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Original Article

Capability of Platelet Factor 4 to Induce Apoptosis in the Cancerous Cell Lines *in Vitro*

Alireza Goodarzi Ph.D., Fatemeh Yari*Ph.D., Mahshid Mohammadipour Ph.D. Mohammad Reza Deyhim Ph.D., Hossin Timori Naghadeh M.D.

Blood Transfusion Research Center, Higher Institute for Research and Education in Transfusion Medicine, Tehran, Iran.

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Background and Aims: Platelet factor 4 (PF4) or CXCL4 is a member of CXC chemokine family which is stored in alpha granules of platelets. The main function known for PF4 is angiostasis which may contribute to prevent tumor metastasis. This feature is mediated by CXCR3 on the endothelial cells. Our principal aim was to study the apoptosis induction in three cell lines treated with PF4 and obtained from human platelet concentrates.

Materials and Methods: We evaluated the apoptotic effect of platelet-derived PF4 on the U266B1 and K562 cell lines expressing CXCR3, compared with Daudi as a CXCR3-negative cell line. PF4 was purified from human platelet concentrates by immunoaffinity chromatography and was concentrated. The quantity and molecular weight of the obtained PF4 was determined by enzyme-linked immunosorbent assay and sodium dodecyl sulfate polyacrylamide gel electrophoresis methods respectively. Cell lines were treated for 72 and 96 h with 90 µg/ml of PF4. Apoptosis was assayed by using CD95, WST-1, active caspase-3, and cell count.

Results: Platelet-derived PF4 was a weak agent to induce apoptosis in U266B1 and K562 cell lines. Our data showed in terms of WST-1 and cell count had a significant difference between control and experiment groups ($p \leq 0.05$), while CD95, LDH, and active caspase-3 did not show such a difference ($p > 0.05$).

Conclusions: We observed that PF4 released from platelets has a weak potential to induce apoptosis in cancerous cell lines. Other factors may also contribute to this process including the applied dose, purification method, cell line type, and its proteoglycan carrier.

***Corresponding Author:** Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran. **Tel:** +982182052238, **Fax:** +982188601555, **Email:** f.yari@ibto.ir

Introduction

Platelet is one of the most important elements in the hemostasis and maintenance of vascular integrity [1]. In addition to different surface receptors interacting with coagulation cascade and endothelial cells, it has internal granules containing growth factors, cytokines, and particularly chemokines. CXCL4, also known under platelet factor 4 (PF4), is a CXC chemokine with heparin-neutralizing activity which encodes by chromosome 4 [2, 3]. Its amino acid sequence was identified in 1977 by using affinity chromatography [4, 5]. Although platelets accounts for the most abundant source of PF4, other cells such as mast cells, monocytes, T cells, and dendritic cells can also secret it [6]. The major receptor for PF4 is CXCR3B, one of G-protein coupled receptors, and there is a high affinity for glycosamino-glycans [3]. Platelets have about 20 μg of PF4 per 10^9 platelets. Surprisingly, the level of PF4 in serum is 10 fold than its plasma concentration and it well reflects the release of PF4 after degranulation of platelets [3, 7]. Many years ago, it had been determined that PF4 is a chemotactic factor for many cells mostly monocytes [8]. Moreover, PF4 acts consensually in the coagulation cascade (procoagulant and anticoagulant) because of its affinity for negative-charged molecules or side chains [3, 9, 10]. On the other hand, PF4 is attached to glycosaminoglycans on the surface of platelets, and monocytes, and probably, endothelial cells are the immunogenic target in heparin-induced thrombocytopenia [10]. Most members of CXC family have angiogenesis, but PF4, like other members of CXC family (MIG, IP-10, and I-TAC), has angiostatic role especially in pathologic states [11, 12]. The first report on angiostatic and anti-tumoral effect of PF4 was reported by Maione et al. in 1990. They showed that recombinant PF4 purified from *Escherichia coli* prevents the endothelial cell proliferation in chorio-allantoic membrane [13, 14]. It has been clearly demonstrated that anigostatic activity of CXC family is mediated by CXCR3B in human micro-vascular endothelial cells [15]. CXCR3B inhibits the DNA synthesis and pro-apoptotic and anti-proliferative effects while other two isoforms of CXCR3 (CXCR3A and CXCR3-alt) mediate prolife-ration, migration, and invasion to the inflammatory sites [16, 17]. In this study, we aimed to evaluate the apoptotic effect of PF4 on the immortalized cell lines *in vitro*. There is no report for the effects of naturally PF4 on diverse cell lines.

Materials and Methods

Extraction and purification of PF4 from human platelet concentrates (PCs)

Preparation of PCs: We obtained PCs directly from the Center for Innovation (Iranian Blood Transfusion Organization, Tehran, Iran). Then the final PC was transferred to -20°C freezer in order to lyse the platelets for a minimum of 24 h. Then, platelet lysate was centrifuged at 16000 g at 4°C for 15 min. for removing both platelets and microparticles.

Gel preparation

0.4 gr of CNBr-activated Sepharose 4B (Sigma-

Aldrich, Germany) powder was swollen in 0.2 M carbonate-bicarbonate (PH=9.2) as the conjugation buffer and incubated at 2-8°C for 24 h. The buffer was removed completely and a small amount was retained. Lyophilized anti-PF4 monoclonal (ThermoFisher Scientific, IL, USA) antibody was solubilized in 200 µl of phosphate-buffered saline (PBS) and 60 µl (which is equal to 30 µg) of it was added to gel. Additionally, 100-200 µl of the conjugation buffer was added to the gel and rotated on a vertical rotator at room temperature for 3 h. This step was done for a covalent binding between the antibody and active groups of the gel.

Preparation of an affinity column for PF4

We used a chromatography column to separate PF4. Nylon wool was moisturized with a distilled water. The narrow bottom of the column was filled tightly with glass wool and connected with a plastic tube to collect the effluent. 2-3 ml of PBS was added to the gel and passed through gently from the wall of the column. The column was washed 3-4 times with binding buffer (PBS). We were careful to avoid the dryness of a column both during working and storage. Initially, loose attachments were removed by using glycine buffer (0.2 M, pH=2.8). The remained active groups of gel were occupied with 0.05 M diethanolamine (DEA) in carbonate-bicarbonate buffer. The equilibrium buffer (PBS) was used in 5-10 fold of gel volume (0.5 ml). If the column was not used for a long period, we used PBS containing 0.05% sodium azide to prevent bacterial contamination during storage at 2-8°C refrigerator.

PF4 separation by immunoaffinity chromatography

Platelet lysate was sent through the column. After finishing the sample, the column was washed with PBS to the extent that the amount of absorption at 280 nm of the liquid coming out of the column became equal zero. After this step, we washed the sample again to make sure that any trace of unwanted protein gets eliminated from the column. Elution step was made with glycine buffer (pH=2.8). Furthermore, 50 µl of Tris buffer (2 M, pH=8.0) was added for each 1 ml of glycine buffer into the collection tubes to prevent the protein denaturation.

PF4 purification and concentration

For condensation of the purified PF4, we used a 20 ml concentrator tube with 5K MWCO (5000 Da molecular weight cut-off) membrane, (Spin-X UF 20, Corning, New York, USA). The cut-off of our tubes was 5000 Da. Therefore, proteins smaller than 5000 Da passed through the column and larger than 5000 Da remained on the top of the column. The concentration tube was filled with the PF4 solution and was centrifuged in 4000 RPM for 15-20 min. at room temperature. After re-centrifugation, the top of column was discarded and the bottom part was centrifuged. This step was repeated until total PF4 was applied on the column. Then, the concentrated protein was stored at -20°C until the time of usage.

Protein dialysis

First, we made up a dialysis buffer comprising of 10 mM ethylene diamine tetra acetic acid and 10 mM sodium bicarbonate. Dialysis tubing (SnakeSkin, Thermo Fisher Scientific, Waltham, MA, USA) with 3.5 K MWCO (3500

Da molecular weight cut-off) and 16 mm internal diameter was boiled for 15 min. in the dialysis buffer. Then, the dialysis tubing was transferred into distilled water and was boiled for 10 min. Lastly, the dialysis tubing was soaked in 20-30% ethanol and when using the alcohol was removed. The dialysis tubing was filled with PF4 and tow sides of it was fastened tightly to avoid any leakage. The filled bag was placed into PBS and the buffer was changed every 3 h. Dialysis bag was stored at a cool room for 24 h. Thereafter, PF4 was sent out from the dialysis bag and aliquoted in desired volume. Bradford assay of total protein: We used Coomasie Brilliant Blue G250 (Bradford reagent) to measure the total protein contained in PF4. Briefly, 10 µl of PF4 (diluted and undiluted) samples were mixed well with 200 µl of Bradford reagent. Optical density was read at the wavelength of 595 nm by a UV/visible Spectrophotometer (WPA Biowave II, Biochrom, Cambridge, UK) against a protein standard of bovine serum albumin (BSA, 2 mg/ml).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and dot blot analysis of PF4

We characterized the purified protein by SDS-PAGE and dot blot techniques. In summary, the resolving and stacking buffers were made and PF4 was loaded into the wells of gel. After applying an electric current (100-120 voltage for 2 h), the separated bands were stained with 0.1% Coomassie Brilliant Blue R-250. For dot blot analysis, PF4 was blotted on the PVDF membrane and placed in a semi-dry blotter (PEQLAB Biotechnologie, Erlangen, Germany). The membrane was soaked in 5% skimmed milk for 2 h in room temperature or as overnight in 2-8°C. Primary antibody (anti-PF4) and secondary antibody (HRP-conjugated anti-mouse IgG) were used in 1:750 and 1:3000 dilutions, respectively and incubated for 1 h at room temperature. Subsequently, PVDF was washed (3×10 min) with PBS buffer including 0.05% Tween 20. To detect the reacted bands, Substrate was prepared exactly before use in the dark room by mixing equal volumes (500 µl of each solution); solution A (luminul) and solution B (H_2O_2). The PVDF was located in the substrate solution in dark room for 10 min. BSA (2%) was used as negative control. Enhanced chemiluminescent (ChemiDoc XRS+ Imaging System, Bio-Rad, USA) was used to capture the images. Obtained data was analyzed with Image Lab software (Bio-Rad, USA).

Determination of PF4 specificity

In order to characterize the purified PF4, we performed an enzyme-linked immunosorbent assay (ELISA) precisely according to the manufacturer's instructions. (PF4 (CXCL4) Human ELISA Kit, abcam, UK). Briefly, the samples and standards were added to the anti-human PF4-coated wells. After incubation and subsequently, washing steps, the biotinylated secondary antibody was added. Afterwards, HRP-conjugated streptavidin, 3,3',5,5'-tetra-methylbenzidine substrate solution, and stop solution were used respectively. Finally, the optical density was measured at 450 nm by a microplate reader (Asys Expert 96, Biochrom, Cambridge, UK).

Cell lines selection and expansion

We used three cell lines (all purchased from the

Pasteur Institute, Tehran, Iran) to assay the apoptotic effect of PF4. U266B1 and K562 (myeloma and CML cell lines, respectively) were considered as the experiment group and Daudi (B lymphoblast; Burkitt's lymphoma cell line) as a control group. Cell lines were expanded in Roswell Park Memorial Institute 1640 (Sigma-Aldrich, Germany) and were completed with 2 mM L-glutamine, 1% penicillin/streptomycin (Gibco, Carlsbad, California) and 10% FBS (Biowest, Nuaillé, France). To ensure the correct selection of cell lines, we evaluated the expression of CXCR3 (CD183) as a surface marker by using flow cytometry. Cells were washed with PBS and resuspended to adjust approximately 10^6 cells/mL. 5 µl of PE anti-human CD183 (CXCR3) (clone G025H7, BioLegend, San Diego, California, USA) was added to the cells and incubation was carried out in a dark place at least for 35 min. at 4°C. In addition, PE mouse IgG1 isotype control (Clone MOPC-21, BioLegend, San Diego, California, USA) was used in the same conditions. Immediately, reaction tubes were analyzed by a flow cytometer (CyFlow Space, Partec, Görlitz, Germany). Data were analyzed by FloMax (version 2.70, Quantum Analysis, Münster, Germany) and FlowJo (version 7.6.2, FlowJo, USA) softwares.

Cell lines treatment with PF4

The cell lines were cultured in 25 cm^2 tissue culture flask (Nunclon, Nunc, Denmark) to get sufficient cell count. Then, cells were counted and their viability was assessed by trypan blue dye exclusion by a hemocytometer (Neubauer improved, Marienfeld, Germany). 50000-100000 cells were counted and seeded in each well of a 6 well tissue culture plate (SPL Life Sciences, China). We treated the cell lines for 24, 48, 72, and 96 h with PF4 in a total volume of 1 ml and incubated in a humidified 5% CO2 incubator (CB 150, Binder, Tuttlingen, Germany).

Lactate dehydrogenase (LDH) assay

The supernatant of the treated cells was collected carefully and transferred into a new 1.5 ml micro-centrifuge tubes (Maxwell, Shanghai, China). LDH levels were measured by a quantitative kit (LDH, DGKC), Pars Azmun, Karaj, Iran) and an automatic chemistry analyzer (Hitachi 911, Boehringer Mannheim, Germany).

CD95 expression

CD95 was used as a maker for apoptotic cells. Cells were washed and suspended in PBS and were adjusted at the concentration of 10^6 cells/mL. 5 µl of FITC-conjugated anti-human CD95 (Fas) antibody, (Clone DX2, BioLegend, San Diego, California, USA) was added to 100 µl of the cell suspension. IgG1 (mouse)-FITC (IOTest, Beckman Coulter, Brea, California USA) was used as isotype control. Cells were incubated at 4°C for 35 min. and were subsequently analyzed by flow cytometer.

Water-soluble tetrazolium-1 (WST-1) assay

WST-1 kit (Cayman Chemical, Ann Arbor, Michigan, USA) was used to evaluate the cells proliferation and viability rates. First, 50 µl of the treated and control cells were transferred into a 96 well tissue culture plate (Maxwell, Shanghai, China). Then, 2.5 µl of the electron mediator and 2.5 µl of the developer were added and incubated for 4 h at 37°C. The plate was shaken gently for 1 min. and optical densities were

measured at 450 nm by the microplate reader.

Active caspase 3

To evaluate the active caspase 3 as a biomarker in apoptotic cells, we assayed the expression of active form of enzyme by using an ELISA kit (active caspase 3 (Asp175) human ELISA, abcam, UK). The test was performed precisely by the kit provider protocol. Standards and extracts of cell pellets were added to the pre-coated microplate. After the washing step, the detector and HRP-labeled antibodies were added consecutively. Afterwards, 3,3',5,5'-tetramethylbenzidine development solution and subsequently, stop solution were added. Finally, optical densities was recorded at 450 nm by the microplate reader. Cell counting was performed before and after treatment with PF4 by using hemocytometer and trypan blue dye exclusion to recognize the percent of viable cells.

Statistical analysis

We used SPSS Statistics (version 24, IBM Corporation, USA) and GraphPad Prism (version 7.04, GraphPad Software, La Jolla, CA, USA) softwares for the data analysis. Paired t-test was used to compare the control and experiment groups. $P \leq 0.05$ was considered as statistically significant.

Results

Determining the concentration of platelet-derived PF4

We obtained four products of PF4 (sample 1-4). Figure 1 shows the concentration of each product.

CXCR3 (CD183) expression on the cell lines

The presence of CXCR3 was identified by a specific anti-CXCR3 antibody and istotype control (anti-mouse IgG antibody). U266B1 and K562 cell lines were used as experiment cells: Daudi was considered as control cell line in terms of CXCR3 expression (Fig. 2).

Characterization of purified PF4

We used PAGE and dot blot analysis procedures to approve the size and antigenicity of PF4 respectively. PAGE showed the correct molecular weight and dot blot also confirmed the specificity based on the reaction with anti-PF4 antibody (Fig. 3).

Apoptosis assessment in the cell lines by using PF4, CD95 analysis, WST-1 assessment and cell count

We evaluated the potential of PF4 to induce the apoptosis in the studied cell lines. For better understanding of the effect of PF4, we used different concentrations of PF4 and different treatment times: 1) 72 h, up to 15 µg/ml; 2) 96 h, up to 15 µg/ml; 3) 96 h, up to 30 µg/ml; and 4) 96 h, 90 µg/ml. The presence of apoptosis was measured by expression, LDH levels, WST-1, active caspase-3 levels, and cell count. All the presented data were related to 96 h treatment of cells with PF4 because there were no substantial effects for 72 h treatment.

We observed that CD95 expression increased considerably for K562 in the concentration of 90 µg/ml of PF4. However, there was no significant difference in the cell groups of the control and experiment ($p>0.05$) (Fig. 4). WST-1 was assessed by reading the optical densities at 450 nm. Any decrease in the optical density after treatment with PF4 indicated the inhibition of cell proliferation in contrast with the untreated cells (Fig. 5). Cell count was evaluated by excluding the dead cells. Thus, viable cells were

calculated in the total cell suspension by considering the volume (Fig. 6).

LDH levels

We used a colorimetric method to quantify the final product of reaction by an autoanalyzer. However, we did not observe any significant increment in the LDH level after treatment with PF4 (data not shown).

Active caspase-3 levels in the cell lines after treatment with PF4

Our data showed that platelet-derived PF4 (in the concentration of 90 µg/ml and for 96 h co-culture) could not lead to a significant increase in active caspase-3 levels as an apoptotic marker in each of the studied cell lines. The comparison between the control and experiment groups showed insignificant changes of active caspase-3 in all of cell groups ($p>0.05$). It explains that PF4 could not cause caspase-3 release as an apoptotic marker (data not shown).

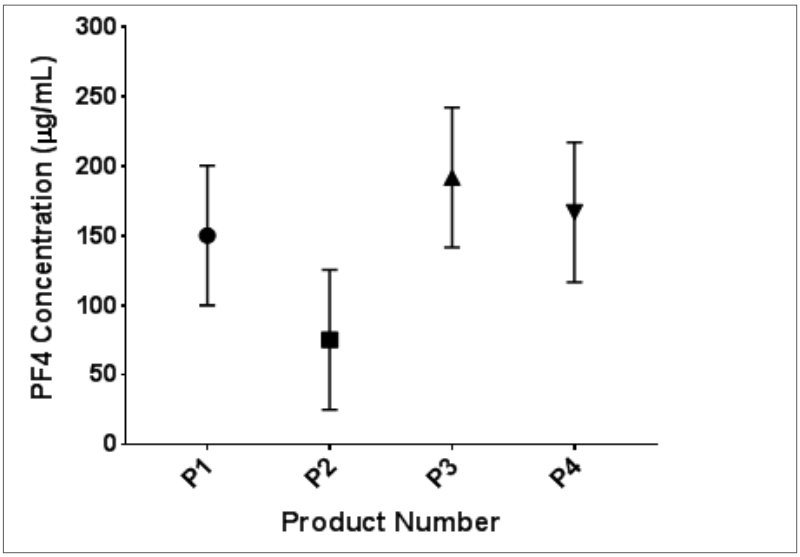

Fig. 1. The concentration (µg/ml) of four products of PF4 (P1-P4) obtained from human platelets. Each point is representative of one product of PF4. Data showed as mean±SD.

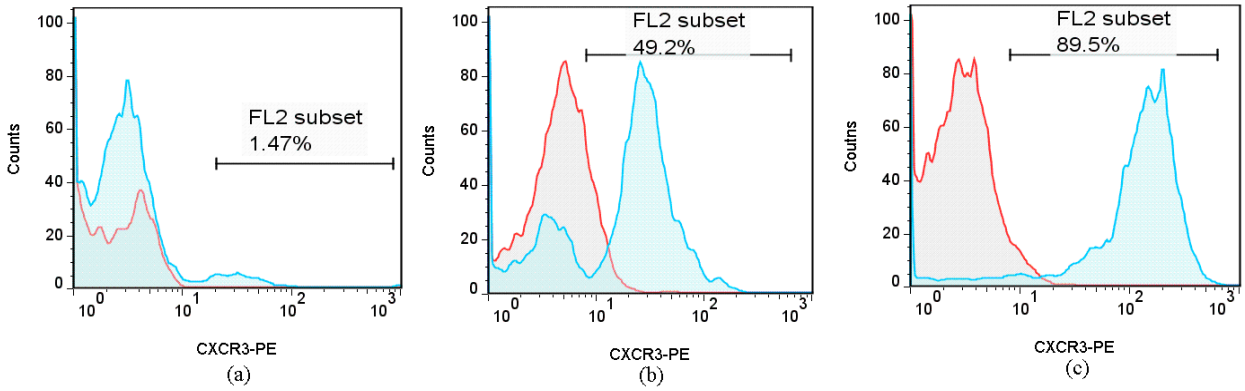

Fig. 2. CXCR3 expression in three cell line before treatment with PF4: Daudi (a), U266B1 (b), and K562 (c). Red and greenish blue histograms are representative of isotypye controls and test samples, respectively. U266B1 (49.2%) and K562 (89.5%) were positive concerning CXCR3 expression.

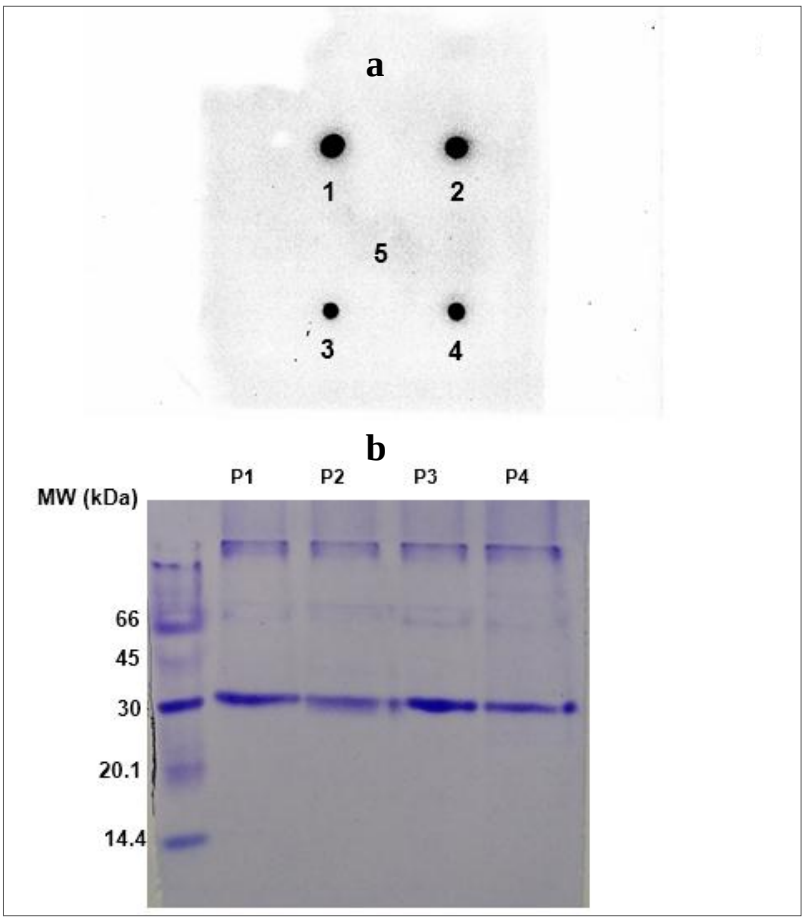

Fig. 3. Dot blot and PAGE analysis of purified PF4. Dot blot (a); black dots are representative of different batches of PF4 obtained from human platelets; 1, 2, 3, and 4. The center (5) was the place of negative control (2% BSA) and there was no observed reaction with anti-PF4 antibody. The bigger zone indicates the higher concentration of PF4. of purified PF4. PAGE (b); the gel shows 4 different products of PF4 from human platelets (P1 to P4). The lane of low molecular weight protein marker (ladder) is specified. The predicted molecular weight for the purified protein was about 30 kDa.

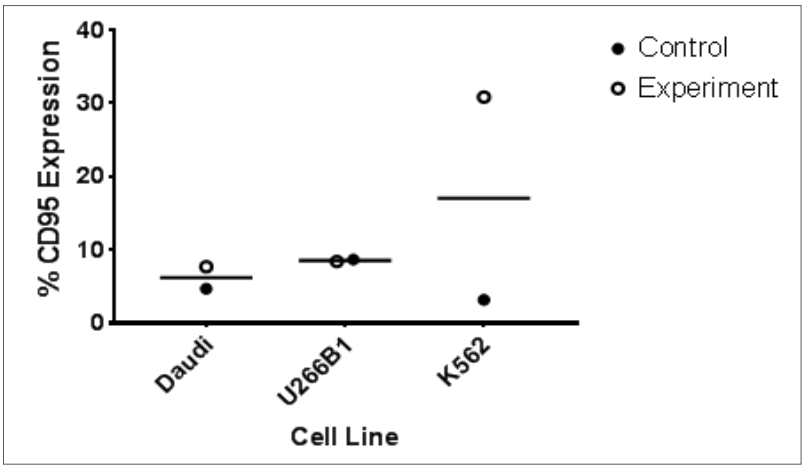

Fig. 4. Mean percentage expression of CD95 in Daudi, U266B1, and K562 after treatment with 90 µg/ml of PF4 for 96 h treatment. We observed that only K562 showed an increase in the expression of CD95 but it was not statistically significant.

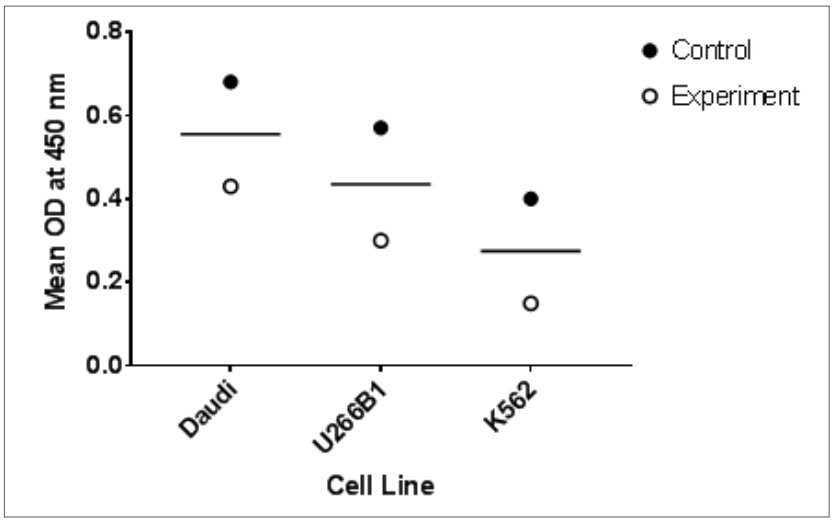

Fig. 5. WST-1 assessment in Daudi, U266B1, K562 after treatment with 90 µg/ml of PF4 for 96 h. Unlike the Daudi cell line, PF4-treated U266 and K562 cells showed a significant decrease ($p<0.05$) in the concentration of 90 µg/ml of PF4.

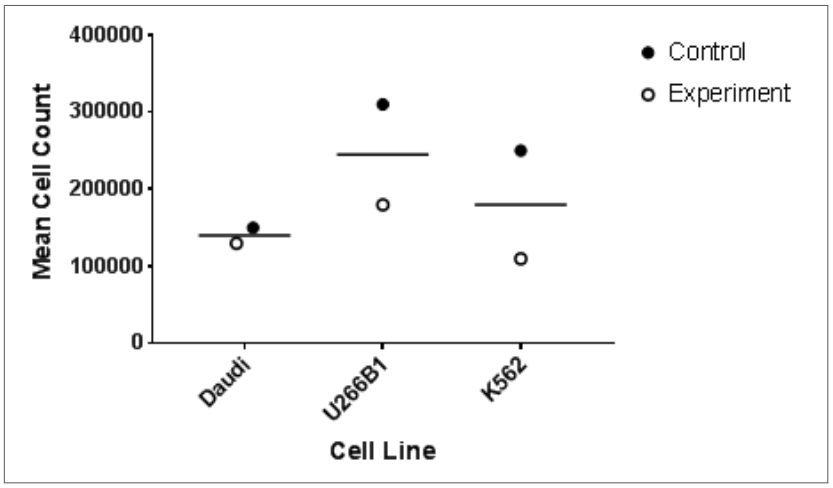

Fig. 6. Mean cell count after treatment with 90 µg/ml of PF4 for 96 h in Daudi, U266B1, and K562. PF4 at 90 µg/mL caused considerable reduction in the cell count of U266B1 and K562 ($p<0.05$) through CXCR3, while this is not the case for Daudi which is CXCR3-negative cell line ($p>0.05$).

Discussion

We assessed the apoptotic effect of PF4 on the cell lines; U266B1 and K562. In addition, Daudi cell line, which was CXCR3-negative, served as a negative control. Before starting treatment, it was necessary to ensure that our cell lines do not secrete PF4 as autocrine. Using ELISA method, we did not detect any release of PF4 from three cell lines (Daudi, U266B1, and K562). The data from this study demonstrated that PF4 in native form is released from platelets and acts as a weak apoptotic agent. We found that WST-1 and cell count results had a significant difference between PF4-treated and untreated cells. However, the results of this

treatment were not significant for active caspase-3, LDH and CD95. Unfortunately, there are a limited number of reports related to PF4 and tumor cells *in vitro*. Liang and colleagues studied the apoptotic effect of PF4 in multiple myeloma cell lines and multiple myeloma patients [18]. They observed a significant apoptosis in U266B1 cells using a very high concentration of recombinant human PF4 (4 µM) while we know its natural concentration in the human serum is approximately 5000-10000 ng/mL (0.9-1.9 µM) [3]. Liang et al. used WST-1, cell number, and caspase-3 activity to evaluate the apoptosis of U266 cells. We showed a weak apoptotic effect for this cell line in our experiments. The main reason for this difference may be due to the use of the native form of PF4, which is released from platelets. This is in contrast with the Liang group who worked on the recombinant form of PF4. It means that platelets secret PF4 complexed with a heavy proteoglycan carrier that recombinant protein lacks it [4, 5, 19-22].

Although, most frequent reports of CXCR3 expression belonged to breast adenocarcinoma cell lines such as MDA-MB-231, MCF-7 and T47D [23], we found that K562 cell line expresses the CXCR3 strongly and can be considered a readily available choice for studying CXCR3 expression. The expression of CXCR3 on the surface of K562 cells was about 90% (Fig. 2c) and even higher than U266B1 cells. The K562 cell line is used as a positive control by many commercial companies producing CXCR3 antibody. Even so, we could not find sufficient data in the literature about the average expression of CXCR3 in K562 and it seems that our work is the first report. CXCR3 is expressed by a subset of normal peripheral blood B cells, some subsets of T cells, natural killer cells and some malignant B-cells such as chronic lymphocytic leukemia, mantle cell lymphoma, and hairy cell leukemia cells [24-26]. We purchased Daudi cell line directly from Pasteur Institute of Iran. Daudi cell line is originally derived from a burkitt's lymphoma patient. Moreover, it was demonstrated clearly that Burkitt's lymphoma is a CXCR3-negative lymphoma by using immunochemistry (IHC) in Jones' study [26] and also our flow cytometric analysis confirmed that Daudi cells did not express CXCR3 (Fig. 2a). Hence, Daudi was considered as a CXCR3-negative control cell line.

The majority of studies which have worked on the PF4 and PF4var (CXCL4L1), have highlighted intensely the angiostatic effect of this CXC chemokine. The core mechanism to inhibit the angiogenesis is mediated by its receptor (CXCR3) on human micro-vascular endothelial cells. This will lead to inducing the apoptosis in the endothelial cells and inhibiting the chemotaxis [13, 27-31]. Morover, it should be pointed out that PF4var (CXCL4L1) inhibits angiogenesis much stronger than PF4 based on some researchers' findings [29, 32]. On the other hand, PF4 may play a role as a chemotactic factor in pathologic conditions and is considered as a prognostic biomarker. For instance, Woller's study demonstrated that PF4-activated monocytes induce apoptosis in the endothelial cell via the release reactive oxygen species. This may involve in some vascular diseases like atherosclerosis and ischemia.

Therefore, it cannot certainly be concluded that PF4 behaves always in favor of the host in diseases [33-36].

Totally, our findings suggest that PF4 has a weak apoptotic agent at the concentration of 90 µg/ml when exposed to CXCR3-expressing cells like U266B1 and K562. We observed that cell count and WST-1 have a significant difference as apoptotic marker but these results were not confirmed by caspase-3, LDH, and CD95. Consequently, it can be concluded that some other factors may bear on the apoptotic effect of PF4 including purification procedure, alterations in steric arrangement, its proteoglycan carrier and the selection of apoptosis markers. Nonetheless, there are hopefully some encouraging reports which make possible the clinical use of PF4 in cancer therapy and metastases monitor in near future [34, 37-39]. This issue is of interest to researchers in the clinical setting particularly as a strategy to treat the cancers in which angiogenesis plays a critical role in tumor progression and metastasis [40, 41]. It can be inferred that further *in vitro* and *in vivo* experiments are needed for utilizing PF4 in the clinic for confronting against cancer and metastasis. Eventually, we strongly recommend the investigations of PF4 and the cell lines originated from endothelial source such as GM7373, because the angiostatic effect of CXCL4 and CXCL4L1 is implemented chiefly on the endothelium [42].

Conclusions

Briefly, PF4 derived from human platelet concentrates can induce apoptosis weakly in U266 and K562 cell lines, but it is more efficient for K562 rather than U266. However, based on our results related to WST-1 assessment and cell counting, we found that PF4 diminishes cell growth and proliferation significantly. Treatment of other different types of cell lines with native PF4 and also with its recombinant form may help perceive more unknown factors that affect apoptotic function of this chemokine *in vitro*.

Conflict of Interest

All authors clearly state that there is not any conflict of interest in financial issues or authorship.

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