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Lys-plasminogen stimulates vitronectin exposure on the platelet surface

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Aim. To study the vitronectin exposure on the platelet surface in the presence of two forms of the plasminogen molecule: Lys- and Glu-plasminogens. **Methods.** Research was conducted on human platelets. Aggregometry was used to check the platelet vitality and cell response towards thrombin. To evaluate the influence of different plasminogen forms on the platelet vitronectin exposure we used the method of flow cytometry. **Results.** It was found that incubation of resting platelets with Lys-plasminogen increased the amount of vitronectin-positive cells, but did not affect significantly their fluorescent intensity. Thrombin stimulation led to an increase in both: the number of vitronectin-positive platelets and the signal of fluorescence at least by two times. The Lys-plasminogen adding to the suspension of washed platelets followed by the thrombin stimulation enhanced the vitronectin exposure on the platelet surface and increased the amount of vitronectin-positive cells as compared to the isolated thrombin stimulation. Glu-plasminogen had no effect on the vitronectin exposure in case of resting or stimulated platelets. **Conclusions.** Lys-plasminogen but not its Glu-form enhances the exposure of vitronectin on the platelet surface. We suggest that the binding of Lys-plasminogen to the surface platelet receptors may generate plasmin that leads to the activation of intracellular signaling cascade.

Keywords: plasminogen, platelets, platelet secretion, vitronectin, flow cytometry.

Introduction

The platelet activation and following thrombus formation involve many adhesive proteins which are secreted from the platelet α -granules including fibrinogen, von Willebrand factor, fibronectin and vitronectin (Vn) [1]. However, it has been recently demonstrated that thrombus formation still occurs in the mice lacking both von Willebrand factor and fibrinogen [2] suggesting that other proteins such as fibronectin, thrombospondin and Vn may be able to mediate the platelet adhesion and aggregation [3]. Among these proteins, a special attention has been paid to Vn, which plays a significant role in platelet physiology. It may regulate thrombosis and fibrinolysis through the interaction with integrins, plasminogen activator inhibitor-1 (PAI-1), collagen, and the urokinase receptor. In plasma, this protein circu-

lates in an inactive folded form, in which most of its ligand-binding domains are hidden. Conversely, in the extracellular matrix and in blood platelets Vn is present in the activated multimeric form [4, 5]. It was suggested that this adhesive protein is a thrombus stabilizer as it has a site (Arg-Gly-Asp) with the potential for binding to integrins [6, 7]. However, the ideas about its exact role in thrombus formation are controversial. It was shown that the platelet-released Vn enhances the platelet aggregation, whereas the plasma Vn inhibits this process. It was reported that Vn has binding site to plasminogen, as monoclonal antibodies against the C-terminal portion of Vn inhibited plasminogen binding [8]. Recently we have shown that Lys-plasminogen but not its Glu-form inhibits the thrombin- and collagen-induced aggregation in the preparations of washed human platelets. In this study, we investigated the effects of two forms

of plasminogen on the vitronectin exposure on the surface of human platelets.

Materials and Methods

Human plasma and platelet preparations were obtained from blood of healthy volunteers ($n = 5$) as it was described before [9]. Research protocols were approved by the Ethical Committee of Palladin Institute of Biochemistry of NASU (from 3rd of November 2014, protocol N 10).

Aggregometry was used to check platelet vitality and cell response towards thrombin. Platelet aggregation was measured by optical aggregometry (aggregometer «Solar AT-02», Belorussia). All assays were performed within 60-180 minutes after platelet collection. Experimental data were statistically analyzed using software «MS Excel» and «Agregometr 2.01». Thrombin (Sigma Aldrich, USA) at final concentration 1 NIH unit/ml was used to stimulate platelets.

Glu-plasminogen was purified from fresh donor plasma and Lys-plasminogen was isolated from Cohn fraction II–III. Lysine-sepharose chromatography was used in both cases [10]. All preparations of plasminogen had no spontaneous plasmin activity.

To evaluate the influence of different plasminogen forms on Vn exposure by platelets the method of flow cytometry was used. The levels of exposed Vn were detected with use of respective antibodies to Vn (Sigma Aldrich, USA). The washed platelets (10^6 cells) were transferred into 100 μ l of 20 mM HEPES buffer, pH 6.8 containing 137 mM NaCl, 4mM KCl, 0.2 mM $MgCl_2$, 0.2 % glucose and 0.2 % bovine serum albumin (BSA). To perform investigations three groups of cells were used: resting platelets (control); platelets incubated with Glu- or Lys-plasminogen (time of exposure 3 min); platelets treated with thrombin (5min), and platelets treated with thrombin after their preliminary incubation with Lys-plasminogen (3min). All procedures with platelets were carried out at 22–25 °C to avoid platelet hyperactivation. Platelets shortly thereafter incubation with plasminogen or resting platelets (control group) were treated with anti-Vn antibodies. To carry out immunochemical detection of Vn we prepared the mixture of antibodies in

0.05 M sodium phosphate buffer (PBS), pH 7.4 with 0.13 M NaCl and 1 % BSA. Antibodies to Vn (2 μ g) were mixed with 100 μ l of PBS and secondary FITC-conjugated antibodies (Sigma Aldrich, USA) (final dilution 1:100). The mixture was incubated in the darkness at 4 °C for 15 min. Platelets were incubated with the obtained mixture in the darkness at room temperature for 30 min. As a control of non-specific antibody binding we used platelets which were incubated only with secondary FITC-conjugated antibodies. Non-bound antibodies were removed by double washing in PBS by centrifugation at 3,000 g for 3 min at 20 °C. The obtained suspension of washed platelets was resuspended in 1 ml of PBS and used for the investigation of Vn exposure at COULTER EPICS XL (Beckman Coulter, USA) equipped with argon laser, excitation $\lambda = 488$ nm. Evaluation of Vn exposure on the platelet surface was done using two parameters: 1) percentage of Vn-positive platelets of the whole amount of investigated cells in the sample; 2) intensity of fluorescence, which was monitored by the channel FL1 (515–535 nm). To sort the

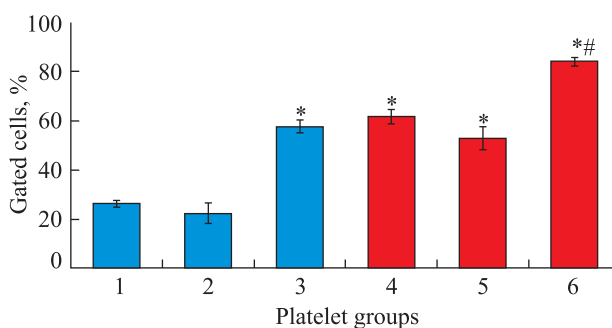


Fig. 1. Effects of Lys- and Glu-plasminogen on vitronectin exposure by intact and thrombin-stimulated platelets: 1 – resting platelets, 2 – resting platelets incubated with Glu-plasminogen, 3 – resting platelets incubated with Lys-plasminogen, 4 – thrombin-stimulated platelets, 5 – thrombin-stimulated platelets preliminary incubated with Glu-plasminogen, 6 – thrombin-stimulated platelets preliminary incubated with Lys-plasminogen. Blue columns – resting platelets, red columns – thrombin-stimulated platelets. The concentrations of plasminogen forms and thrombin were 1.2 μ M and 1.0 NIH units/ml respectively in all experiments. Difference in the compared groups is statistically significant ($p < 0.05$): * – compared to the group of ‘resting platelets’, # – compared to the group of «thrombin-stimulated platelets»

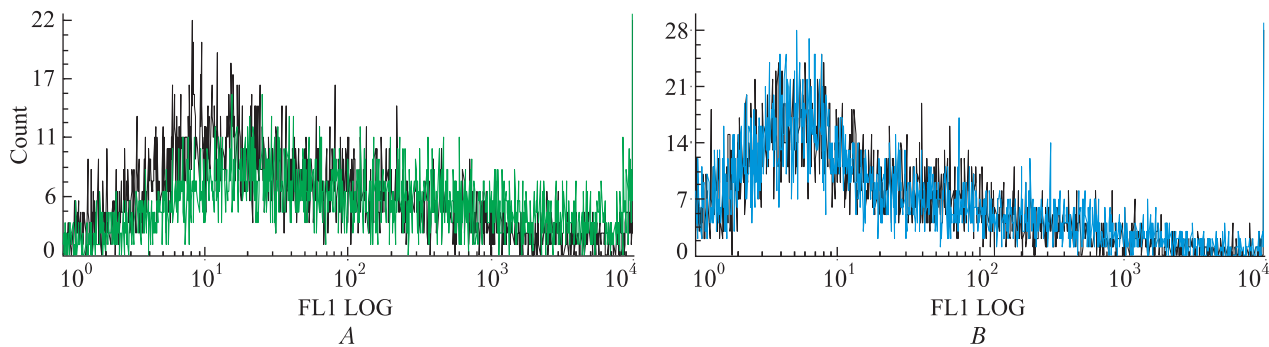


Fig. 2. Influence of Lys-plasminogen (*A*) and Glu-plasminogen (*B*) on vitronectin exposure by resting human platelets. Count – the amount of events, FL1 LOG – logarithm of fluorescence intensity with FITC. Black colour-resting platelets, green colour-platelets incubated with Lys-plasminogen, blue colour-platelets incubated with Glu-plasminogen

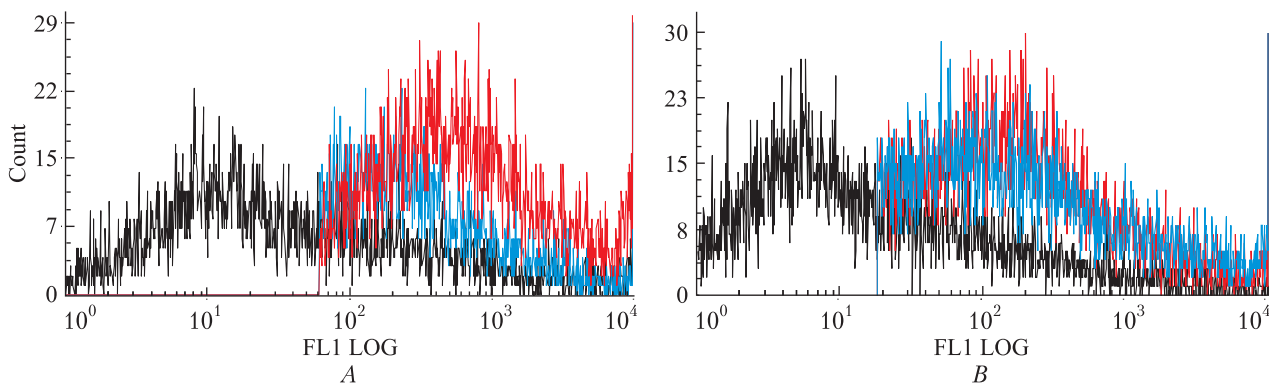


Fig. 3. Influence of Lys-plasminogen (*A*) and Glu-plasminogen (*B*) on vitronectin exposure by stimulated human platelets. Count – the amount of events, FL1 LOG – logarithm of fluorescence intensity with FITC. Black colour-resting platelets, blue colour-thrombin-stimulated platelets, red colour-thrombin-stimulated platelets preliminary incubated with Lys-plasminogen (*A*) or Glu-plasminogen (*B*)

platelets the corresponding gates were established. The changes in signal of fluorescence, which were observed in case of the impact of the investigated molecules, were defined according to the displacement of fluorescence curves relative to the marker, which limits the fluorescence curve of the intact platelets. The quantitative changes of the fluorescence were expressed in arbitrary units (AU) that mean the common logarithm according to scale Log FL1. To obtain statistically significant results at least 10 thousand events were analyzed.

The measurement of cytometric parameters in each group of cells was fulfilled in two parallels. Then, a mean value for the corresponding data obtained from all donors was calculated. The results were graphically presented using «FCS Express V3» (De Novo

Software, USA). The data were expressed as mean \pm SD. Statistical treatment of the results was made with Student's t-test, the difference was considered as significant with $p < 0.05$.

Results and Discussion

Using the flow cytometric analysis we determined the amount of Vn-positive cells and their fluorescence intensity. The presented data characterize the vitronectin exposure on the surface of resting and thrombin-stimulated washed platelets (Fig. 1). The appearance of Vn-positive cells in the population of resting platelets can be due to the spontaneous activation of a part of cells taken in the experiment. It is recommended to use the population of platelets, the level of spontaneous activation of which does not exceed 50–55 % [11]

that is in accordance with our data. When Lys-plasminogen was introduced into the incubation medium with intact platelets, an increase of vitronectin-positive cells was noted. The introduction of Glu-plasminogen had no effect (Fig. 1). It is known that the platelet affinity for Lys-plasminogen is higher as compared to Glu-plasminogen [12]. In its turn, the bound Lys-plasminogen may be considered as the preferable adhesive ligand for Vn, since the affinity of Lys-plasminogen towards vitronectin is significantly higher ($K_d = 100$ nM and $K_d = 1$ mM respectively) [8].

We established that thrombin activation caused an increase of Vn-positive cells at least by 2 times as compared to the population of resting platelets. It is known that thrombin activation leads to secretion of alpha-granules, which contain vitronectin. According to the previous data, the activated platelets secreted highly adhesive multimeric Vn, half of which remained the platelet bound [13]. If Lys-plasminogen was added to the incubation medium prior to the thrombin activation, we noticed a significant increase of Vn-positive cells. In case of Glu-plasminogen, significant changes in the amount of Vn-positive cells were observed in comparison with the case of thrombin stimulation without prior plasminogen incubation. This difference may be also explained by different affinity of Glu- and Lys-plasminogens towards vitronectin.

The changes in intensity of the fluorescence signal of intact and thrombin-activated platelets have the same tendency as it was demonstrated for the quantitative rearrangement of the cells. The resting platelets incubated with Glu- or Lys-plasminogen do not reveal any significant changes of the fluorescence signal as compared to the control curve (Fig. 2). In case of the thrombin-activated platelets it was an essential shift of the peak of the fluorescence curve to the right of the control curve (Fig. 3), which agrees with the appearance of vitronectin-binding sites on the surface of thrombin-activated platelets. The preliminary incubation of stimulated platelets with Lys-plasminogen leads to the shift of the peak of fluorescence to the right (Fig. 3, A), whereas the incubation with Glu-plasminogen did not cause any changes as compared to the curve obtained for thrombin-stimulated platelets (Fig. 3, B).

The obtained results may be interesting considering the role of plasminogen in cellular signaling. As it has been recently shown on peripheral monocytes and macrophages, the plasminogen binding to the annexin A2 S100 A10 may induce the plasmin generation, which results in the activation of intracellular signaling cascade and leads to a pro-inflammatory response [14]. It is quite possible that the binding of Lys-plasminogen (which has open conformation and therefore is more readily converted into plasmin) to the platelet membrane may serve as an initial step of the cellular response. However, the further investigations should be done to prove this suggestion.

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Lys-плазминоген стимулирует экспонирование витронектина на поверхности тромбоцитов

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Цель. Исследовать экспонирование витронектина на поверхности тромбоцитов в присутствии двух форм плазминогена: Lys- и Glu-плазминогена. **Методы.** Исследования проводили на тромбоцитах человека. Для проверки жизнеспособности тромбоцитов и клеточного ответа на тромбиновую стимуляцию использовали метод агрегометрии. Для оценки влияния разных форм плазминогена на экспонирование витронектина тромбоцитами использовали метод проточной цитометрии. **Результаты.** Было показано, что инкубация интактных тромбоцитов с Lys-плазминогеном приводила к увеличению количества витронектин-позитивных клеток, но не оказывала существенного влияния на их интенсивность флуоресценции. Стимуляция тромбином сопровождалась по меньшей мере двукратным увеличением как числа витронектин-позитивных тромбоцитов, так и интенсивности сигнала флуоресценции. Добавление Lys-плазминогена к суспензии отмытых тромбоцитов с последующей активацией тромбином усиливало экспонирование витронектина на тромбоцитарной поверхности и увеличивало количество витронектин-позитивных клеток по сравнению с изолиро-

ванной стимуляцией агонистом. Glu-плазминоген не оказывал влияния на экспонирование витронектина в случае интактных или активированных тромбоцитов. **Выводы.** Lys-плазминоген, но не его Glu-форма, усиливает экспонирование витронектина на поверхности тромбоцитов. Мы предполагаем, что связывание Lys-плазминогена с рецепторами на тромбоцитарной поверхности может способствовать образованию плазмина, который, в свою очередь, запускает процессы внутриклеточного сигналинга.

Ключевые слова: плазминоген, тромбоциты, тромбоцитарная секреция, витронектин, проточная цитометрия.

Lys-плазміноген стимулює експонування вітронектину на поверхні тромбоцитів

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Мета. Дослідити експонування вітронектину на поверхні тромбоцитів за присутності двох форм плазміногену: Lys- та Glu-плазміногену. **Методи.** Дослідження проводили на тромбоцитах людини. Для перевірки життєздатності клітин та клітинної відповіді на стимуляцію тромбіном використовували метод агрегометрії. Для оцінки впливу різних форм плазміногену на експонування вітронектину тромбоцитами було використано метод протокової цитометрії. **Результати.** Було показано, що інкубація інтактних тромбоцитів з Lys-плазміногеном призводила до зростання кількості вітронектин-позитивних клітин, але суттєво не впливала на їх інтенсивність флуоресценції. Стимуляція тромбіном супроводжувалася принаймні двократним зростанням як кількості вітронектин-позитивних тромбоцитів, так і інтенсивності сигналу флуоресценції. Додавання Lys-плазміногену до суспензії відмитих тромбоцитів з наступною активацією тромбіном підсилювало експонування вітронектину на тромбоцитарній поверхні та збільшувало кількість вітронектин-позитивних клітин порівняно з ізольованою стимуляцією агоністом. Glu-плазміноген не спричиняв впливу на експонування вітронектину в разі інтактних та активованих тромбоцитів. **Висновки.** Lys-плазміноген, але не його Glu-форма, підсилює експонування вітронектину на поверхні тромбоцитів. Ми припускаємо, що зв'язування Lys-плазміногену з рецепторами тромбоцитарної поверхні може сприяти утворенню плазміну, який, в свою чергу, запускає процеси внутрішньоклітинного сигналинга.

Ключові слова: плазміноген, тромбоцити, тромбоцитарна секреція, вітронектин, протокова цитометрія.

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