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Cytotoxic protein from human platelets

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Abstract The 14 kDa protein was purified from human platelets. It displays high cytotoxic activity to the human ACL cells at 10 M concentration (21.8 ± 7.1%). Its N-terminal sequence is YAPQXQFGP–, being highly homologous to region 241–249 residues of the human Cls complement component.

Key words: Platelet; Cytotoxic protein; Cell cytotoxicity

1. Introduction

Human platelets take part in immune response processes. They initiate cytolysis of endotheliocytes [1,2] and autologous cytolysis of red blood cells [3]. In addition, they have IgE-, IgG-cytokine-dependent cytotoxicity against microorganisms [4,5] and are cytotoxic to human cancerous cells like other cells in the immune system [6,7]. For example, human platelets possess high specific cytotoxicity to ACL cells [8]. The cancerous cells have thrombospondine adhesion domain receptors on the cell membrane [9]. Interleukines and other endogenic immune response modifiers stimulating platelet aggregation also induce platelet cytotoxicity [10,11]. The platelet activation factor (PAF) stimulating bioactive substances released from secretion granules of blood plates increases the platelet cytotoxicity activity at concentrations ranging from 10^{-12} to 10^{-6} M [7]. All this suggests that some cytotoxic protein mediators are located within human platelets. Our paper deals with purification of these proteins and determination of their N-terminal sequences.

2. Materials and methods

2.1. Preparation of platelets

Venous blood (1000 ml) containing heparin (15 ml/ml) taken from healthy donors was centrifuged at 200×*g* for 10 min. Blood plasma enriched with platelets was centrifuged at 400×*g* for 10 min. The sediment containing platelets was resuspended in the RPMI-1640 cell culture medium, then twice centrifuged at 1000×*g* for 10 min to remove serum proteins. The platelet sediment was stored at −70°C.

2.2. Preparation of supernatant of platelets

The platelet sediment was resuspended in buffer containing 0.1% TFA, 2 M NaCl, then frozen and thawed 3 times for cell membrane destruction. Cell debris and unbroken cells were centrifuged at 5000×*g* for 30 min for removal.

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Abbreviations: ACL, adenocarcinoma cell line; PAF, platelet activation factor; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse-phase high-performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PVDF, polyvinylidene difluoride

2.3. Protein purification

The supernatant was immediately applied to the C_4 column (Hi Pore RP-304, Bio-Rad, 4.6×250 mm), equilibrated with 0.1% TFA and eluted with the 60-min linear acetonitrile gradient from 0 to 90%. The flow rate was 1 ml/min; UV detection wavelength was 220 nm. Each peak aliquot (50 ml) was tested to establish cytotoxic activity. The fractions were lyophilized and stored at −4°C.

Active fractions were analyzed by SDS-PAGE (12%) according to Laemmli [12]. The proteins were electroblotted on the Immobilon-P membrane using a semi-dry procedure in 0.02 M sodium bicarbonate buffer at 1 mA/sm current density for 1.5 h. They were then developed by Coomassie R-250 staining.

To measure protein the PVDF membrane was scanned by Ultraspec 2202 laser densitometer (LKB) at 600 nm.

2.4. MTT cytotoxicity assay

Aliquots of HPLC fractions were lyophilized, resuspended in 100 ml of RPMI-1640 cell culture medium and incubated with 5% CO_2 at 37°C for 18 h. MTT cytotoxicity assay was performed according to [13]. The cytotoxicity (C, %) was counted with the equation: $C = [(OD_c - OD_{cx})/OD_c] \times 100\%$. Here, OD_{cx} is the optical density of the experimental solution and OD_c of the control solution.

The statistic method [14] was applied to analyse the results of the five identical experiments.

The cytotoxicity of certain proteins was measured after elution of protein band from the PVDF membrane as described by Chertov et al. [15].

2.5. Amino acid sequence determination

Amino acid sequencing was performed from the PVDF membrane using a Model 470A Applied Biosystems gas-phase sequencer.

3. Results

We obtained 15 fractions from RP-HPLC of the supernatant containing human platelets (Fig. 1). The fractions were tested to establish the cytotoxicity to the ACL target cells. Fraction 5 eluted from 33 to 36% of acetonitrile demonstrated the highest cytotoxicity (24.1 ± 7.4%) (Fig. 2).

We observed only one band of 14 kDa protein (p14) after separating this fraction by SDS-PAGE. According to densitometric analysis of the p14 SDS-PAGE band, 100 ml of plaque plasma contained more than 10 pmol of p14. The protein was electroblotted and eluted from the PVDF membrane, its cytotoxicity was measured at various concentrations (Table 1), thus being inactive at a concentration of 10^{-8} M. The highest cytotoxicity was observed at 10^{-10} M (21.8 ± 7.1%).

The p14 N-terminal sequence appeared to be gomologous to region 241–249 residues of the human Cls complement component: p14, -^{1}YAPQXQFGP^{9}- and Cls, -^{241}VAGDRQFGP^{249}.

4. Discussion

The cytotoxic activity of human platelets has been described by many authors. Soper et al. [16] outlined the platelet's role in the mechanism of antibody-dependent cellular cytotoxicity.

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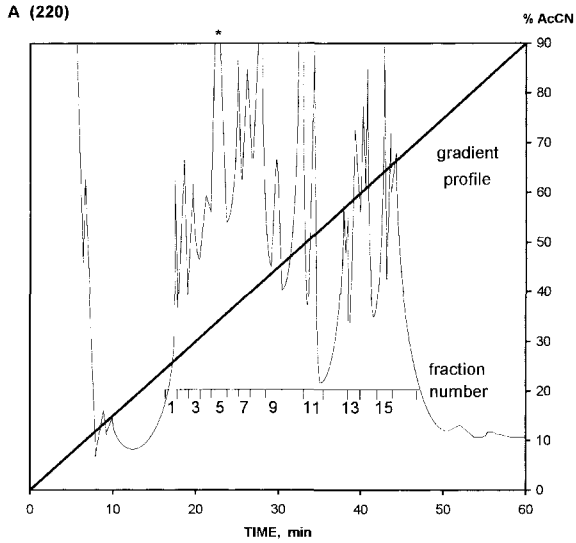

Fig. 1. Elution profile of a human platelet extract (2 ml) on Hi Pore RP-304 column (250×4.6 mm, Bio-Rad). First eluent was 0.1% TFA. The linear acetonitrile gradient ranged from 0 to 90% for 60 min. The flow rate was 1 ml/min. UV detection wavelength was 220 nm. *Highest cytotoxic (21.8±7.1%) fraction.

Shin et al. [17–19] showed that platelets were involved as effectors in the cytotoxic antibody-dependent immune response in vivo. According to Slezak et al. [20] the antibody-dependent cellular cytotoxicity system is arranged on the platelet cell membrane because the supernatant obtained after platelet lysis and centrifugation showed much less activity against chicken erythrocytes than did the sediment containing platelet membranes.

Ibele et al. [6] described the platelet cytotoxic action to the finite cell lines of kidney melanoma and adenocarcinoma. The supernatant obtained after incubation (5% CO_2, 4 h, 37°C) of

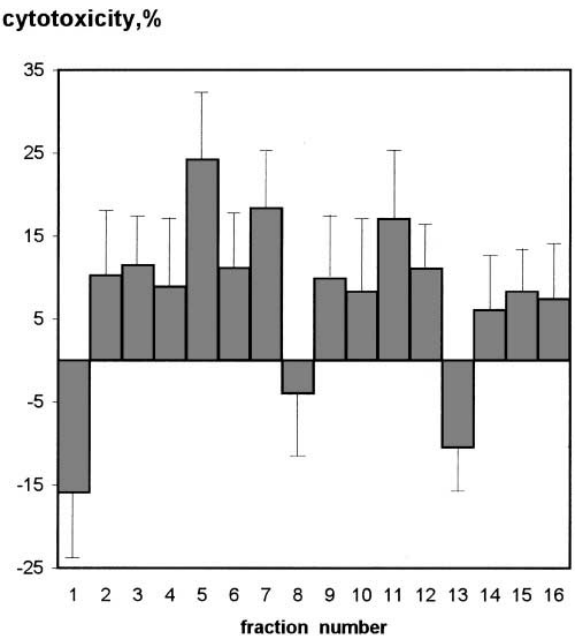

Fig. 2. Cytotoxic activity of HPLC fractions (Fig. 1) of the supernatant of human platelets.

Table 1
Cytotoxic activity of p14

Concentration	Cytotoxic activity	Probability level
10^{-8} M	6.3%±6.8%	>0.05
10^{-10} M	21.8%±7.1%	<0.05
10^{-12} M	14.2%±8.8%	<0.05

human platelets in multiwell plates containing the RPMI cell culture medium was cytotoxic to cancer cells. The platelets were activated after adhesion to the plate bottom and their granules with a cytotoxic factor were dissolved in the supernatant. Our results confirmed data described by Ibele et al. However, the supernatant that we prepared was used to obtain the cytotoxic protein by 3-fold freezing and thawing with subsequent centrifugation at 5000×*g* for 30 min. As shown by Slezak et al. [20] the platelet granules were completely dissolved in the supernatant.

The 14 kDa protein that we purified displayed cytotoxic activity to the ACL cells in the absence of antibodies. However, its high homology to a region of the human Cls complement component still requires an explanation.

According to Segava et al. [21] the platelets activated by thrombin and those nonactivated are cytotoxic to K-562 cells. The supernatant containing secret products of platelets activated by thrombin is not, however, cytotoxic to K-562 cells. This fact apparently indicated the specificity of the platelet cytotoxic factor action and the dependence on a target cell type. Different cytotoxic factors were probably involved in cytolysis of various target cells. In this case, the factor investigated by Segava et al. had a high lability and lost its activity in the supernatant.

The above data testify to the existence of not less than two different mechanisms of platelet cytotoxicity: platelets (1) cause antibody-dependent cellular cytotoxicity with their mediators disposed on the platelet cell membrane and (2) display cytotoxic activity in the absence of antibodies, although this does not depend on the type of target cells and the cytotoxic factors are thus contained in platelet granules.

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