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Original Article

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An affinity-based method for the purification of platelet factor 4 from outdated platelet concentrates

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Abstract

***Background & Aims*:** Platelet factor 4 (PF4) is considered as a chemokine mainly strode in the granules of platelets. Its important role in heparin-induced thrombocytopenia (HIT) was the basis of many investigations about this chemokine. High affinity to heparin was used to extract of PF4 from platelet concentrates (PCs). Despite many advantages of recombinant PF4 (rPF4), some researchers prefer to purify rich proteins of platelets from outdated PCs mainly because of cost effectiveness. The main aim of this study was introducing a home-made method to purify PF4 from PCs in blood banks.

***Materials & Methods*:** In this experimental study, we presented a customized procedure based on immunoaffinity chromatography to isolate PF4 that may be useful for laboratory access of PCs from blood bank centers. Briefly, platelet lysate (PL) was extracted from PCs by freeze/thaw cycles and then treated with anti-PF4 antibody to elute PF4 extract in the final product. Data were analyzed by SPSS software (version 26). Student *t*-test was used to compare the results. A probability of < 0.05 was accepted as significant statistically.

***Results*:** Our experiments showed that immunoaffinity chromatography might be considered as an alternative source to provide PF4 particularly in view of cost effectiveness.

***Conclusion*:** PF4 is one of the most bulk growth factors stored in platelets. It has the versatile applications both in diagnosis of HIT and in the study of platelet biology. A home-made protocol presented in this investigation can be helpful to obtain PF4 from blood bank in a reasonable scale.

***Keywords*:** Immunoaffinity Chromatography, Platelet concentrate, Platelet factor 4, Purification

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Introduction

Platelet factor 4 (PF4) or CXCL4 is one of the major platelet chemokines which is released upon platelet activation. Its amino acid sequence was identified in 1977 by Deuel and colleagues (1). Although other cells can produce this chemokine in a lesser amount, the most frequent source always produced and stored in the platelets (2). The high affinity to glycosaminoglycans (GAGs) particularly heparin caused to set up the purification methods based on heparin column

chromatography (3, 4). PF4 is released from platelets along with a proteoglycan carrier (chondroitin 4-sulfate) leads to a high molecular weight around 90 kDa in the whole protein (5). Although many recombination systems also developed to produce PF4 in high yield expression (6), it may not be cost-effective for the developing countries with limited resources.

In this study we demonstrated a procedure to purify PF4 from the expired human PCs in the blood bank. We assume that this protocol can help to access PF4 in the laboratories that provide PCs from blood bank services.

Materials & Methods

Preparation of anti-PF4 antibody:

100 µg of lyophilized powder of human anti-PF4 antibody (ThermoFisher Scientific, USA) was gently reconstituted in 200 µL of phosphate buffered saline and homogenized.

Sepharose 4B gel:

0.4 g of Sepharose 4B (Sigma-Aldrich, Germany) was dissolved in a vial containing carbonate-bicarbonate buffer and then incubated in at 4°C for 24 h. The supernatant of buffer was removed from the top of it and just a small volume was left. 60 µL of anti-PF4 antibody was mixed with the gel and 100-200 µL of carbonate-bicarbonate buffer was added. The solution of gel and antibody was transferred to a smaller vessel and placed on the vertical rotator at room temperature (RT). The covalent binding of antibody and active group was performed in this step.

Immunoaffinity Chromatography Column:

We used a customized glass tool with the progressive decreasing of diameter from to head bottom. First, some glass wool was immersed in a beaker containing distilled water and left for some hours. Moisturized glass wool was inserted from the wide end of the column and filled completely until the glass wool was exited from the tight end. Tight end of the column was connected to a cord of blood bag and a sliding clamp was added to adjust the flow.

The column was rinsed 3-4 times with 2-3 mL of PBS. We avoid the complete drying of the column because of destroying it. Then, gel solution was passed from the column and again 3-4 mL of PBS was passed from the column. This work was repeated many times. The weak links were dissociated with 0.1 M glycine (pH= 2.8). The rest of the active groups in the gel was filled with 0.05 M diethanolamine (DEA) in carbonate-bicarbonate buffer. PBS was added in 5-10 times of gel volume (500 µL). PBS including 0.05% sodium azide was left in the column to avoid the contamination if the column was not used for a long period. The upper opening of the column was sealed with parafilm and the column was stored at 4°C.

Procurement of platelet (PL) lysate from human platelet concentrates (PCs):

PCs were obtained from the Innovation Center (Iranian Blood Transfusion Organization, Tehran, Iran) with a platelet-rich plasma (PRP) method from volunteer donors. PC bag was transferred into -80°C deep freezer for 24 h in order to the lysis and degranulation of platelets. The lysed PC was placed in a 37°C water bath to thaw the contents. The thawed PL was centrifuged in 16000 g for 20 min at 4°C to remove the platelet bodies and microparticles. The clear supernatant of PL was collected and aliquoted in the desired volumes. PLs was stored ate -80°C until the day of use.

PF4 separation by immunoaffinity chromatography:

Applying of PL sample on the column:

PL was passed from the stabilized column at RT in a small and after that increasing volume. The column was rinsed many times with PBS until the optical density (OD) of effluent was equal to zero at 280 nm.

Elution:

0.1 M glycine (pH = 2.8) was used to elute the column and effluent was collected in a new and clean tube. 50 µL of tris buffer (pH = 8.0) was added in the collection tube for each 1 mL of glycine to protect the protein from denaturation. The OD of the collected samples were read at 280 nm to measure the PF4 concentration.

Purification and Concentration of the Separated PF4:

We used a 20 mL concentration tube (Spin-X UF 20, Corning, New York, USA) with 5 kDa molecular weight (MW) cut-off. First, the concentration tube was filled

with PF4 solution and centrifuged at 2000*g* for 20 min at RT. The lower part of the tube was discarded and the upper part centrifuged again. The concentrated PF4 was obtained in a smaller volume and stored at -20°C.

Protein Dialysis:

The SnakeSkin dialysis tubing (Thermo Fisher Scientific, Waltham, Massachusetts, USA) protein with 3.5 kDa MW cut-off was boiled for 15 min in the dialysis buffer and 10 min in distilled water. Finally, dialysis tubing was immersed in a 20-30% ethanol and the alcohol was removed while using.

Dialysis tubing was filled with PF4 and two sides of it was fastened firmly with the cotton to stop any leakage. The filled tubing was placed in a beaker containing PBS. We also used shaker and magnet for a better exchange (Figure 1).

Fig. 1. Protein dialysis in a beaker containing PBS.

The buffer was changed every 3 h. The dialysis tubing was transferred to a 4°C room. The protein was brought out from the dialysis tubing and aliquoted in the desired volumes.

Measurement PF4 Concentration by Bradford Method:

Bovine standard albumin (BSA) with 2 mg/mL concentration was considered as protein standard and serial dilutions were made consequently. The unknown PF4 samples were assayed against BSA at 595 nm. The concentrations of PF4 were calculated by the standard curve.

Characterization of PF4:

Polyacrylamide gel electrophoresis (PAGE):

The resolving and stacking gels were provided based on our optimized recipe and applied in an electrophoresis apparatus. PF4 samples were diluted five times with the sample buffer and boiled for 5 min to denature proteins. The samples were loaded in 5 to 10 µg on the wells of gel. Protein ladder (size marker) similarly was applied separately. Electric force was run for 2 h in 100-120 V and was diminished to 80 V at the last 30 min.

The gel was dyed with Coomassie Brilliant Blue R-250 for 2 h at RT. For longer storage of the colored gel, it was stored at the refrigerator in 30% acetic acid.

Dot Blot Analysis:

The polyacrylamide gel was transferred to polyvinylidene difluoride (PVDF) membrane and the primary and secondary antibodies were added in the next day. Semidry blotter was our blotting device. Electric force was run for 1.5 h in 5 V. The primary (anti-PF4) and secondary (HRP–anti-mouse) antibodies were diluted 1:750 and 1:3000 times respectively before use. The final reaction between the substrate and HRP was enhanced chemiluminescent (ECL) and recorded by ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, California, USA). The captured image was analyzed by Image Lab software.

The Specificity Determination of PF4:

We used an enzyme-linked immunosorbent assay (ELISA) to assure the specificity and quantity of PF4. All steps were performed by following the instructions of the kit manufacturer (Abram, UK). Simply, PF4 samples and standards were added to the wells and washings were carried out where indicated. Lastly, the color reaction was recorded at 450 nm by a microplate reader.

Results

PAGE of PF4 samples:

We demonstrated that our PF4 samples had the MW approximately 30 kDa. This data showed that the proteoglycan carrier of PF4 was removed in the chromatography separation. Because PF4 has a homotetramer structure with each chain 7.8 kDa, therefore the PAGE confirmed the accuracy of related protein (Figure 2).

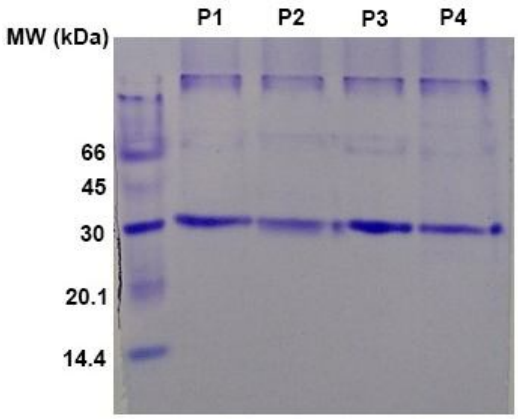

Fig. 2. PAGE analysis of the purified PF4. The gel shows four separate products of PF4 by immunoaffinity chromatography. As the protein was laid on nearly 30 kDa band, PF4 was extracted as homotetramer. The left lane is related to a size marker of protein based on kDa. It is noteworthy that there was no any indication of PF4 carrier since the isolated bands correspond with a tetramer PF4 (about 31 kDa)

Dot Blot:

This assessment helped us assure the specificity of purified PF4. As we used the same antibodies for chromatography and dot blot, the data were complementary (Figure 3).

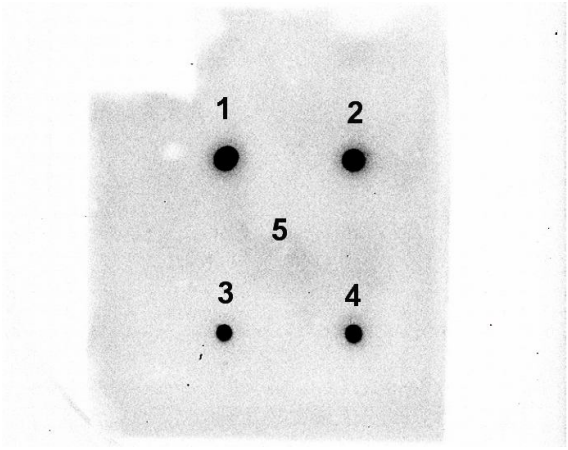

Fig. 3. Dot blot assay of PF4. Number 1 to 4 are representative of four PF4 with different concentrations. Bigger zone is equal to the higher concentration of PF4. Number 5 in the center is the place of control (BSA) having no reaction with the specific anit-PF4 antibody.

ELISA:

Another way to characterize PF4 was ELISA. Antigen-antibody reaction confirmed the specificity of PF4. Also, this assay gave the quantification of protein against the known concentration of standards (Figure 4).

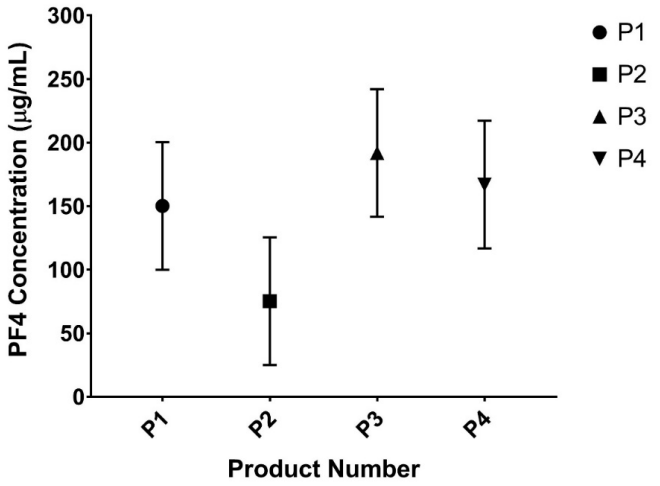

Fig. 4. ELISA results of four PF4 products. Data are shown as ± SD.

Discussion

PF4 is one of the most frequent chemokines stored in α-granules of platelets. This play mainly as an angiostatic factor in the tumor conditions (7, 8). However, PF4 levels of platelet can give a picture of primary tumor growth (9). These data collectively demonstrate that it needs to be studied the effect of PF4 in the health and disease. The best instance for this explanation can be presented by the major role of PF4 in the pathogenesis of heparin-induced thrombocytopenia (HIT) (10, 11). This unique characteristic also was the basis of PF4 isolation and purification from many years ago in the heparin column by affinity chromatography (12). There are also some reports to purify PF4 using affinity and ion-exchange chromatography (13). The majority of chemokines are removed due to very low affinity to heparin compared to PF4 in the elution step applying high concentration of NaCl (3). Many common methods used heparin column to purify PF4 from PCs (3, 4). However, the advantage of our study was a new method by using anti PF4 antibody and immunoaffinity chromatography.

Furthermore, more developments in molecular biology lead to producing a recombinant form of PF4 in *E. coli* having the similar properties to human PF4 (14). However, because of the main source of PF4 in alpha granules of platelets (15), there is an encouraging interest to extract PF4 from outdated PCs (16, 17). Our data confirmed that PF4 purification by immunoaffinity chromatography can maintain the conformational features of the molecule since specific ELISA showed the positive reaction with protein. But, as shown in Fig. 2, our method cannot purify PF4 as a whole molecule with proteoglycan carrier and it was resulted from the PAGE analysis based on MW of PF4 bands. Regarding growth factor nature of PF4, there are some promising reports to use it as a therapeutic agent (18). When PF4 is considered in the clinical settings, the bearing of function should also be assured other than antigenicity. Therefore, it will be more reliable to assay heparin

neutralizing feature of PF4, though we could not to perform it due to shortage of resources.

There are some agonists such as thrombin to activate and degranulate of platelets and PF4 secretion (19). In addition, some researchers used deep freezing method at -80°C or liquid nitrogen leading to rupture the platelet and lysate extract. We believe the PL product may contain more growth factor because of the complete degranulation (20). We recommend our customized procedure to obtain PF4 from PCs in research centers which they avail the unused platelets from blood banks. The main limitation in our study was the inability to perform functionality test against heparin due to shortage of resources. We strongly suggest that it shall be considered for future experiments on PF4.

Acknowledgments

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Data Availability

The raw data supporting the survey are available from the authors upon reasonable request.

Conflict of interest

The authors of this work clearly declare that there is not any apparent conflict of interest in either funding or authorship issues.

Ethical statement

The research team upheld strong ethical standards throughout the study. (IR.TMI.REC.1395.009)

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