EXPERIMENTAL ARTICLES

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PRODUCTION AND CHARACTERIZATION OF ANTIBODIES TO TISSUE PLASMINOGEN ACTIVATOR: APPLICATION FOR THE PLATELET FLOW CYTOMETRY ASSAY

E. I. Yusova[1]
T. V. Grinenko[1]
T. A. Goncharenko[1]
L. G. Kapustyanenko[1]
A. A. Sobchenko[2]
A. A. Tykhomyrov[1]

[1]Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine

[2]Blood Center of the National Military Medical Clinical Center "Main Military Clinical Hospital", Ukraine, Kyiv

E-mail: yusova07@gmail.com

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Tissue plasminogen activator (tPA) is one of the key protein of plasminogen/plasmin system that converts plasminogen in the active proteinase plasmin. Platelets are able to bind both tPA and plasminogen on their surface, thus providing stimulatory effects on activation of zymogen. The present study was aimed to produce polyclonal antibodies against tPA and characterize their immunochemical capacities for further application in flow cytometry assay to study interaction between tPA and platelets. The experimental methods involved immunization of rabbit with tPA, collection of immune serum, synthesis of tPA-containing immunoaffine sorbent, ELISA, and flow cytometry. Polyclonal monospecific antibodies against tPA with high affinity to the antigen ($K_d = 4.05 \cdot 10^{-9}$ M) were obtained. Flow cytometry assay based on the use of the produced antibodies showed the presence of binding sites for tPA on the plasma membrane of inactive platelets. Moreover, agonist-stimulated platelets were revealed to expose more binding sites than their resting counterparts. Certain subpopulations of platelets that differ in the ability to bind tPA on their surface were also identified. Obtained data are of significant importance for further investigation of mechanisms underlying the role of platelets to regulate fibrinolytic rates.

Key words: plasminogen activator of tissue type (tPA), immunization, polyclonal antibodies, platelets, flow cytometry.

Tissue plasminogen activator (tPA, EC 3.4.21.68) is a serine proteinase that plays a key role in the regulation of blood fibrinolysis via converting the proenzyme plasminogen into its proteolytically active form plasmin (EC 3.4.21.7), which is responsible for hydrolysis of the fibrin network of the blood clot [1]. Recent researches demonstrate that tPA is involved not only in fibrinolysis, but also takes part in the regulation of proliferation of endotheliocytes and cancer cells of pancreas, modulation of apoptosis of neurons and interstitial renal fibroblasts, and also greatly contributes to the loss of blood-brain barrier integrity [2–6]. At present, extensive experimental material has been accumulated indicating that tPA acts as a cytokine that binds to plasma membrane receptors, activates a wide range of intracellular signaling pathways, and controls gene expression [7]. It was shown on NRK-49F cells (interstitial rat kidney fibroblasts) that tPA binds to the cell membrane receptor LRP-1, induces its phosphorylation at tyrosine residues, triggers the phosphorylation of intracellular signal messengers Mek 1 and Erk-1/2, thus leading to

overexpression of matrix metalloproteinase-9 (MMP-9) gene [8].

tPA is one of the components of the plasminogen activation system. It is known that the initiation of the process of plasmin formation depends on the colocalization of the proenzyme and its activators on polymer fibrin, extracellular matrix proteins, and cell surface. Particular interest is presented by data that platelets, which are involved in ensuring hemostasis and in pathological thrombus formation, are able to be accumulated on the surface plasminogen and its activators and thus engaged in localization and regulation of fibrinolytic process. According to the literature, recombinant tPA interacts with intact and thrombin-stimulated platelets, K_d 340 ± 25 nM and 800 ± 60 nM, respectively [9]. However it was shown that plasminogen on the surface of platelets is transformed into plasmin not by tPA, but by urokinase, which is exposed on monocytes or microparticles of endothelial cells [10]. In this context, the question of the functional significance of the binding of tPA to platelets requires further research.

Antibodies are widely used for studying interaction of tPA with other proteins and cells, identifying its receptors, and establishing the molecular mechanisms of its functioning in various physiological and pathophysiological processes. The aim of this work was to obtain and characterize the basal immunochemical properties of polyclonal antibodies against tPA and to validate their suitability for application in flow cytometrical detection of tPA presented on the platelet surface.

Materials and Methods

Preparation of affinity sorbent. Synthesis of immunoaffinity sorbent with covalently cross-linked tPA was performed with the use of Sepharose 4B (GE Healthcare Bio-Science, Sweden), activated with BrCN, according to the manufacturer's protocol. 1.7 g of BrCN-Sepharose dry powder was stirred for 15 min in 1 mM HCl and was washed on a glass filter with the same solution (total volume — 200 ml per 1 g of powder). The gel swelled in a small volume of 1 mM HCl (10–15 ml). After swelling, the gel was washed with buffer for binding (0.1 M $NaHCO_3$ with 0.5 M NaCl, pH 8.3) in a volume of 50 ml per 1 g of the dry preparation. The preparation of protein for immobilization was carried out in a binding buffer. The BrCN-Sepharose was mixed with a solution of tPA at the rate of 10 mg of protein per 1 ml of gel. The protein carrier binding reaction was carried out for 10–12 hours at 4 °C with the careful mixing. After immobilization, the sorbent was pressed on a glass filter and washed with 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3 (total volume 100 ml). Active BrCN-Sepharose groups that did not bind the protein were blocked by 1 M ethanolamine, pH 8.0, for 10–12 hours at 4 °C with careful mixing. Residual ethanolamine was washed with 0.1 M $NaHCO_3$ (pH 8.3), double-distilled water and 0.1 M Na-acetate buffer (pH 4.0). The last washing of the sorbent suspension was carried out with 0.05 M Tris buffer, pH 7.4, and 0.15 M NaCl. To determine the amount of tPA that binding with BrCN-Sepharose, 0.3 ml of suspension was collected and hydrolyzed in 0.9 ml of 1 M NaOH. The protein concentration in the hydrolyzate was measured on a spectrophotometer at λ = 280 nm. Obtained affinity sorbent had the ligand binding to BrCN-Sepharose of 90%. The working column had volume of 4.5 ml, capacity per ligand 2.35 mg/g of Sepharose.

Rabbit immunization. To obtain the immune antiserum from laboratory animals, two rabbits weighing 4 kg, which were kept on a standard diet in the animal house of the Palladin Institute of Biochemistry of NAS of Ukraine, were immunized with tPA (Actilise, Boehringer Ingelheim, Germany). All ethical procedures were followed during the whole experiment. 0.9 mg of antigen was dissolved in 0.9 ml of 0.05 M Tris-HCl buffer with 0.13 M NaCl, pH 7.4, and emulsified with an equal volume of complete Freund's adjuvant (Sigma-Aldrich, USA). The emulsion was injected intracutaneously into six points along the paravertebral area. Three weeks later, animals were boosted under the same conditions, but with the use of incomplete Freund's adjuvant (Sigma-Aldrich, USA). Blood sampling was carried out at the day 12 after antigen administration. Blood was collected from the ear vein into glass centrifuge tubes and kept at 4 °C for 16 hours. The clot was removed, and the serum was centrifuged at 1,000 *g* for 15 min.

Antibody isolation and purification procedures. To isolate the globulin fraction, a saturated solution of ammonium sulphate was added to the serum in equal volume and incubated at 4 °C for 16 hours and centrifuged at 1,000 *g* for 30 min. The supernatant was removed, the precipitate dissolved in 0.05 M Tris-HCl buffer with 0.13 M NaCl, pH 7.4, dialyzed against the same buffer at 4 °C. After dialysis, a proteinase inhibitor 4-nitrophenyl 4-guanidinobenzoate hydrochloride (Sigma-

Aldrich, USA) was added to the globulin solution to a final concentration of 10^{-4} M.

To isolate IgG pool from the globulin fraction, a column with protein A-sepharose (Protein A-sepharose from *Staphylococcus aureus*, Sigma-Aldrich, USA) in a volume of 2 ml, which was equilibrated with a tenfold volume of 0.05 M Tris-HCl buffer with 0.13 M NaCl, pH 7.4, was applied. To remove proteins that did not specifically bind with sorbent, the column was washed with the same buffer solution. IgG bound with protein A-Sepharose, were eluted with 0.1 M glycine buffer, pH 2.8. The fractions were collected in 1 ml plastic tubes and pH was immediately neutralized by adding 1 M Tris, pH 8.5. The protein concentration in the fractions was monitored spectrophotometrically at $\lambda = 280$ nm. The IgG-containing fractions were combined, dialyzed against 0.05 M Tris-HCl buffer with 0.13 M NaCl, pH 7.4 (200 ml for 30 min for 3 times at 4 °C) and concentrated by centrifugation with the use of 100 kDa cut-off Amicon Ultra filters (Millipore Ltd, Ireland).

To obtain antibodies on a column with tPA-Sepharose, a solution of IgG in the ratio of 1.2–1.4 ml per 1 ml of gel was applied. Then, the column was washed with 0.05 M Tris-HCl buffer with 0.13 M NaCl, pH 7.4 to remove non-specifically bound proteins. Elution of specific IgG was performed with 0.2 M glycine buffer, pH 2.8. The fractions were collected in 1 ml-tubes and immediately neutralized with 1 M Tris-HCl solution, pH 8.5. The concentration of immunoglobulins in fractions was monitored spectrophotometrically at $\lambda = 280$ nm. IgG-containing fractions that specifically bound to tPA-Sepharose were pooled, dialyzed against 0.05 M Tris-HCl buffer with 0.13 M NaCl, pH 7.4 (200 ml at 30 min × 5 times) at 4 °C and concentrated with the use of Amicon Ultra 50 kDa cut-off centrifuge filter. The protein concentration was determined by the difference in the optical density of solutions at 280 and 320 nm. The following values of extinction coefficients (E_{280}, 1%, 1 cm) and molecular weight were used for the calculation of concentration: 14 and 150 kDa for the antibodies [11]. The antibodies were stored in 50% glycerol at −20 °C.

Immunoassay analysis (ELISA) of immune serum and isolated antibodies was carried out in MaxiSorp microplates ("Nunc", Denmark). Antigen — tPA, urokinase or Glu-plasminogen (10 μg/ml in 0.1 M $NaHCO_3$, pH 9.6, V = 0.1 ml) was absorbed on the microplates wells and incubated for 16 h at 4 °C. Unbound proteins were washed with 0.05 M Tris-HCl buffer with 0.13 M NaCl and 0,05% Tween-20, pH 7.4 (TBST, 0.2 ml × 5 times) and 0.05 M Tris-HCl buffer with 0.13 M NaCl, pH 7.4 (TBS, 0.2 ml × 5 times). To block nonspecific binding, bovine serum albumin (BSA) was added to the wells (2%, 0.2 ml per well), incubated for 2 h at 37 °C. After washing out BSA, immune serum in dilution series (1/200–1/102,400) or IgG to tPA (0.25–25 mcg) was added in a volume of 0.1 ml per well and incubated for 2 h at 37 °C. Unbounded antibodies were washed out with TBST for 10 times. Secondary antibodies against rabbit IgG conjugated with alkaline phosphatase (EC 3.1.3.1) (Sigma-Aldrich, USA) diluted in TBS at the ratio of 1:30,000, were added in a volume of 0.1 ml microplate wells and incubated for 1 hour at 37 °C. Washing from unbound secondary antibody were carried out in 5 times with distilled water, 5 times with TBST, and 5 times with TBS. An alkaline phosphatase substrate, para-nitrophenyl phosphate disodium salt (1 mg/ml of 10% diethanolamine, pH 9.8), 0.1 ml per well, was used to detect antigen-antibody complexes. The optical density was measured after 20 min of incubation with the substrate S2251 (H-D-Val-L-Leu-L-Lys-*p*-nitroanilide) on a Multiskan EX reader (Thermo Electron Corporation, Finland) at $\lambda = 405$ and 492 nm. Purity of isolated specific IgG pool was monitored electrophoretically in 8% PAGE-SDS [12].

Platelet isolation. Human platelets were obtained by differential centrifugation of donor blood, mixed with anticoagulant (0.1 M sodium citrate, 0,08 M citric acid, 0.11 M glucose) in a ratio of 9:1 (blood : anticoagulant). The blood was centrifuged for 20 min at a room temperature at 160 *g* to isolate the platelet-rich plasma fraction. The resulting supernatant was centrifuged twice for 20 min at a room temperature at 340 *g*. After each centrifugation, the cells was resuspended in a buffer for washing platelets (0.02 M HEPES, 0.137 M NaCl, 0.004 M KCl, 0.0002 M $MgCl_2$, pH 6.8, with 0.2% glucose and 0.2% BSA). The volume of buffer was equal or be (at last stage) 1/2 of platelet-rich plasma fraction volume. Washed platelets were stored at 37 °C. Platelets viability and the adequacy of the cellular response to the action of the agonist (thrombin) were checked by aggregometry as described earlier [13].

Flow cytometry assay. The interaction of tPA with platelets was determined by flow cytometry with the use of Coulter Epix XL cytometer (Beckman Coulter, USA). The following groups of cells were taken: 1) intact platelets;

2) platelets incubated with tPA; 3) thrombin-activated platelets; 4) platelets incubated with tPA after their treatment with thrombin. Platelet activation (6.5×10^7 cells/0.2 ml) with thrombin (1 unit NIH/ml) was performed for 5 min at 22–25 °C in accordance with previously selected conditions. After incubation, the cells were washed twice after centrifugation in 0.02 M HEPES, 0.137 M NaCl, 0.004 M KCl, 0.0002 M $MgCl_2$, pH 6.8, with 0.2% glucose and 0.2% BSA and resuspended in 0.2 ml of the same buffer solution. The control group of cells was subjected to the same procedures, but without activation by thrombin. After incubation, anti-tPA antibodies were added to the cell suspension 1:200 diluted, pre-incubated for 30 min at 37 °C in the dark with 0.2 ml of the secondary FITC-labeled anti-rabbit IgG (Sigma-Aldrich, USA) 1:200 diluted. The cells were incubated for 30 min at room temperature in the dark and the unbound antibodies were removed by double washing with a HEPES buffer by centrifugation at 1,000 *g* for 5 min at 20 °C. The resulting cell pellet was resuspended in 1.0 ml of HEPES buffer and used for cytometry analysis. For each analysis, the device collected at least 10,000 events for optimum data analysis. The cell flow rate mode was medium or low, and the fluorophore excitation wave length was 488 nm. The fluorescence intensity was recorded as a logarithmic scale using the FL1 channel. The FITC fluorescence intensity was expressed in arbitrary units to evaluate the signal difference between different platelet groups. Quadrants (Q) III and IV (upper right and lower right) were set to limit the populations of tPA-FITC-positive platelets. Events in any quadrant other than Q III and IV are indicative of tPA non-binding platelets. All data were corrected for nonspecific fluorescence of controls quantified in the same way (native platelets not incubated with tPA and FITC-labeled antibodies). Quantitative assessment of the redistribution between different platelet populations was performed using two parameters of the flow cytometer: granularity (by the side scattering SS) and fluorescence intensity. Measurement of cytometric parameters in each group of cells was performed in two parallels. Graphical presentation of the results was made with the use of FCS Express V3 program (De Novo Software, USA).

Quantitative data of immunochemical analysis are expressed as mean (M) ± standard error of mean (m). The results of flow cytometry assay are presented as medians of relative fluorescence intensity values and assigned as arbitrary units.

Results and Discussion

Immunoassay results indicate that antigen injections induced synthesis of specific antibodies with a serum titer up to 1:100,000 (Fig. 1, *1*). The specificity of the immune response was confirmed by the absence of binding of IgG from non-immune serum with the correspondent antigen (Fig. 1, *2*).

Polyclonal antibodies to tPA were isolated from the antiserum as described [14,15] with some modifications. A suspension of the total fraction of serum globulins obtained by salting out proteins with a saturated solution of ammonium sulfate was repeatedly passed through a column with protein A-sepharose. IgG bound to the sorbent was eluted with 0.2 M glycine buffer, pH 2.8. Then the IgG solution was repeatedly passed through a column with tPA-sepharose to isolate the pool of specific antibodies to tPA. The final yield of antibodies was approximately 20% of the total amount of IgG fraction. The purity of the obtained antibody preparation was checked electrophoretically in 8% PAAG in the presence of 0.1% SDS. Densitometry analysis of electrophoregram (Fig. 2) showed that 150 kDa band corresponding to IgG molecules is presented with 99% purity.

According to the literature data, the tPA molecule consists of five structural modules, including a finger domain (Ser1-His44), an epidermal growth factor-like domain (Ser50 — Asp87), two kringle domains (Cys92 — Cys173

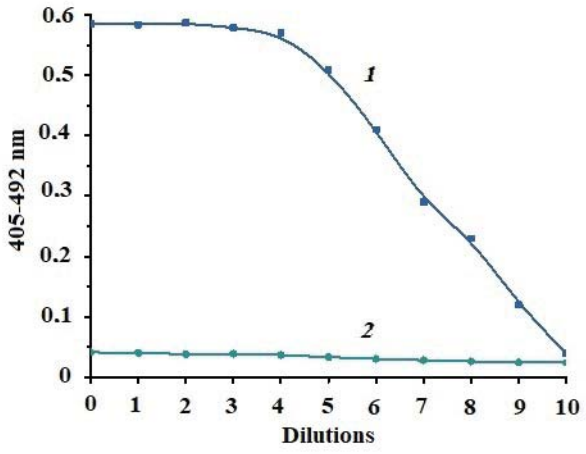

Fig. 1. **Binding profile of antibodies from the immune anti-tPA rabbit serum with antigen**
Dilutions: *1* — 1:200; *2* — 1:400; *3* — 1:800; *4* — 1:1,600; *5* — 1:3,200; *6* — 1:6,400; *7* — 1:12,800; *8* — 1:25,600; *9* — 1:51,200; *10* — 1:102,400.
Averaged curves are typical for series of repeated experiments ($n = 3$)

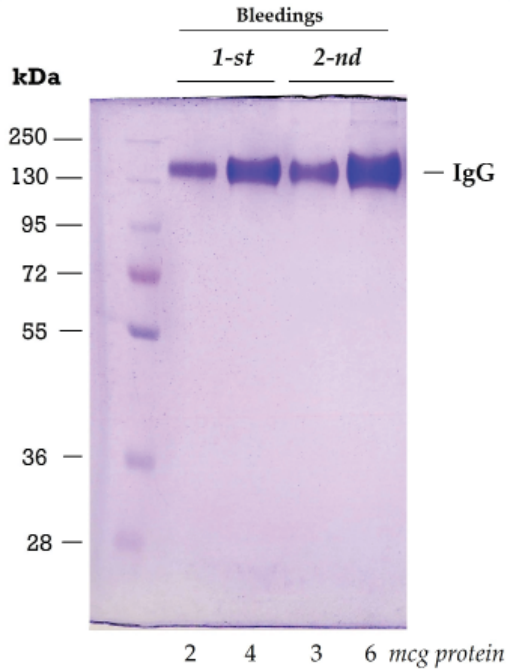

Fig. 2. **Electrophoregram of affinity purified antibodies against tPA eluted from tPA-sepharose** (8% PAAG, Coomassie R-250 staining)

and Cys180 — Cys261), and C-terminal serine proteinase domain (Ser262 — Pro527), similar to the catalytic domains of other serine proteinases, where His322, Asp371, and Ser478 form the catalytic triad of tPA active center [16]. Structurally, kringle domains of tPA are homologous to similar domains of such proteins as plasminogen, urokinase-type plasminogen activator, prothrombin, factor XII, and apolipoprotein, but the greatest homology is observed between the second kringle domains of tPA and urokinase molecules [17]. Using the BLAST-UniProt platform, a comparative analysis of the amino acid sequences of these structural units of the molecules of both proteins was performed, which revealed that the homology between them is more than 50% (Table 1). To verify cross-reactivity of obtained anti-tPA antibodies with the homologous kringle-containing proteins, plasminogen and urokinase, is an important task, which is necessary to be solved for an adequate application of these antibodies in the ongoing and further studies of tPA-involved protein-protein and protein-cell interactions, as well as precise detection of this enzyme in biological material.

The interaction of anti tPA antibodies with tPA, urokinase and plasminogen was investigated by ELISA. Dissociation constant K_d of protein-protein complexes formation was calculated using plots of the ratio [concentration/level of ligand binding to immobilized protein] according to the equation:

$$A = A_{max}/(1+ K_d/[L]),$$

where A — a p-nitroanilide absorption level that is proportional to the amount of bound ligand on the linear region of the curve; A_{max} — an absorption at the ligand saturated concentration; [L] – a molar concentration of ligand at A; K_d — a dissociation constant [18].

Anti-tPA IgG demonstrate a high affinity for immobilized tPA ($K_d = 4.05 \cdot 10^{-9}$ M) and lower to urokinase ($K_d = 1.26 \cdot 10^{-8}$ M) and plasminogen ($K_d = 1.01 \cdot 10^{-8}$ M). Saturation of tPA binding sites for antibodies was observed at the concentration of $3.3 \cdot 10^{-8}$ M, while antibodies taken in such concentrations did not saturate binding sites on urokinase and plasminogen (Fig. 3). Of note, at the maximum used concentration of IgG ($1.67 \cdot 10^{-7}$ M), the level of binding of antibodies to tissue activator appeared to be by 4 folds higher than that with both urokinase and plasminogen. Therefore, the obtained polyclonal antibodies are apparently monospecific to tPA.

Further, produced antibodies were used for investigation of the interaction of tPA with native (non-stimulated control)

Table 1. **Amino acid sequence of kringle domains of tissue activator and urokinase**

Kringle domain of urokinase	TCYEGNGHFYRGKASTDTMGRPCLPWNSATVLQQTYHAHRSDALQLGLGKH-NYCRNPDNRRRPWCYV
Second kringle domain of tPA	TCYEDQGISYRGTWSTAESGAECTNWNSSALAQKPYSGRRPDAIRLGL-GNHNYCRNPDRDSKPWCYV
Identical amino acid residues	TCYE..G..YRG..ST...G..C..WNS....Q..Y...R.DA..LGLG.HNYCRNPD ....PWCYV

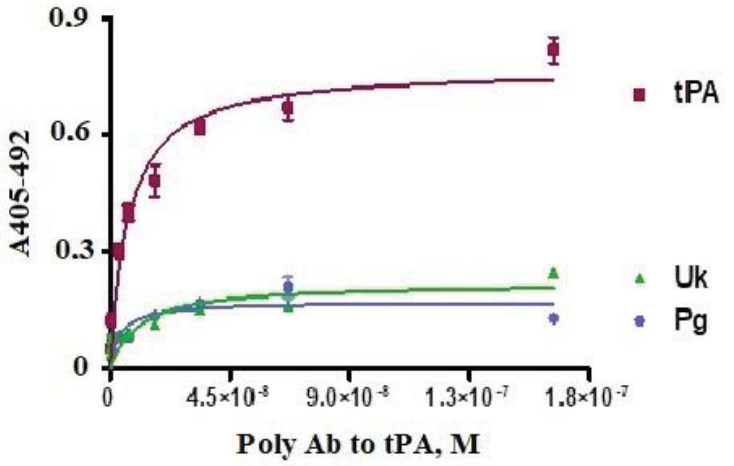

Fig. 3. **Binding of anti-tPA antibodies with tPA, urokinase and plasminogen**
The averaged curves ($n = 3$) are typical for series of repeated experiments

and thrombin-stimulated platelets by flow cytometry assay. The results of data analysis are shown in Figs. 4 and 5. The gates that separated the population of non-fluorescent cells were set based on the intensity of fluorescence level in the control groups of cells (washed non-activated and thrombin-stimulated platelets not incubated with proteins) (Figs. 4, *A* and 5, *A*).

Incubation of both intact (control) and thrombin-stimulated platelets with the cocktail of anti-tPA IgG and secondary anti-rabbit IgG-FITC lead to 3- and 4-fold increase in the number of events with a FITC-positive signal, respectively, while the fluorescence intensity values increased by 1.4-folds in the both cases (Table 2). These results indicate binding and presence of the antigen on the platelet surface.

These data confirmed our earlier observation of the exposure of specific binding sites for tPA on the plasma membrane of platelets circulating in the bloodstream, the number of which increases as a result of their agonist-induced stimulation [16]. In the case of the treatment of thrombin-stimulated platelets with tPA followed by the incubation with anti-tPA antibodies, certain sub-populations of activated cells (about 30% of the total number of platelets), characterized by high FITC-fluorescence level, were discovered (Fig. 4, Table 3). These data strongly indicate interaction of exogenous tPA with membrane-associated binding cites on the platelet surface. These results are in a good agreement with the literature data [19] showing reversible and concentration-dependent binding for tPA to the surface of non-activated platelets, which is realized through a single class of low affinity sites and can be enhanced with agonist-induced cell stimulation.

It is known that platelet activation induces appearance of several phenotypic subpopulations of cells, which may perform

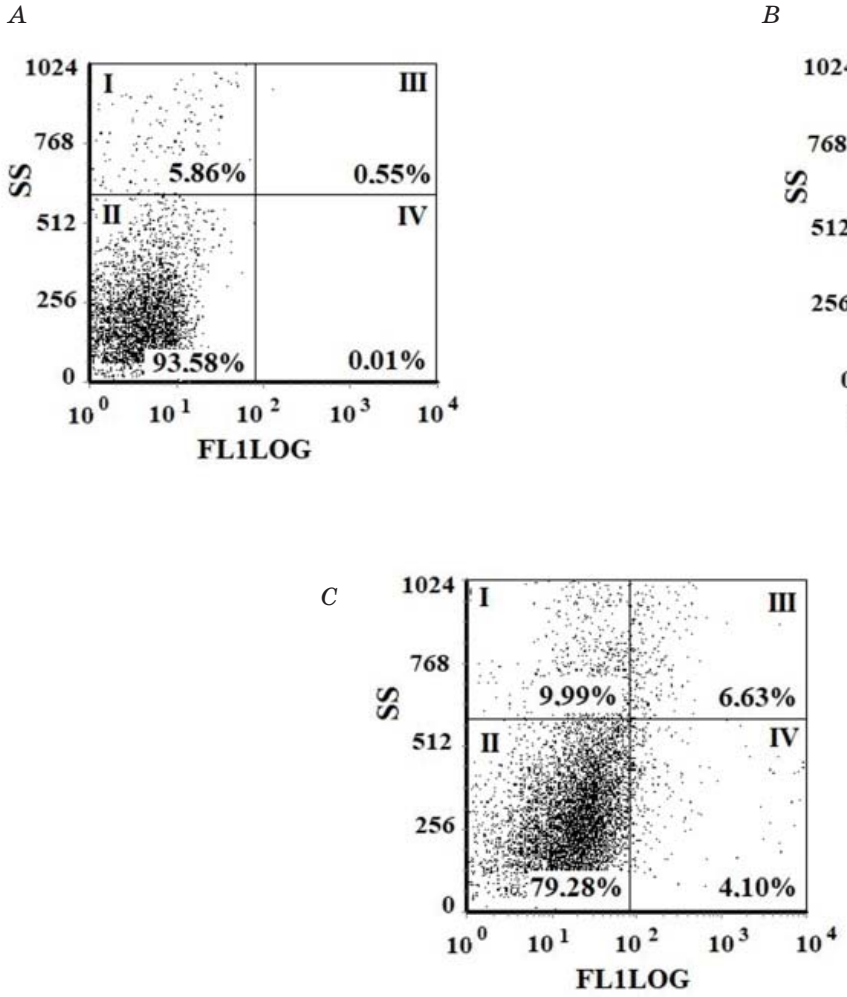

Fig. 4. **Representative scatter dot plots of inactive (resting) platelet population: intact control cells (*A*); intact cells after incubation with anti-tPA: antibodies and correspondent anti-rabbit FITC-conjugated IgG (*B*), intact cells after incubation with tPA: anti-tPA antibodies and correspondent anti-rabbit FITC-conjugated IgG (*C*):**
FL1 LOG — the logarithm of the FITC fluorescence intensity;
SS — cell granularity expressed as arbitrary units

Table 2. **Flow cytometry data on binding of tPA with non-stimulated (resting) platelets**

Group of cells	Gated cells,%		Median fluorescence intensity	
	Q I-II	*Q III-IV*	*Q I-II*	*Q III-IV*
Non-activated platelets	99.44	0.57	10	232
Non-activated platelets +anti-tPA IgG+IgG-FITC	96.46	3.54	26	337
Non-activated platelets +tPA +anti tPA IgG+IgG-FITC	89.27	10.73	48	367

Table 3. **Flow cytometry data on binding of tPA with thrombin-stimulated platelets**

Group of cells	Gated cells,%		Median fluorescence intensity	
	Q I-II	*Q III-IV*	*Q I-II*	*Q III-IV*
Activated platelets	95.87	4.13	12	232
Activated platelets+anti- tPA IgG+IgG-FITC	85.37	14.62	25	329
Activated platelets+tPA+anti-tPA IgG+IgG-FITC	67.18	32.82	30	861

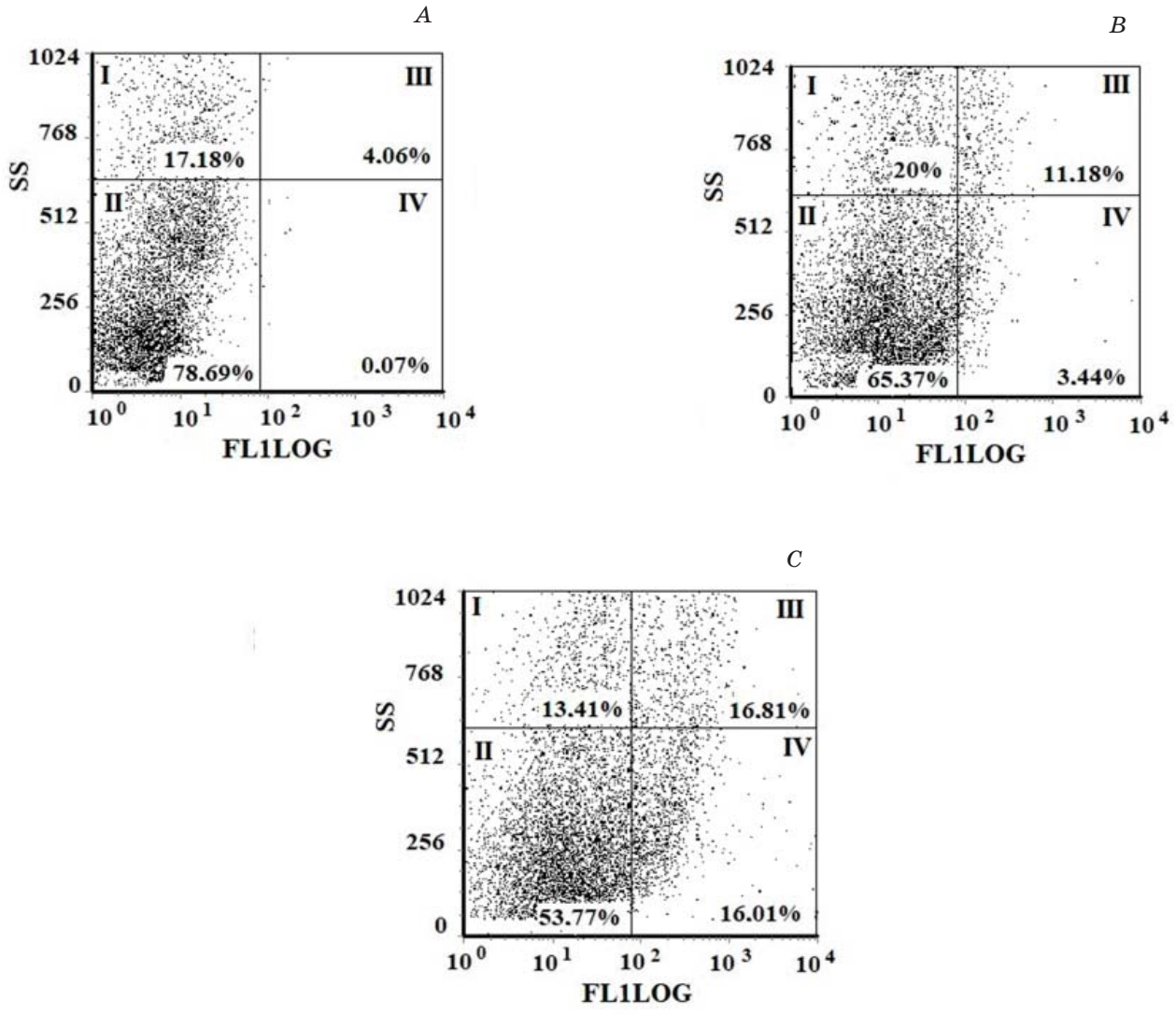

Fig. 5. **Representative scatter dot plots of the thrombin-stimulated platelet populations: activated cells (*A*), activated cells after incubation with antibodies to tPA and anti-species IgG-FITC (*B*), activated cells after incubation with tPA, antibodies to tPA and anti-species IgG-FITC (*C*):**
FL1 LOG — the logarithm of the FITC fluorescence intensity; SS — cell granularity expressed as arbitrary units

different functions in the thrombus microenvironment [20]. There are highly activated procoagulant platelets, on the surface of which phosphatidylserine is exposed and coagulation factors are associated. Such platelets have been shown to increase the formation of tenase and prothrombinase complexes by almost 1000 times. A population of platelets formed after cell stimulation with strong agonists, such as collagen or thrombin, and capable of irreversibly binding a number of alpha-granular proteins, including factor V, thrombospondin, fibrinogen, fibronectin, and von Willebrand factor was also identified [21]. They were referred to as "coated platelets". Aggregating platelets are characterized by the presence of the active form of integrin aIIbβ3 on their surface and fibrinogen binding, providing them a primary role in a clot retraction. Studies of a model of a blood clot formed in a stream by fluorescence microscopy revealed that in a blood clot, plasminogen is localized in "caps" located on the surface of phosphatidylserine-exposing platelets [20]. It is likely that platelet subpopulations also differ in the availability of tissue activator binding sites on their surface.

Our results provide direct evidence that activated platelets expose additional binding sites for tPA. Vaughan et al. [22] have demonstrated that unstimulated platelets bound 120,000 ± 24,000 molecules per platelet with an apparent K_d of 340 ± 25 nM, whereas thrombin-stimulated platelets bound 290,000 ± 32,000 molecules per platelet with an apparent K_d of 800 ± 60 nM. These observations suggest that the platelet surface bears a large number of specific, but low affinity binding sites for tPA and may provide important evidence for the role of platelets in localization and regulation of fibrinolysis. Application of immunochemical detection of tPA on platelet surface in cytometry assay may have an essential clinical significance. Since tPA infusion was shown to significantly affect platelet aggregation [23], monitoring of platelet-associated pool of tPA can be included as an eligible criterion during tPA-based thrombolytic therapy in order to improve the safety and efficacy of such treatment.

The present paper describes the methodological approaches for obtaining polyclonal antibodies against tissue plasminogen activator (tPA). Monospecific antibodies with high affinity to the antigen (K_d = $4.05 \cdot 10^{-9}$ M) were obtained and characterized. As an example of promising practical application of the produced antibodies, they were tested in flow cytometry assay for determination of tPA levels associated with platelet surface. The presence of binding sites for tissue activator on the plasma membrane of unstimulated platelets was shown, while agonist-induced stimulation resulted in increase of exposed binding sites for tPA. Subpopulations of platelets that differ in their ability to bind tPA on their surface were identified. The data obtained are important for elucidating the mechanisms of platelet involvement in the regulation of the rate of fibrinolytic process.

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The authors declare that they have no conflict of interest associated with this manuscript.

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ОТРИМАННЯ І ХАРАКТЕРИСТИКА АНТИТІЛ ДО ТКАНИННОГО АКТИВАТОРА ПЛАЗМІНОГЕНУ: ЗАСТОСУВАННЯ ДЛЯ ДОСЛІДЖЕННЯ ТРОМБОЦИТІВ ЗА ДОПОМОГОЮ ПРОТОКОВОЇ ЦИТОФЛУОРИМЕТРІЇ

О. І. Юсова[1], *Т. В. Гриненко*[1], *Т. О. Гончаренко*[1], *Л. Г. Капустяненко*[1], *А. О. Собченко*[2], *А. О. Тихомиров*[1]

[1]Інститут біохімії ім. О. В. Палладіна НАН України, Київ
[2]Центр крові Національного військово-медичного клінічного центру «Головний військовий клінічний госпіталь», Україна, Київ

yusova07@gmail.com

Тканинний активатор плазміногену (ТАП) — один з ключових протеїнів плазміноген/плазмінової системи, який здійснює перетворення плазміногену на активну протеїназу плазмін. Тромбоцити, з поверхнею яких зв'язуються плазміноген і тканинний активатор, забезпечують стимулювальний ефект на реакцію активації. Метою роботи було отримати поліклональні антитіла до ТАП, вивчити їхні імунохімічні властивості та дослідити взаємодію тканинного активатора з тромбоцитами методом протокової цитофлуориметрії. Застосовували такі експериментальні підходи: імунізація кроля тканинним активатором, отримання імунної антисироватки, синтез імуноафінного сорбенту на основі тканинного активатора, імуноензимний аналіз (ELISA), протокова цитофлуориметрія. Одержано поліклональні моноспецифічні антитіла до тканинного активатора, що характеризувалися високою афінністю до антигену ($K_d = 4{,}05 \cdot 10^{-9}$ М). За допомогою антитіл методом протокової цитофлуориметрії показано, що на плазматичній мембрані неактивованих тромбоцитів є сайти зв'язування тканинного активатора, кількість яких збільшується за агоністіндукованої стимуляції клітин. Виявлено субпопуляції тромбоцитів, що відрізняються за здатністю зв'язувати на своїй поверхні тканинний активатор. Отримані дані мають важливе значення для з'ясування механізмів залучення тромбоцитів до регулювання швидкості фібринолітичного процесу.

Ключові слова: тканинний активатор плазміногену (ТАП), імунізація, поліклональні антитіла, тромбоцити, протокова цитофлуориметрія.

ПОЛУЧЕНИЕ И ХАРАКТЕРИСТИКА АНТИТЕЛ К ТКАНЕВОМУ АКТИВАТОРУ ПЛАЗМИНОГЕНА: ПРИМЕНЕНИЕ ДЛЯ ИССЛЕДОВАНИЯ ТРОМБОЦИТОВ С ПОМОЩЬЮ ПРОТОЧНОЙ ЦИТОФЛУОРИМЕТРИИ

Е. И. Юсова[1], *Т. В. Гриненко*[1], *Т. А. Гончаренко*[1], *Л. Г. Капустяненко*[1], *А. А. Собченко*[2], *А. А. Тихомиров*[1]

[1]Институт биохимии им. А. В. Палладина НАН Украины, Киев
[2]Центр крови Национального военно-медицинского клинического центра «Главный военный клинический госпиталь», Украина, Киев

yusova07@gmail.com

Тканевой активатор плазминогена (ТАП) — один из ключевых протеинов плазминоген/плазминовой системы, который осуществляет превращение плазминогена в активную протеиназу плазмин. Тромбоциты, с поверхностью которых связываются плазминоген и тканевой активатор, оказывают стимулирующий эффект на реакцию активации. Целью работы было получение поликлональных антител к ТАП, изучение их иммунохимических свойств и исследование взаимодействия тканевого активатора с тромбоцитами методом проточной цитофлуориметрии. Использовали следующие экспериментальные подходы: иммунизация кролика тканевым активатором, получение иммунной антисыворотки, синтез иммуноаффинного сорбента на основе ТАП, иммуноэнзимный анализ (ELISA), проточная цитофлуориметрия. Получены поликлональные моноспецифические антитела к тканевому активатору, характеризующиеся высокой аффинностью к своему антигену ($K_d = 4{,}05 \cdot 10^{-9}$ М). С помощью полученных антител методом проточной цитофлуориметрии показано, что на плазматической мембране неактивированных тромбоцитов есть сайты связывания тканевого активатора, количество которых увеличивается при агонистиндуцированной стимуляции клеток. Выявлены субпопуляции тромбоцитов, отличающиеся по способности связывать на своей поверхности ТАП. Полученные данные имеют важное значение для выяснения механизмов вовлечения тромбоцитов в регуляцию скорости фибринолитического процесса.

Ключевые слова: тканевой активатор плазминогена (ТАП), иммунизация, поликлональные антитела, тромбоциты, проточная цитофлуориметрия.