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POLYCLONAL ANTIBODIES AGAINST HUMAN PLASMINOGEN: PURIFICATION, CHARACTERIZATION AND APPLICATION

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The plasminogen/plasmin system plays a crucial role in both fibrinolysis and the of cell functions in a wide range of normal and pathological processes. Investigation of plasminogen/plasmin functions requires the availability of well-characterized and effective molecular tools, such as antibodies. In the present work, the isolation and characterization of rabbit polyclonal antibodies against human plasminogen are described, and approaches for the identification of plasminogen and its fragments using the purified antibodies are demonstrated. For the antibodies isolation, standard animal immunization and blood collection procedures, serum isolation, protein salting out and affinity chromatography were performed. For the antibodies characterization and application, the following methods were used: enzyme linked immunoassay (ELISA), Western blotting, FITC-protein conjugation, flow cytometry and spectrofluorometry. Obtained polyclonal rabbit anti-human plasminogen antibodies interact with human Glu- and Lys-plasminogen, kringles 1–3 and 1–4 of plasminogen, mini-plasminogen, the heavy and light chain of plasmin. We propose the application of anti-plasminogen antibodies for the direct ELISA, Western blot analysis, and for flow cytometry and spectrofluorometric analysis of plasminogen binding with cells. Produced anti-plasminogen antibodies may represent promising tools for the investigation of plasminogen/plasmin system functions, either fibrinolytic or signaling.

Key words: plasminogen, rabbit polyclonal antibodies, affinity chromatography, ELISA, Western blotting, FITC-coupling, flow cytometry, spectrofluorometry.

Plasminogen is a key component of the fibrinolytic system and an inactive precursor of the main fibrinolytic enzyme plasmin (3.4.21.7). Plasminogen conversion to plasmin is mediated by plasminogen activators, either tissue-type or urokinase-type ones, on cells and fibrin surface. Plasmin regulates fibrin clot lifetime, maintains normal circulation and blood vessels passability [1], mediates extracellular matrix proteolysis during cell migration, and influences cell signaling [2].

Human plasminogen is a one-chain glycoprotein with a molecular weight of 92 kDa and 791 amino acid residues. Intact zymogen, normal for the circulation, contains glutamic acid on N-terminus (Glu-plasminogen). Partially degraded plasminogen molecule contains lysine (Lys-plasminogen) and appears on primary stages of fibrinolysis or under pathological conditions as a result of proteolytic activity in blood [3].

Plasminogen molecule consists of N-terminal domain, 5 kringle domains (K1-5) and serine-protease domain. Glu-plasminogen molecule is folded due to the N-terminal peptide interaction with K4 and K5, whereas N-terminal peptide cleavage in Lys-form of the zymogen leads to deployment of the molecule. Kringle domains are responsible for the substrate and cell receptor binding and serine-protease domain after the zymogen activation is able to cleave substrates. Degradation of plasminogen molecule by proteases (elastase, trypsin, plasmin, etc.) leads to plasminogen kringle-containing fragments formation,

which have antiangiogenic properties and thus are called angiostatins [4].

Plasminogen/plasmin interacts with a wide range of cell types. Plasminogen receptors were found on platelets, neutrophiles, macrophages, endotheliocytes, smooth muscle cells, fibroblasts, neuronal cells and different types of cancer cells [5]. Plasminogen/plasmin mediates cell signaling during inflammation, inducing proinflammatory cytokines production and macrophages chemoattraction, as well as metastasis, cell migration and proliferation, angiogenesis, tissue remodeling, myogenesis, neuritogenesis [6–8].

Plasminogen fragments, known as angiostatins, have mostly opposite function than parent molecule, inhibiting cell proliferation, migration, wound healing, and sometimes exhibit their functions through distinctive cell receptors [9, 10].

Investigation of plasminogen/plasmin functions requires an availability of well-characterized and effective instruments, such as antibodies. Polyclonal antibodies against plasminogen can be used for measurement of plasminogen level and identification of the zymogen/enzyme and its degraded forms in different type of samples, studying of plasminogen/plasmin interaction with cells and tissues, blockage of plasminogen functional activity, etc.

The aim of the present work was to isolate and characterize rabbit polyclonal antibodies against human plasminogen and to develop approach for the identification of plasminogen interaction with cells.

Materials and Methods

Proteins isolation

Glu-plasminogen was purified from citrate donor blood plasma using the Lysine-Sepharose 4B affinity chromatography in the presence of 1000 IU/ml aprotinin (AWD pharma, Germany) with subsequent salting out [11]. Salting out was performed with $(NH_4)_2SO_4$ (0.4 g per 1 mL of eluted protein solution) overnight at 4 °C, precipitate was centrifuged at 2,000 *g* and 4 °C for 30 min and then dissolved in 50 mM tris/HCl buffer with 150 mM NaCl (TBS), pH 7.4, in the presence of 100 mcM *p*-nitrophenyl guanidine benzoate to avoid plasmin activity. Plasminogen solution was dialyzed against TBS, pH 7.4, using 12 kDa dialysis tubing (Merck-Millipore, Germany) and stored at −20 °C.

Lys-plasminogen was purified from the blood plasma fraction $III_{2,3}$ by Cohn using the abovementioned method.

Plasmin was obtained by activation of Glu-plasminogen with urokinase-Sepharose 4B [12] and stored at −20 °C in TBS, pH 7.4, with 50% glycerol.

Plasminogen fragment kringle 1-3 was isolated from plasminogen hydrolysate, obtained by elastase-mediated hydrolysis, using affinity chromatography as described elsewhere [13].

Plasminogen fragments kringles 1–3 + 1–4 and mini-plasminogen were kindly provided by Dr. Artem Tykhomyrov (Palladin Instiute of Biochemistry of the National Academy of Sciences of Ukraine).

Tissue-type plasminogen activator used in the study was from Boerhinger Ingelheim (Actilyse).

Plasminogen-Sepharose synthesis

Plasminogen conjugation to Sepharose was performed using CNBr-activated Sepharose 4B (Sigma Aldrich, USA) according the manufacturer's recommendation. CNBr-activated Sepharose was swelled in cold 1 mM HCl for 30 min and washed with 0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl, pH 8.3–8.5 (coupling buffer). Plasminogen was dialyzed against coupling buffer and conjugated with CNBr-Sepharose during 8 h(5 mg protein per cm^3 of gel), then washed with 5 gel volumes of coupling buffer. Sepharose uncoupled CNBr-groups were blocked with 100 mM glycine in coupling buffer for 2 h at 4 °C and washed with TBS, pH 7.4. Plasminogen-Sepharose was stored in TBS, pH 7.4, containing 100 mcM *p*-nitrophenyl guanidine benzoate to avoid possible plasmin activation and conservated with 0.02% sodium azide.

Rabbit immunization and antiserum preparation

Rabbit immunization was performed in accordance with the recommendations [14]. Male rabbits weighing about 3 kg were kept on the standard diet in the animal house of Palladin Institute of Biochemistry of NASU. Each rabbit was immunized by an emulsion containing 0.1 mg of human plasminogen in 0.5 ml TBS, pH 7.4, and 0.5 ml of complete Freund`s adjuvant (Sigma Aldrich, USA). Six subcutaneous injections were given symmetrically in dorsal thoracic and lumbar regions. In 2 weeks, animals had boost immunization with incomplete Freund`s adjuvant (Sigma Aldrich, USA). In 10 days, the blood serum was tested for ELISA and the presence and titer of antibodies specific to the introduced antigen. In 12–15 days, blood was collected for 3 times.

Blood was collected from marginal ear vein by venipuncture into glass tubes and incubated at 4 °C for 16 hours for clot formation. After the clot removal serum was centrifuged to remove the debris at 200 *g* for 15 min at 4 °C. The supernatant was used for the antibody purification.

Anti-plasminogen antibodies isolation

The globulin fraction of blood proteins was isolated by salting out from blood serum using saturated solution of $(NH_4)_2SO_4$ in volume ratio 1:1 during 16 hours with subsequent centrifugation 15 min at 1,000 *g* and 4 °C. The precipitate was dissolved in TBS, pH 7.4, and dialyzed. Total immunoglobulin G fraction was isolated from globulin solution using affinity chromatography on Protein A-Sepharose (Sigma Aldrich, USA) as described elsewhere [14].

Immunoglobulin G solution, purified on Protein A-Sepharose, was applied on Plasminogen-Sepharose equilibrated with TBS pH 7.4 in volume ratio of protein solution : affinity gel 1.5:1, then the column was washed with TBS pH 7.4. Plasminogen-specific antibodies were eluted with 200 mM glycine, pH 2.8, and immediately mixed with 1 M tris-HCl, pH 8.5, in volume ratio 100:8. Protein concentration in eluate was controlled by light absorption at 280 nm. Fractions, containing the antibodies, were collected, mixed, dialyzed against TBS, pH 7.4, and concentrated using Amicon 100 centrifugal devices (Merck-Millipore, Germany). Purified antibodies were stored with glycerol (volume ratio 1:1) at −20 °C.

FITC-labelling of plasminogen-specific antibodies

Antibodies-FITC conjugation was carried out using FITC (fluorescein isothiocyanate, Sigma Aldrich, USA) [15]. FITC stock solution 1 mg/ml in DMSO was added to antibodies (2 mg/ml in 0.1 M sodium carbonate buffer, pH 9.0) in volume ratio 50: 1000 and the reaction mixture was incubated for 8 hours at 4°C in the dark, than the excess of the label was blocked by NH_4Cl in a final concentration of 50 mM during 2 hours 4 °C in the dark and dialyzed against 0.05 M sodium-phosphate buffer with 0.15 M NaCl (PBS), pH 7.4, overnight at 4 °C. F/P ratio was 3.2. FITC-labeled antibodies were stored with 25% glycerol at −20 °C.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (Fluka, Germany) was performed using standard method [16]. For the protein purity evaluation, gels after the procedure were stained with 0.025% Coomassie Brilliant Blue R250 in 25% isopropanol with 10% acetic acid (w/v/v) and washed with 7% acetic acid overnight. For Western blot analysis gels were washed twice with distilled water to remove SDS without staining.

Western and far-blot analysis

Western blotting was performed using standard approach [17]. Electrophoresis was performed without reducing agents, except plasmin sample, which contained 1% β-mercaptoethanol. Proteins were transferred from polyacrylamide gel onto 0.45 mcm pore size nitrocellulose membrane (GVS, Italy). The membrane then was blocked with 5% fat-free skim milk solution during 2 h at 37 °C, incubated 2 h with 1 mcg/ml anti-plasminogen antibodies in PBS pH 7.4 and then 1 h with anti-rabbit secondary peroxidase-conjugated antibodies (Sigma Aldrich, USA) diluted according to the manufacturer recommendations. After each antibody, the membrane was washed 5 times × 5 min with PBS, pH 7.4, containing 0.1% Tween-20 and 5 times × 5 min with PBS, pH 7.4. Washed membrane was stained with 4-chloro-6-naphtol (Sigma Aldrich, USA) methanol solution 30 mg/mL and 3% hydrogen peroxide in PBS, pH 7.4 (volume ratio 10 : 90 : 2).

Enzyme linked immunoassay

Proteins (1 mcg in 100 mcL of PBS, pH 7.4) were immobilized in 96-wells high-sorption microplates (NUNC Maxi-Sorp, Denmark) for 2 h at 37 °C and thoroughly washed by PBS, pH 7.4, containing 0.1% Tween-20 and PBS, pH 7.4. Then wells were blocked by 200 mcL of 1% BSA solution in PBS, pH 7.4, for 1 h at 37°C. After blocking, plasminogen-specific antibodies (0-5 mcg/100 mcL of PBS, pH 7.4) were added into the wells and incubated for 1 h at 37 °C and washed. For the determination of the primary antibodies interaction with the immobilized proteins, goat secondary anti-rabbit antibodies conjugated with alkaline phosphatase (Sigma Aldrich, USA) were used as recommended by the manufacturer, and after washing, developed for 30 min using chromogenic phosphatase substrate — 500 mcg/mL *p*-nitrophenyl phosphate (Sigma Aldrich, USA) in 10% diethanolamine, pH 9.8, and measured by Multiscan microplate reader (Thermo Scientific, USA) at 405 nm.

Platelet preparation

Blood was collected from two healthy volunteers was collected by venipuncture and

immediately mixed with anticoagulant (3.2% sodium citrate) in 9:1 volume ratio. Platelets were isolated from blood using the approach described elsewhere [18]. Platelets count was performed using an aggregometer (Solar AT-02, Belarus).

Platelets (5×10^6 cells) were incubated with Glu-plasminogen (1 mcM final concentration) in 20 mM HEPES buffer, containing 137 mM NaCl, 4 mM KCl, 0.2 mM $MgCl_2$, pH 7.4, for 10 min at 37 °C, washed twice by centrifugation (1,000 *g* for 10 min at 25 °C), and then incubated with 5 mcg/mL of FITC-labeled anti-plasminogen antibodies in PBS with 1% BSA, pH 7.4, for 20 min at 37 °C and washed twice.

Spectrofluorometric analysis

Binding of FITC-labeled plasminogen-specific antibodies was analyzed using flow cytometry and steady-state spectrofluorometry.

Flow cytometry was performed using Coulter Epics XL flow cytometer (Beckman Coulter, USA) via FL1 channel (515–535 nm) for FITC label. For each sample 30,000 events were chosen as sample size. Population of untreated platelets was used as a control.

Steady-state spectrofluorometry was performed using QuantaMaster spectrofluorometer (Photon Technology International, Canada), excitation wavelength was 490 nm, emission was measured at 520 nm.

Data analysis

Data was analyzed and presented using standard tools of GraphPad Prism 7 software. The flow cytometry results are presented using "FCS Express V3" software.

Results and Discussion

Glu-plasminogen used for rabbit immunization and Plasminogen-Sepharose synthesis was electrophoretically pure (Fig. 1, *a*). Blood serum of immunized animals contained 150-kDa protein fraction, complying the immunoglobulin G molecular weight (Fig. 1, *b*, Ser). The antibodies purified with subsequent use of protein A- and Plasminogen-sepharose were electrophoretically pure (Fig. 1, *b*, AB). Specific antibodies yield was 7.1%.

Analysis of the purified antibodies binding to Glu- and Lys- forms of human plasminogen demonstrates the same affinity: saturation of 1 mcg of immobilized plasminogen is reached at the antibody concentration 0.5 mcg/ml, for Glu-plasminogen K_d = 5.02 ± 1.20 nM, for Lys-plasminogen K_d = 5.40 ± 1.27 nM (Fig. 2). Recommended antibodies dilution for the direct ELISA is 0.5 mcg/mL. Tissue-type plasminogen activator was used in the assay as negative control to discern possible antibodies cross-reaction with kringle domain-containing non-plasminogen proteins and it is confirmed that the obtained antibodies have specificity to plasminogen.

To clarify which plasminogen domain contains antigenic determinant for the antibodies, Western-blot analysis was applied (Fig. 3, *a*). Optimal antibodies dilution (1 mcg/mL) was chosen using dot-blot analysis (data not shown). The ability of the antibodies to interact with plasminogen, heavy and light chains of plasmin, plasminogen fragments K 1-3, K 1-4 and mini-plasminogen was demonstrated. Moreover, the use of anti-plasminogen antibodies allows to distinct different forms of plasminogen fragments in the mixture (Fig.3, track K1-4+K1-3), which can represent different angiostatins [4, 19, 20]. Western blot analysis of platelet lysate (whole cell, 10^6 cells/track, in 5 x Laemmli buffer) confirms plasminogen and its fragments to be presenced in platelets (Fig. 3, *b*), among which is typical one with molecular weight near 50 kDa complying angiostatin that can be released by platelets [21]. Therefore, the obtained antibodies can be applied for the determination either plasminogen or plasminogen fragments, including angiostatins.

Plasmin sample contains β-mercapto-ethanol to divide heavy (63 kDa) and light (26 kDa) chains. Western blotting was applied for the analysis.

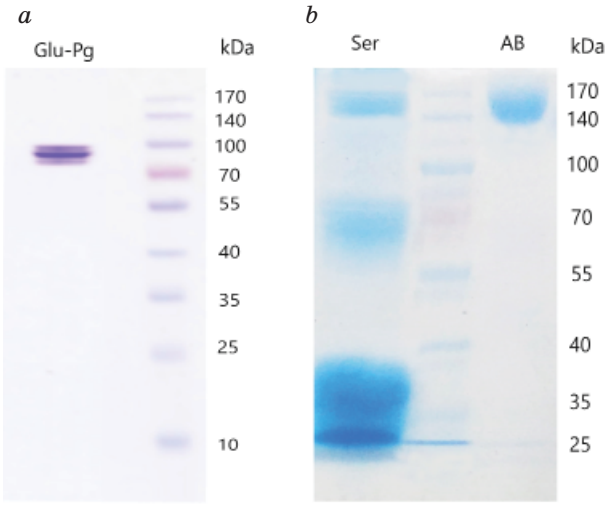

Fig. 1. **Electrophoregram of (*a*) purified human Glu-plasminogen, used for the animal immunization and (*b*) the rabbit serum (Ser) and affinity purified anti-plasminogen antibodies (AB)**

Investigation of plasminogen or plasminogen fragments interaction with cells can be carried out using fluorometric techniques. For this purpose, the anti-plasminogen antibodies are conjugated with different fluorescent labels and applied for the fluorometric analysis. To test the obtained antibodies suitability for these applications, we performed FITC-labeling of the antibodies and demonstrated Glu-plasminogen binding to platelets with flow cytometry and steady state spectrofluorometric analysis.

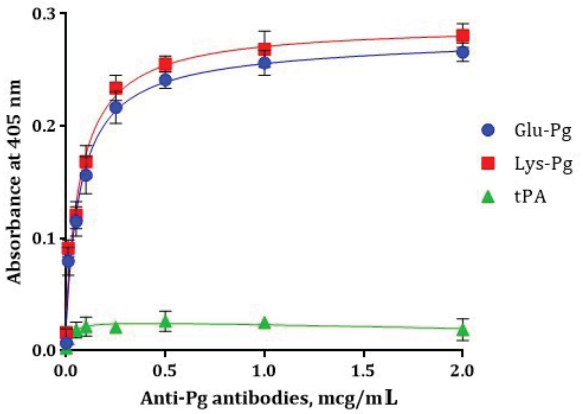

Fig. 2. **Binding of purified polyclonal antibodies against plasminogen with Glu-plasminogen (Glu-Pg), Lys-plasminogen (Lys-Pg), and tissue-type plasminogen activator (tPA) evaluated by ELISA**

Fig. 4 demonstrates typical fluorescence histograms of native washed human platelets, bound with anti-plasminogen FITC-labeled antibodies before and after incubation with exogenic plasminogen. Data obtained with flow cytometry and presented with FCS Express 3 software. As a control, platelet autofluorescence was monitored. Results indicate that washed platelets absorb plasminogen from plasma on their surface and, when isolated, can bind additional exogenous zymogen amount (median value is 4.7 for the platelets-FITC-antibodies interaction, and 8.3 for the platelets-plasminogen-FITC-antibodies interaction, comparing with 1.03 of platelets autofluorescence).

Steady-state fluorometry analysis indicates the same results (Fig. 5, *a*): washed platelets surface contains plasminogen or its fragments and can bind additional exogenous plasminogen. Washed platelets can bind additionally FITC-antibodies after the preincubation with exogenous plasminogen comparing with the labeled antibodies binding level without plasminogen addition (Fig. 5, *b*).

Platelets autofluorescence serves as a control. Mean values obtained for washed platelets of two healthy volunteers.

It is well established that platelets are able to bind plasminogen, and the cells washed from platelet reach plasma interact with the zymogen through different partner proteins, which can be removed from the cell surface by gel-filtration on Sepharose 2B, but not washing by centrifugation [22–25],

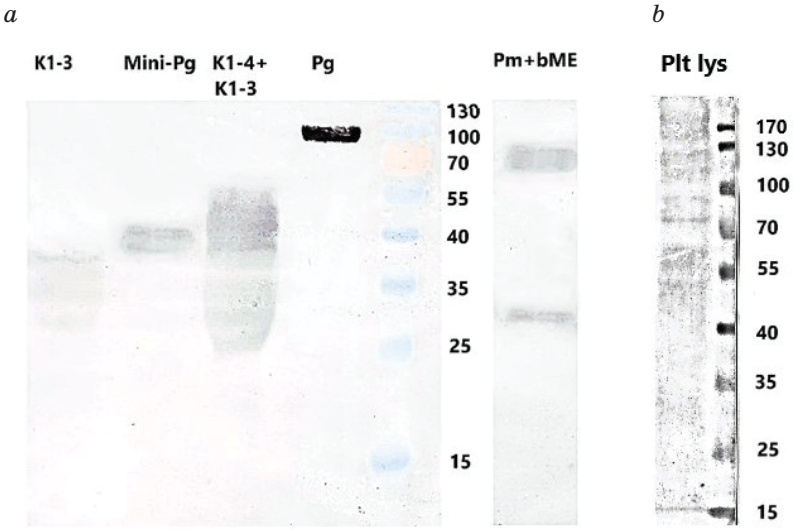

Fig. 3. **Interaction of purified polyclonal antibodies against plasminogen:**
a — Glu-plasminogen (Glu-Pg), mini-plasminogen (Mini-Pg), plasminogen kringles 1-4+1-3 (K14+K1-3), kringles 1-3 (K1-3) and plasmin (Pm+bME); *b* — platelets lysate (Plt lys).

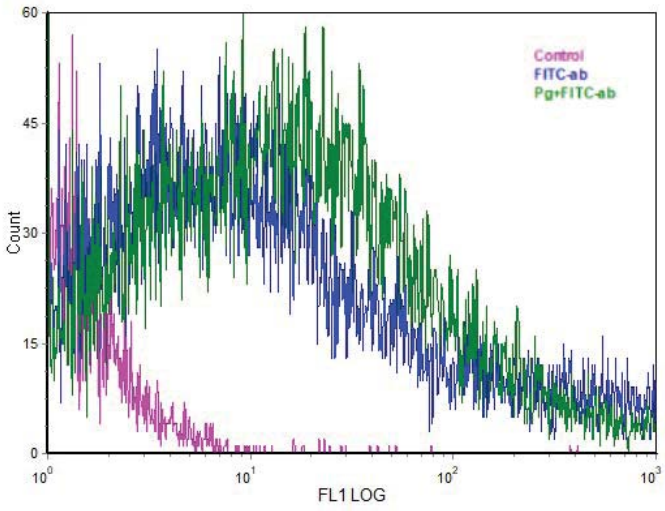

Fig. 4. **Typical fluorescence histograms of platelets distribution of untreated (Control), only FITC-antibodies treated (FITC-ab) and plasminogen and FITC-antibodies treated (Pg+FITC-ab) platelets**

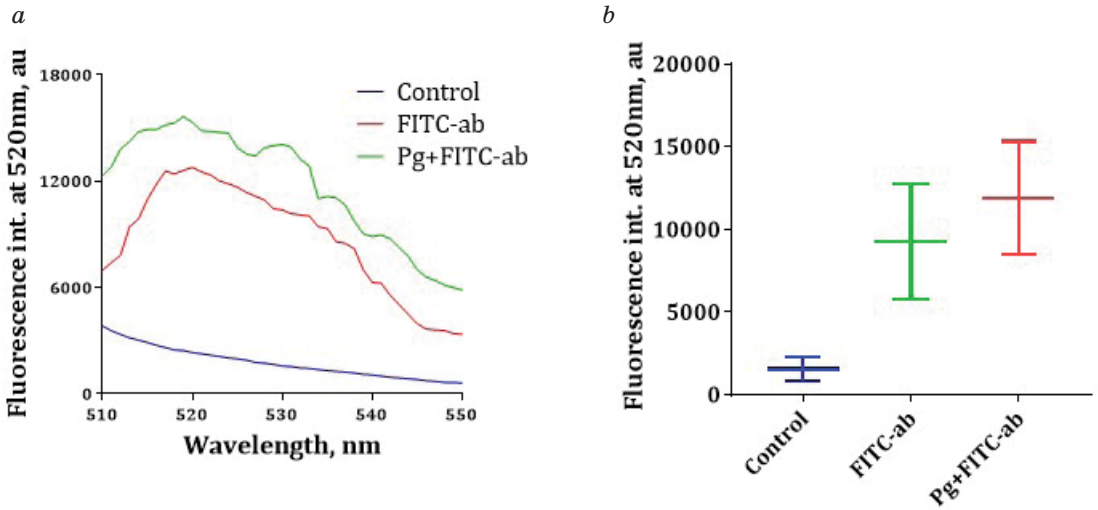

Fig. 5. **Spectrofluorometric analysis of Glu-plasminogen binding to intact platelets using FITC-labeled anti-plasminogen antibodies:**
a — Typical fluorescence spectra of untreated — autofluorescence (Control), only FITC-antibodies (FITC-ab) treated and plasminogen and FITC-antibodies treated (Pg+FITC-ab) platelets; *b* — Fluorescence intensity FITC-antibodies (FITC-ab) treated and plasminogen and FITC-antibodies treated (Pg+FITC-ab) platelets at maximum FITC emission wavelength (520 nm)

thus the results obtained using FITC-labeled anti-plasminogen antibodies comply with the previously published data.

The results of the present work demonstrate that rabbit polyclonal antibodies against human Glu-plasminogen can be used for the detection of full-length molecules of Glu- and Lys-forms of zymogen, as well as kringle- and protease-domain containing fragments. FITC-labeled anti-plasminogen antibodies are suitable for plasminogen detection on cells using fluorometric approaches and allow evaluating changes in bound plasminogen levels depending on the different functional states of cells. To perform the identification of intact or fragmented forms of the zymogen, the Western blot analysis can be applied.

The obtained antibodies can represent a promising tool for the investigation of plasminogen/plasmin system functions, either fibrinolytic or signaling, for example platelet physiology, cell migration, tumor growth, angiogenesis, inflammation, etc.

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ПОЛІКЛОНАЛЬНІ АНТИТІЛА ДО ПЛАЗМІНОГЕНУ ЛЮДИНИ: ОЧИЩЕННЯ, ХАРАКТЕРИСТИКА ТА ЗАСТОСУВАННЯ

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Плазміноген/плазмінова система відіграє вирішальну роль у фібринолізі та регулюванні функцій клітин у широкому діапазоні нормальних та патологічних процесів. Дослідження ролі плазміногену/плазміну потребує добре охарактеризованих та ефективних інструментів, зокрема антитіла. У роботі описано виділення поліклональних антитіл кроля проти плазміногену людини та надано їх характеристику, а також запропоновано підходи до ідентифікації плазміногену та його фрагментів з використанням одержаних антитіл. З метою виділення антитіл застосовували стандартну процедуру імунізації тварин і забору крові, виділення сироватки, висолення протеїнів та афінну хроматографію. Для характеристики та застосування антитіл використовували такі методи: імуноензимний аналіз, вестерн-блот, кон'югацію протеїнів, протокову цитометрію та спектрофлуорометрію. Показано, що антитіла взаємодіють з Glu- і Lys-плазміногеном людини, кринглами 1-3 та 1-4 плазміногену, міні-плазміногеном, важким та легким ланцюгами плазміну. Пропонується застосування антитіл до плазміногену у прямому імуноензимному аналізі, вестерн-блот-аналізу та після мічення флуоресцентною міткою у протоковій цитометрії та спектрофторометричному аналізі зв'язування плазміногену з клітинами. Отримані антитіла до плазміногену є перспективними інструментами для дослідження як фібринолітичних, так і сигнальних функцій плазміноген/плазмінової системи.

Ключові слова: плазміноген, поліклональні антитіла кроля, афінна хроматографія, імуноензимний аналіз, вестерн-блот, FITC-мічення, протокова цитометрія, спектрофлуориметрія.

ПОЛИКЛОНАЛЬНЫЕ АНТИТЕЛА К ПЛАЗМИНОГЕНУ ЧЕЛОВЕКА: ОЧИСТКА, ХАРАКТЕРИСТИКА И ПРИМЕНЕНИЕ

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Плазминоген/плазминовая система играет решающую роль в фибринолизе и регуляции функций клеток в широком диапазоне нормальных и патологических процессов. Исследование роли плазминогена/плазмина требует наличия хорошо охарактеризованных и эффективных инструментов, таких как антитела. В работе описано выделение поликлональных антител кролика против плазминогена человека и приведена их характеристика, а также предложены подходы к идентификации плазминогена и его фрагментов с использованием полученных антител. Для выделения антител применяли стандартную процедуру иммунизации животных и забора крови, выделение сыворотки, высаливание протеинов и аффинную хроматографию. Для характеристики и применения антител были использованы следующие методы: иммуноэнзимный анализ, вестерн-блоттинг, конъюгация FITC-протеинов, проточная цитометрия и спектрофлуорометрия. Показано, что антитела взаимодействуют с Glu- и Lys-плазминогеном человека, кринглами 1-3 и 1-4 плазминогена, мини-плазминогеном, тяжелой и легкой цепями плазмина. Предлагается использование антител к плазминогену для прямого иммуноэнзимного анализа, вестерн-блот-анализа и после мечения флуоресцентной меткой для проточной цитометрии и спектрофлуорометрического анализа связывания плазминогена с клетками. Полученные антитела к плазминогену являются перспективными инструментами для исследования как фибринолитических, так и сигнальных функций плазминоген/плазминовой системы.

Ключевые слова: плазминоген, поликлональные антитела кролика, аффинная хроматография, иммуноэнзимный анализ, вестерн-блот, FITC-мечение, проточная цитометрия, спектрофлуориметрия.