs to pore formation by inserting α-helixes within the lipid membrane (10-12). *Fragaceatoxin C* (FraC) belongs to an actinoporins family with a molecular weight of 20 kDa that mainly binds sphingomyelin in membranes and forming 2nm in diameter cation-selective pore (12,13). The crystal structure of the pore of FraC is resolved to 3.1Å resolution (11). Each FraC monomer is instructed from an β-sandwich core flanked on two sides by α-helices (14). The FraC crystal structure displays four various stages consisting of water-soluble monomer, monomer attached to the membrane, oligomeric intermediate or pre-pore, and functional pore (15). The expression of recombinant FraC in *E. coli* revealed that the structural and functional features of the native and recombinant proteins are identical (16). FraC is a potent hemolysin and requires particular residues for its activity in the membrane (12,17). Conserved residue Phe16 of FraC is essential for formation of a pore in cholesterol-rich cell membranes like RBC (17). Moreover, Val60 is a critical residue participating in the oligomerization of the functional pore in RBC (18). The results of an investigation showed that the recombinant FraC protein has potent cytotoxic effects on both HL-60, and KG-1 cell lines (4). While PFTs have shown encouraging results in cancer studies, because of the absence of specificity in subjugation to the goal membranes, these proteins are not still a genuine strategy to control the cancers (19, 20). Treatment based on targeting peptides is a route to make proprietary in a toxic molecule, which should be a tumor treatment (19). PBMCs are broadly used in research and toxicology applications. For the toxicity assessment of anticancer compounds on humans, especially on the immune system, researchers utilize PBMCs (21). The aim of current work is the examination of recombinant FraC on PBMCs in vitro.

Materials & Methods

Materials

Isopropyl-b-D-thiogalactopyranoside (*IPTG*) was procured from Fermentas. Ni-NTA (nitrilotriacetate) resin was from Novagen Inc. Tryptone, yeast extract, and agar were obtained from Scharlau Company. All sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) chemicals were purchased from Merck. Kanamycin was from Sigma-Aldrich. Fetal calf serum and *RPMI-1640* medium (DMEM) were purchased from GIBCO/life technologies Inc. (Gaithersburg, MD). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), neutral red (NT), ficoll-Hypaque, dioxin, and dimethyl sulfoxide (DMSO) were procured from Sigma-Aldrich (St. Louis, MO).

FraC production

Production of the recombinant toxin was performed using the *pET* system and *E. coli* as a host. The *pET-28a(+)* is a bacterial expression plasmid developed for the cloning and high expression of recombinant proteins in *E. coli*. The gene encoding FraC had previously been cloned into pET28a by Dr. Imani in the Biochemistry Laboratory of the Faculty of Veterinary Medicine and was used for this purpose. The expression of the target gene is induced by IPTG or lactose. It carries His tag sequence at N-terminal and C-terminal region, thrombin protease digestion site, and kanamycin resistance gene sequence (22). In this research, the FraC coding sequence, which already cloned between two restrictions enzymes, *NcoI* and *HindIII*, was used for the recombinant production of FraC.

Competent cell preparation and transformation

The preparation of competent cells was performed with the chemical method by CaCl2 and MgCl2. An aliquot of an overnight *E. coli BL21* culture was subjected into the LB medium including 50 µg/ml kanamycin and incubated at 37 °C till the OD600 nm reached to 0.35–0.4. When reached suitable growth, the culture was incubated on ice for 30 min. To neutralize negatively charged components of foreign DNA and bacteria surface, ice-cold CaCl2 and MgCl2 were added to the cells followed by centrifugation to collect the cell pellet. The transformation was performed with the thermal shock method. Briefly, 1 µl of pET28a-vector solution harboring FraC coding sequence was mixed to 100 µl competent cells and cultured on ice for 30 min. In the next step, cells were transferred to 42 °C temperature for 45 sec, then on ice. Transformed cells were grown in 800 µl of Super optimal broth with catabolite repression (SOC)

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medium for one hour at 37 °C. Finally, cell pellets were incubated on LB agar plates including 50 µg/ml of kanamycin and cultured at 37 °C overnight (22).

FraC Protein induction

Expression and purification of FraC were performed according to our previously published work. Briefly, an overnight culture of transformed BL21 cells containing kanamycin (50 µg/ml) was used to inoculate with 100 ml LB medium and incubated at 37 ºC until the OD600 nm was 0.6-0.8. Bacteria were induced by 10 mM lactose and 1mM IPTG at 30 ºC temperature for 16 hours. To down bacteria pellet, bacteria culture was centrifuged at 4000 rpm at 4 ºC for 15 min. The cell pellet was washed in lysis buffer (pH 7.8), followed by cells that were disrupted by sonication at 20 cycles of 1 minute on ice. Cell Suspension was centrifuged at 13000 rpm at 4 ºC for 15 min. Finally, the bacterial supernatant containing the total protein of cells was collected (4).

Purification of FraC

The crude protein solution was applied to affinity chromatography column (Ni-NTA) equilibrated with lysis buffer pH 7.8 (containing 50 mM Tris, 300 mM NaCl, and 10 mM imidazole). To wash non-recombinant proteins, the washing buffer (50 mM Tris, 300 mM NaCl, 20 mM imidazole, and 4mM DTT) was loaded onto the column. Eventually, recombinant FraC was separated by adding elution buffer (containing 50 mM Tris, 50 mM NaCl, and 300 mM imidazole) (23).

Protein electrophoresis and determination

SDS-PAGE was used to analyze the extracted protein. The higher the gel content, the smaller the pores created, and the lower the molecular weight of the separated proteins. Because the molecular weight of the FraC protein is low (20 KD), 16% gel was used. Protein staining was carried out with Coomassie blue dye. Protein estimation was performed by Bradford assay using a standard protein (bovine serum albumin) (24).

Hemolytic activity of FraC

Human red blood cells (RBC) were freshly separated and washed thrice with 25mM PBS. Suspension opacity was adjusted with an apparent volume of PBS to OD700 nm corresponds to 0.9. Then they were incubated with variable concentrations of purified toxin for 15 min at room temperature with periodic shaking, and RBC lysis was monitored spectrophotometrically by the decrease in turbidity at 700 nm. The percentage of hemolysis was monitored as follows:

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

Where A_{obs} corresponds to the observed absorption for each well. A_{max} and A_{min} represent the absorbance of the intact and hemolysis RBC. For positive and negative controls, 100µl of distilled water, and PBS corresponds to A_{min}, and A_{max} were added to RBC cells (22).

Evaluation of the cytotoxic activity of Fra C

PBMC isolation

All of the experimental manners were performed in accordance with the guidelines Ethics Committee of Urmia University (Code Number: A/90/1003). The blood samples were given after approval with the institutional Health Research. More importantly, the consent form was signed by donors. Heparinized blood samples (20 ml) were isolated from the five volunteers. To isolate a buffy-coat layer, bloods were centrifuged for ten min at 1800 rpm at 4 ºC. The isolated cells were diluted 1: 2 in PBS and centrifuged over a Ficoll gradient at 2500 rpm in 18 ºC for 30 min. After re-suspension, the hypotonic lysis was applied to omit contaminant erythrocytes. PBMCs were rinsed three times and then resuspended in DMEM, which contained 10% fetal calf serum (25).

Assessment of cell viability

Trypan blue staining was applied to forejudge between viable and non-viable cells. Briefly, 1000 µl of the PBMCs suspension (1×10^6 cell/ml) was mixed to each well of 24-well microplates and pulsed with Phytohemagglutinin (PHA) solution (1 mg/ml) for 24h with a serial dilution of FraC (100, 200, 400, 800, 1000 NM). Afterward, the PBMCs suspension was diluted in an equal volume of 0.4% trypan blue dye, and the cells were counted in a Neubauer chamber (26).

Cell membrane activity

Cell membrane activity was evaluated by the Neutral red (NR) uptake assay, similar to the procedures described earlier. In brief, 200 µl of the PBMCs suspension (1×10^6 cell/ml) was added to each well of 96-well microplates and stimulated with 50 µl PHA solution (1 mg/ml) for 24h with a serial dilution of FraC (100, 200, 400, 800, 1000 NM). Afterward, each well was primed with 20 µl of the NR solution (3.3mg/ml) at 37 °C. The medium was deleted after 4h, and the cells were rinsed three times in PBS. 200 µl

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10% acetic acid plus 40% ethanol solution was added to each well for solubilizing the internalized dye. Finally, the optical density was monitored at 550 nm (27).

Evaluation of mitochondrial activity

The MTT reduction assay was used to evaluate the metabolic activity of cells. In brief, The PBMCs were plated in 96-well flat-bottomed plates (1×10^6 cell/ml) in a volume of 200 µl and stimulated with 50 µl PHA solution (1 mg/ml) or medium alone. The cells were incubated for 24h with a serial dilution of FraC (100, 200, 400, 800, 1000 NM). After incubation, cells were primed with 20 µl of a 5 mg/mL solution of MTT in PBS for 4h at 37 °C. Next, the medium was omitted, and 150 ml dimethyl sulfoxide was admixed. To dissolve Formosan crystal, the plate was shaken vigorously. The optical density was monitored by using a microplate reader (Dynatech, Denkendorf, Germany) at 570 nm. The examinations were done in triplicate. The findings were expressed as the survivability present according to the ratio of (OD550 of stimulated cells with PHA to OD550 of non-stimulated cells with PHA) × 100 (28, 29).

Statistical analysis

All experiments were repeated in triplicate. The IC50 was evaluated by linear regression analysis by using Minitab software (version 18, State College PA 16801-3210, USA). Statistical analysis was done by using one-way ANOVA plus Tukey's post hoc test. All data were presented as mean±SD. Values of $P<0.05$ were inspected as the significance level.

Results

To confirm the purification of FraC, reducing SDS-PAGE was conducted. Once FraC was purified by affinity chromatography, the protein quantity of all fractions was estimated and the 20 µL of fraction contained protein was run on SDS-PAGE, and the results are shown in Fig 1. A sharp protein band with molecular weight of 20 kDa in lane A, and protein marker (lane B) was expected FraC (Figure 1).

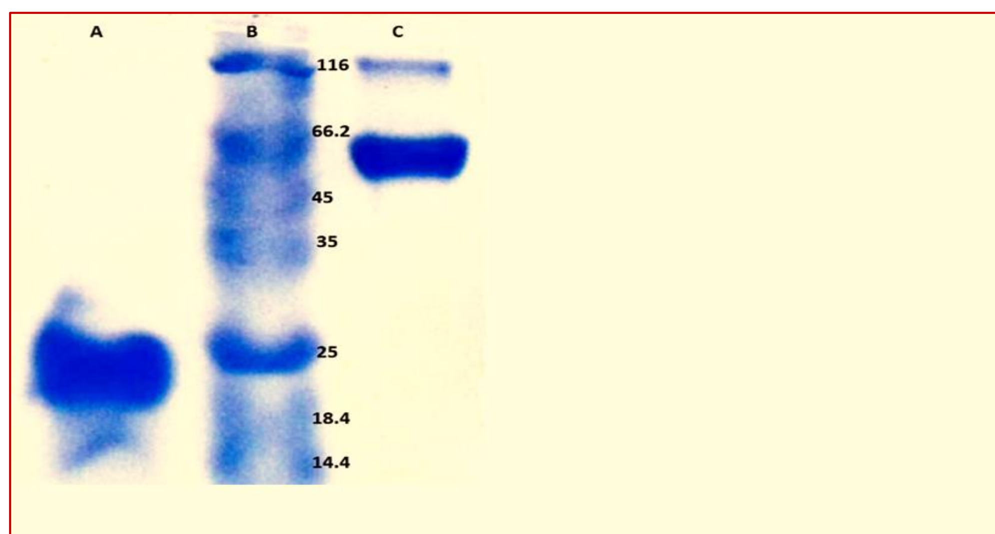

Figure 1. Confirmation of purity of FraC. Lane A contains a band at approximately 20 kDa which indicates the presence of purified toxin. Lane B shows protein marker and lane C is 66 kDa BSA protein as another marker along with protein marker. The gel stained with Coomassie Brilliant Blue R-250.

Various dilutions of *FraC* were applied on RBCs, and cytotoxic activity was analyzed by recording OD700 nm by using a spectrophotometer. The results showed that by increasing the concentration of toxin, the absorption of RBC decreased, indicating the cell lytic function of purified FraC. The hemolytic assessment indicated that the *FraC* had a remarkable lytic activity on RBC so that at a concentration of 24 NM, almost 50% of RBCs were damaged. These data indicated that not only purified toxin sustained its potential during expression and purification producers but also put its role at lower concentrations. (Chart 1)

To evaluate the potential cytolytic activity of purified FraC toxin, we employed PBMCs and incubated them with different concentrations of toxin for 24 h. As depicted in figure 3, the vitality of PBMCs was not affected by low

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concentrations of toxin, but at the higher concentrations (1000 NM), the vitality of cells was lost. In this regard, the Trypan Blue test indicated that only 50% of cells were alive at the concentration of 1000 nM (Chart 2).

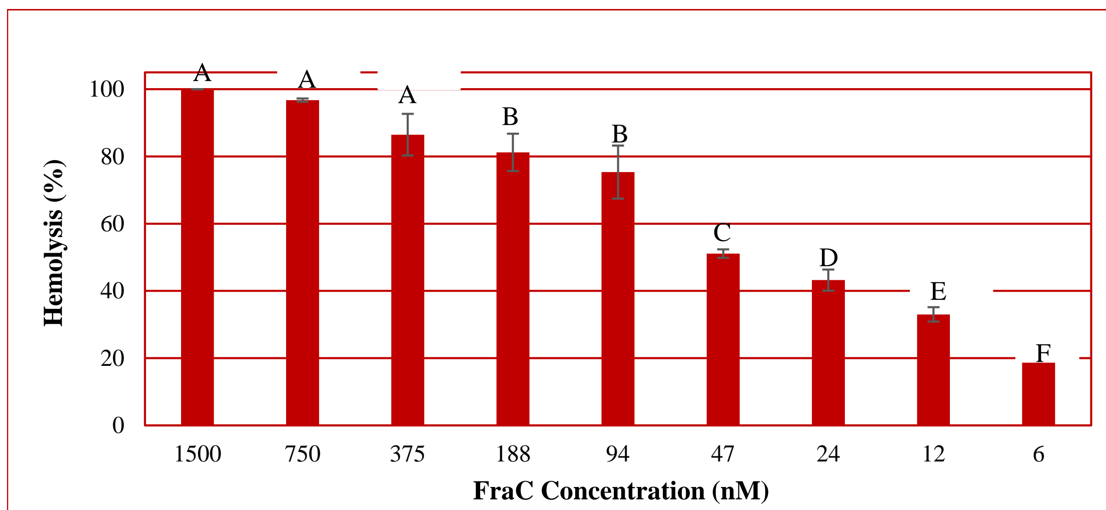

Chart 1. Hemolytic function of purified toxin. RBC suspension was adjusted to OD700 and incubated with variable concentration of toxin and finally the OD700 of cell suspension were recorded by spectrophotometer after 15 min. Different letters indicate a significant difference at the level of P<0.05.

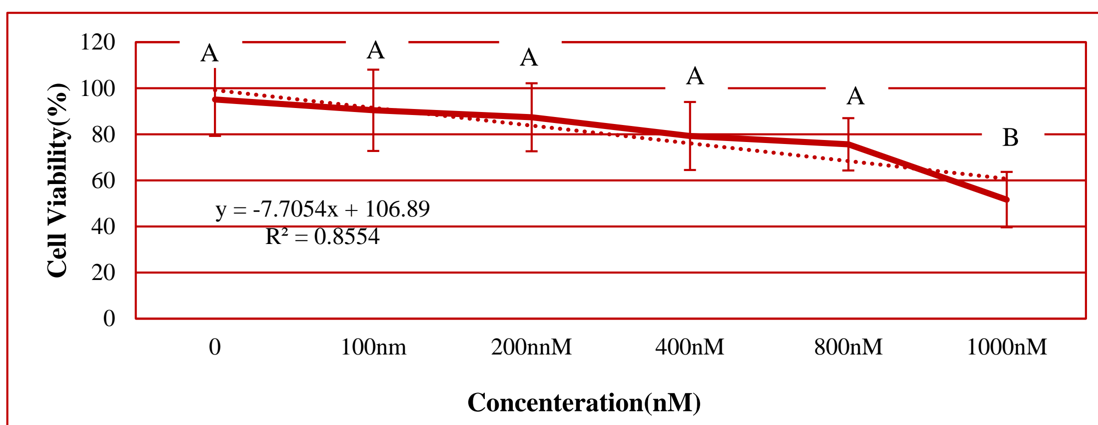

Chart 2. PBMCs viability after treatment with increasing concentrations of toxin. Different letters indicate a significant difference at the level of P<0.05.

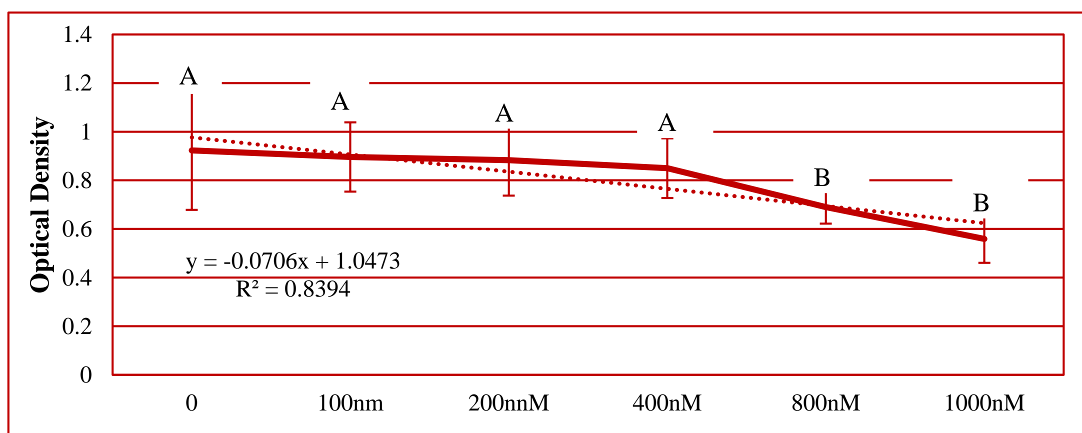

Chart 3. PBMCs viability after treatment with increasing concentrations of toxin. Different letters indicate a significant difference at the level of P<0.05.

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The results of the MTT test showed that toxin did not exert significant cytotoxicity at low concentrations. However, the metabolic activity of living PBMCs was significantly decreased at the higher concentration (800 and 1000 NM) of toxin (Chart 3).

Obtained data indicated that the Neutral red uptake assay was significantly reduced in PBMCs in a dose-dependent manner, compared to cells without treatment (Chart 4).

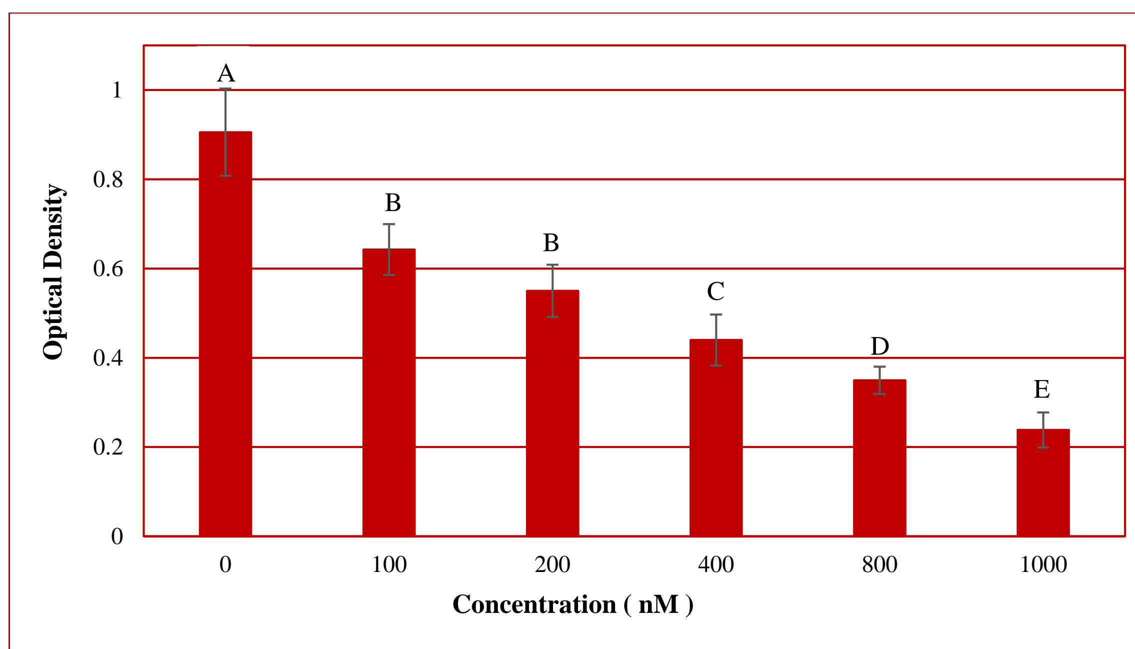

Chart 4. Effect of toxin on cell viability by neutral red uptake assay. Different letters indicate a significant difference at the level of $P<0.05$.

Discussion

Discovering and introducing new useful antitumor compounds or approaches has been one of the top precedencies of many research projects. Chemotherapy, and radiation therapy are effective but have many side effects. Therefore, it is necessary to extend new treatments that are more effective and specific (1,2). Targeted transfer of therapeutic drugs into cancer cells is considered as a new strategy to tackle cancer (30). Peptides and antibodies can be conjugated to a cytotoxic compound to deliver it to a cancer cell (31). Several PFTs have been investigated for improvement of the medications to serve as therapeutic factors with anti-cancer potential (3). PFTs were causing cell-damage by opening pores in lipid membranes, eventually, leading to cell death (29, 32). Although PFTs have shown promising results in cancer studies, the more significant benefit of these toxins (including invading the membrane, which is the most widespread target in the alive cells), is also their momentous weakness (33). Using targeting peptides is a good strategy to make proprietary in a toxic molecule, which should be a tumor treatment (19). In an investigation, Kheirandish et al. have engineered PFT from listeriolysin O for breast cancer cells by adding a luteinizing hormone-releasing hormone (LHRH) targeting peptide to its N-terminus (34). Furthermore, the first immunotoxins produced via conjugating a monoclonal antibody binding IOR-T6, an exclusive antigen demonstrated on the surface of immature T-cells and a hemolytic pore-forming toxins from *Stichodacthyla helianthus* by Avila et al. Later, the same group produced a new immunotoxin with a monoclonal antibody against carcinoembryonic antigen (33). Hemolytic activity of the FraC had been previously explored using the native protein extracted from the sea anemone and recombinant protein. In the present study, IC50 of the recombinant FraC in human RBC hemolysis assay was 24 NM comparable to native protein, and the other previously studied recombinant form (4, 16, 22). Findings of the study showed that recombinant FraC has cytotoxic activity against PBMCS. These results were comparable to the cytotoxic activity of FraC on both HL-60

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and KG-1 cell lines and other actinoporins on leukemic cells (4, 6, 35). Purified FraC had a minor cytotoxic effect on peripheral blood mononuclear cells, which was confirmed by three different methods: Trypan Blue staining, MTT, and Neutral red uptake assays. The results of the Trypan Blue staining indicated that low doses of toxin impacted a little membrane damage, but applying higher doses of FraC damage of cell membrane and cellular toxicity was quite evident. According to the MTT assay, toxin did not exert significant cytotoxicity at low doses despite the membrane damage. However, mitochondrial function and total cellular activity still preserved; PBMCs are sensitive to the high dose of toxin. At the last two doses (800 and 1000 NM), cytotoxicity is almost apparent. The difference between two IC50 by Trypan Blue and MTT test is significant due to the survival of the cells in Trypan Blue test while they are reduced when the MTT test is used. Therefore, in the MTT test, by measuring the tetrazolium salt recovery by the mitochondrial succinate dehydrogenase enzyme, the metabolic activity of the cell is measured, but in the Trypan Blue test, membrane damage and pore formation are investigated. Furthermore, we used Neutral red uptake assay for assessment of cytotoxic activity of FraC. Neutral red uptake sharply reduced at high concentrations of toxin (400-1000 NM). The reason for this considered at the level of the ATP, and ultimately, its decrease will affect the membrane function. These results are well in line with the results of the Trypan Blue test so that the highest percentage of dead cells were observed in the higher doses of toxin. At lower doses, although the membrane integrity and active endocytosis has been reduced slightly, however, due to the lack of mitochondrial damage and ATP synthesis, the viability of cells has not changed significantly compared with control cells.

Conclusions

The assessment of cytotoxicity of FraC through viability studies like Trypan Blue staining indicated that high concentrations of toxin significantly decreased the percentage of viability of PBMCs. Further, data obtained using the Neutral red uptake and MTT assays exhibited even though the integrity of the cell membrane was partially lost, but the mitochondrial function remained. Due to their cytotoxic capacity, engineering of PFTs by targeting peptides could make it much more specific to tumor cells.

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Conflict of Interests

The authors have no conflict of interests.

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مقاله پژوهشی

اثر سایتوتوکسیک فراگاسیاتوکسین C نوترکیب روی سلول های تک هسته ای خون محیطی

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چکیده

زمینه و هدف: اکتینوپورین ها پروتئین ها و پپتیدهای سیتولیتک هستند که توسط شقایق دریایی تولید می شوند و باعث تشکیل منفذ در غشای لیپیدی می شوند. فراگاسیاتوکسین C یکی از اعضای این خانواده با وزن مولکولی ۲۰ کیلودالتون می باشد که به طور خاص به اسفنگومیلین در غشای پلاسمایی متصل و منافذ کاتیونی انتخابی به قطر دو نانومتر را در غشا تشکیل می دهد. هدف اصلی از این مطالعه بررسی فعالیت زیستی سم روی سلول های تک هسته ای خون محیطی(PBMCs) می باشد.

مواد و روش ها: وکتور بیانی pET28a دربرگیرنده توالی کد کننده FraC به سلول مستعد اشرشیا کلی سویه BL21 ترانسفورم گردید. سپس تخلیص پروتئین بوسیله کروماتوگرافی تمایلی انجام شد. تخلیص پروتئین توسط الکتروفورز ژل پلی آکریل آمید تایید شد. غلظت های مختلف از سم روی سلول های قرمز خون (RBC) تست شد و فعالیت همولیزی آن، با ثبت در طول موج ۷۰۰ نانومتر آنالیز شد. به منظور بررسی فعالیت ضدسلولی سم تخلیص شده، PBMCs با استفاده ازغلظت های متفاوت سم مورد تیمار قرار گرفتند. فعالیت سیتولیتیک پروتئین FraC روی سلول های تک هسته ای خون محیطی با استفاده از روش (دی متیل تیازول– دی فنیل تترازولیوم بروماید MTT)و نوترال رد سنجیده شد.

نتایج: ارزیابی فعالیت همولیتیک نشان داد سم در تمام مراحل تخلیص فعالیت خود را حفظ می کند. نتایج بررسی فعالیت سیتولیتیک روی PBMCs نشان می دهد در غلظت های پایین FraC، نسبت زنده مانی سلولها به گروه شاهد تغییر معنی داری نیافته است. فعالیت متابولیکی PBMCs فقط در غلظت های بالاتر توکسین (۸۰۰ و ۱۰۰۰ نانومولار) به طور قابل توجهی کاهش یافت. با توجه به نتایج تست تریپان بلو، داده های حاصل از روش نوترال رد نشان داد با افزایش غلظت سم، سنجش جذب نوترال رد در PBMCs به طور قابل توجهی کاهش می یابد.

نتیجه گیری: با وجود فعالیت ضد سلولی سم روی RBC، FraC در غلظت های پایین دارای فعالیت سایتوتوکسیک روی سلولهای تک هسته ای خون محیطی نمی باشد.

کلمات کلیدی: اکتینوپورین، فراگاسیاتوکسین C، سلول های تک هسته ای خون محیطی

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