Surface-exposed actin binds plasminogen on the membrane of agonist-activated platelets: a flow cytometry study

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Aim. To elucidate whether actin exposed on the surface of agonist-activated platelets is responsible for plasminogen binding. **Methods.** Human washed platelets were obtained by gel-filtration on Sepharose-2B. The levels of exposed actin on the outer surface of plasma membrane of intact and thrombin- or collagen-stimulated platelets were monitored by flow cytometry. The binding of plasminogen fluorescent conjugate (Pg-FITC) with platelets treated with anti-actin antibody or non-immune IgG (control) was analyzed cytometrically. **Results.** Thrombin or collagen exposure resulted in the dose-dependent actin expression on the surface of stimulated platelets. Pre-treatment of stimulated platelets with specific anti-actin antibodies was shown to significantly prevent the binding of Pg-FITC with the platelet surface. **Conclusion.** The results of the present study indicate that actin exposed on the platelet surface at the agonist-induced activation is involved in the plasminogen binding.

K e y w o r d s: platelets, surface-exposed actin, plasminogen, flow cytometry.

Introduction

Platelets play a key role in hemostatic process and regulate inflammatory and proliferative events through releasing adhesive and coagulation proteins, growth factors and angiogenesis inhibitors. The platelet activation triggers cytoskeletal rearrangement that provides morphology changes, exocytosis, adhesion, aggregation, and retraction [1]. Activation also stimulates the expression of subcellular structures and novel proteins not detectable in the quiescent state on the outer surface of plasma membrane. There are several lines of independent evidences indicating exposition of some cytoskeletal proteins on the surface of agonist-stimulated platelets [2, 3]. Actin, a principal component of platelet cytoskeleton microfilaments, is among them [4]. However, the molecular mechanisms of actin translocation on the activated membrane and "external" functions of this major cytoskeletal protein are still obscure.

Plasminogen, a 92 kDa single-chain glyco-protein, is an inactive proform of proteinase plasmin that dissolves fibrin in blood clots, acts as a proteolytic factor in tissue remodeling, tumor invasion, and inflammation [5]. It has been well-documented that the binding of plasminogen to platelets is augmented by thrombin stimulation, suggesting exposure of additional binding sites on the activated membrane [6]. It has been demonstrated during the experiments with pure proteins and with the use of cell culture models that plasminogen and actin interact with high extent of affinity. According to the data obtained by various research groups, K_d for the plasminogen-actin complex is about $70 - 140$ nM [7]. These values are consistent with those describing the high-affinity interaction of plasminogen with actin exposed on PC12 cells [8]. Previously, actin has been identified on the surface of various normal and transformed cell types [see 9, 10, 11 for summary]. For example, externally exposed actin has been observed on the sperm head, where it takes part in the process of membrane modification during sperm capacitation and initiation of acrosomal reaction [12]. Moreover, actin located on the outer side of the plasma membrane has been found as a binding site for plasminogen. The Surface-expressed β/γ actin isoforms of catecholaminegic cells are directly involved in the plasminogen activation, and resultant plasmin promotes the formation of bioregulatory inhibitory peptides, thus modulating neurotransmitter release [13]. Finally, β-actin expressed on the surface of some tumor cell lines (PC-3, HT1080, MDA-MB321), mediates plasmin autoproteolysis resulting in the angiostatin AS 1–4.5 formation [7, 14]. It is logical to suppose that if the amounts of cell-surface actin are related to its possible functional activity, the surface actin expression may provide a subtle mechanism for regulation of plasminogen binding. It should be further tested what kind of functional activities actin-bound plasminogen expresses on the platelet surface: is it involved in platelet signaling or does it undergo proteolytic fragmentation, resulting in the biologically active fragments (angiostatins) formation? It is important to establish due to the fact that the interaction of plasminogen/plasmin system and platelets is considered as a crossroad between hemostasis, tissue reparation and remodeling, wound healing, cell invasion, angiogenesis, and inflammation. Therefore, this issue raises many questions, the dissection of which may shed light on a putative role of the actin-plasminogen interactions on platelets in norm and their contribution to different pathological processes.

We have previously developed and used flow cytometry assay to measure actin exposition on the platelet surface and observed that dynamic changes of this parameter directly depend on the agonist (thrombin) concentration [4]. The aim of this study is to test the hypothesis that the surface-exposed platelet actin can be involved in plasminogen binding, and thus to provide further evidence for of functional significance of platelet actin in such an unexpected location.

Materials and Methods

Chemicals

All chemicals and reagents were from Sigma Aldrich (USA) except for those specified.

Plasminogen purification and plasminogen-fluorescein isothiocyanate (Pg-FITC) conjugation

Native (Glu-) plasminogen was purified from fresh blood plasma of healthy donors by means of affine chromatography on lysine-sepharose, as described elsewhere [15]. Electrophoretically pure plasminogen was labeled with fluorescein isothiocyanate (FITC) to obtain fluorescent plasminogen conjugate (Pg-FITC), as previously described [16]. Neither plasminogen nor Pg-FITC displays spontaneous proteolytic activity, as measured using the S2251 chromogenic assay. Research protocols were approved by the Ethical Committee of Palladin Institute of Biochemistry of NASU (3rd November, 2014, protocol no. 10).

Platelets

Washed platelets were obtained from human platelet-rich plasma by gel-filtration on Sepharose 2B as described elsewhere [17]. Informed consent was obtained from all donors (n = 3) in accordance with the Declaration of Helsinki, and ethical approval for the study was provided by the local ethics committee as mentioned above. To assess platelet viability and functional activity, platelet aggregation was measured by optical aggregometry (aggregometer Solar AT-02, Republic of Belarus). All assays were performed within 60–80 min after platelet collection. Thrombin at final concentrations 0.1, 0.5 or 1.0 unit NIH/ml and collagen (Tekhnologia Standard, Russian Federation) at final concentrations 0.125 mg/ml, 0.6 mg/ml or 1.25 mg/ml were used to stimulate platelets (5×10^6 platelets per sample). Platelets were incubated with agonists for 5 min at 37 °C. The same amount of untreated (resting) platelets was used as a control.

Flow cytometry

Flow cytometry assay was used as a sensitive and direct method for detection of actin surface expression and monitoring Pg-FITC binding with platelet surface. Platelet activation was stopped with 1 % paraformaldehyde in sodium phosphate buffered saline (PBS), pH 7.4, for 20 min at room temperature, to prevent internalization of surface antigens and antibodies. Fixed platelets were washed with sodium-citrate (3.8 %)-PBS and sodium-citrate (3.8 %)-PBS containing 1 % BSA. Anti-actin antibody and anti-rabbit FITC-IgG were mixed in PBS in 1:80 and 1:200 dilutions, respectively (according to manufacturer recommendations) and left for 30 min at 37 °C. Then, platelets were incubated with antibody mixture for 30 min at 4 °C. Platelets incubated only with secondary FITC-conjugated antibody were used as a control for non-specific IgG binding (spontaneous, or background fluorescence).

In this study, we prevented interaction of platelet-surface actin with Pg as a ligand by an anti-actin-antibody. Fixed platelets were treated with anti-actin antibodies for 30 min at room temperature, washed with PBS and labelled with Pg-FITC (in saturated concentration 100 μg/ml) in the same conditions. One sample of platelets was treated with non-immune IgG as an isotype control. All procedures with labelled proteins were performed in the dark place to avoid photobleaching. After staining, platelets were washed from unbound antibodies or Pg-FITC twice with PBS by centrifugation at 1000 g for 3 min at 4 °C. Then, platelets were resuspended in 1 ml of PBS, and the percentage of FITC-positive platelets and intensity of specific FITC fluorescence were monitored using of Coulter
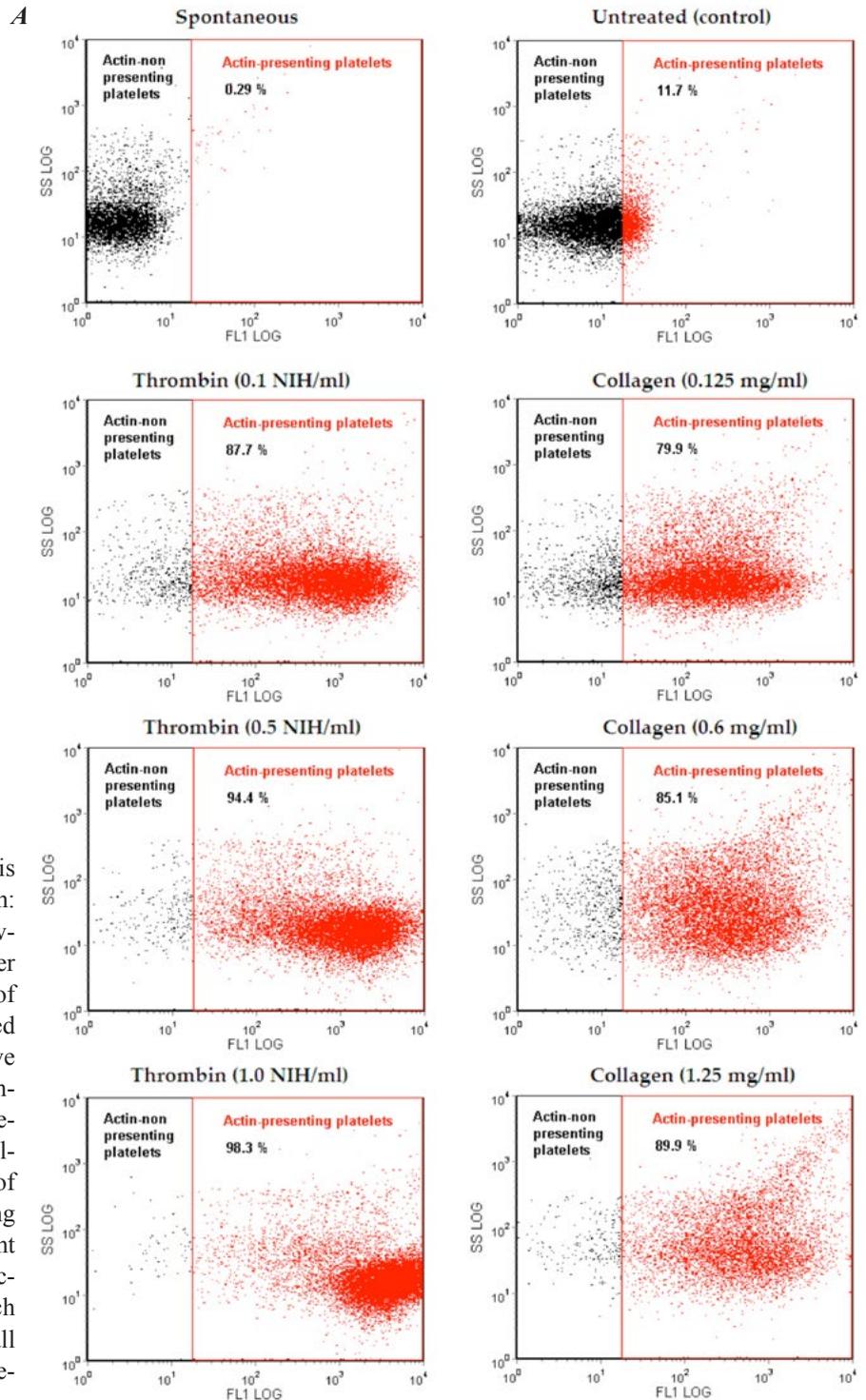
A

Fig. 1. Flow cytometric analysis of platelet surface-exposed actin: *A* – representative dot plots showing actin exposition on the outer side of plasma membrane of thrombin- or collagen-stimulated platelet; *B* and *C* – representative flow cytometry histograms of anti-actin antibody binding to platelets activated by thrombin or collagen, respectively; *D* – values of FITC-signal intensities showing agonist concentration-dependent expression of platelet surface actin (the differences between each group vs. resting control are all statistically significant with *P* level no higher than 0.01).
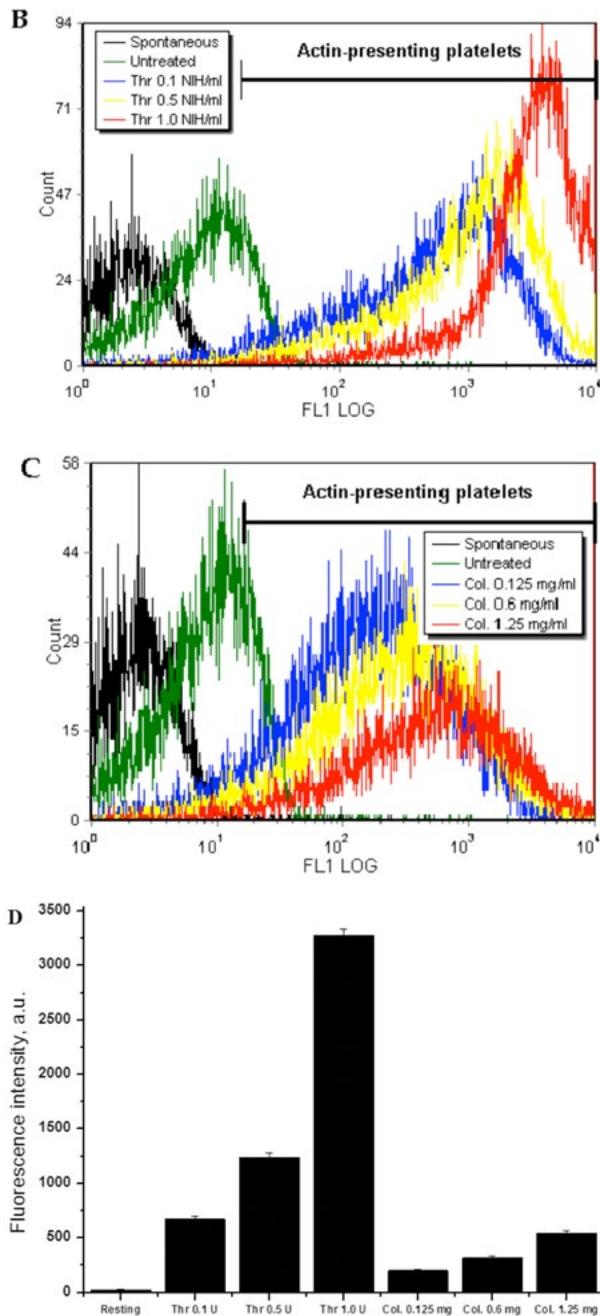
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value setting. Surface actin- or plasminogen-positive platelet populations were gated separately from the specific fluorescence-negative platelet population on the basis of sideward scattering (SSC). For control samples and thrombin-activated platelets, 15000 events were analysed, whereas for collagen-activated platelets at least 3000 events were analyzed due to high ability of collagen to cause platelet aggregation. Then the FITC-stained events found in the positive gate were expressed as the percentage of definition platelets. The measurement of cytometric parameters in each group of platelets was carried out in duplicate, and the mean value was calculated based on the values obtained from all donors. Intensity values of FITC fluorescence were expressed as arbitrary units. The results were analyzed and presented using "FCS Express V3" software system (De Novo Software, USA).

Statistical analysis

The results were expressed as mean ± standard error of mean (M ± m) or as medians for non-normal distributions. The statistics were performed with the use of Student's *t*-test. Differences between parameters studied in each group were considered to be significant at P less than 0.05.

Results and Discussion

This study was performed to determine for the first time the role of surface-exposed actin in plasminogen binding by platelets. Earlier, we have performed flow cytometry assay to determine if thrombin-activated platelets are able to expose actin on their surface [4]. Here, we extend that study and established the platelets, activated by thrombin or other potent natural

Epics XL device (Beckman Coulter, USA) via FL1 channel (515–535 nm). Population of untreated platelets was chosen for threshold

agonist, collagen, to represent actin-positive outer membrane. The results of cytometry sorting of platelets, presented in Fig. 1A, indicate an increase in percentage of the specific fluorescence actin-positive platelets in response to the agonist action. If the samples of resting platelets contained up to 12 % of actin-presented species, the platelets after submaximal (0.1 unit NIH/ml thrombin) stimulation represented 88 % of actin-positive population. The percentage of actin-positive platelets reaches up to maximal value 94 and 98 % after incubation with 0.5 or 1.0 units NIH/ml of thrombin, respectively. The mean number of actin-presented platelets raised from 80 to 90 % after stimulation with increasing collagen concentration (from 0.125 mg/ml to 1.25 mg/ml). It is seen from Fig. 1B and C that thrombin, used in the indicated concentrations, induced more intense actin surface expression compared to collagen. Measurements of fluorescence signal values revealed a strong agonist dose-dependent character of the actin exposition in platelets treated by thrombin in concentration of 0.1, 0.5 or 1.0 unit NIH/ml, which was respectively 30-, 54- and 143-fold higher in comparison with this level for untreated platelets (Fig. 1D). However, collagen stimulation caused a lesser extent of the actin exposition that maximally reached only 24-fold increment in the case of application of the highest concentration (1.25 mg/ml). These differences between the thrombin- and collagen-induced actin expositions are probably related to the activation of distinct signal pathways responsible for cytoskeleton rearrangement in platelets.

In the present work, we unequivocally established that the agonist-induced exposition of actin contributes to the plasminogen interaction with activated platelets. Interestingly, there is only limited population of resting platelets ($\sim$ 35 % of total amount of platelets), which appeared to be able to bind plasminogen (Fig. 2A). In contrast, both thrombin and collagen in their maximal concentrations (1.0 U NIH/ml and 1.25 mg/ml, respectively) dramatically increase the quantity of Pg-FITC-positive platelets (in average, 94.4 and 95.2 %, respectively). However, as shown by the intensity signal curve analysis, the plasminogen-binding ability of the collagen-exposed platelets appeared to be much weaker than that for of thrombin-activated counterparts (Fig. 2 B and C). Notably, actin blockade by inhibitory antibodies insignificantly lowered the quantity of Pg-FITC-binding platelets. Thus, 93.8 and 84.9 % of thrombin- or collagen-stimulated platelets, respectively, remained to be gated as FITC-positive events. However, the intensity signal from fluorescence conjugate bound to platelets decreased 4- and 2.5-fold in the cases of thrombin- or collagen-activated platelets, correspondently, Fig. 2D). These data mean that, apart from actin, other molecular targets also mediate the plasminogen interactions with the surface of activated platelets. It has been earlier reported that there are several molecules on the platelet surface, which ensure plasminogen binding, including integrins (αMβ2, α5β1, αVβ3, αIIbβ3), fibrinogen, fibronectin, vitronectin, and laminin 18.

The presence of cytoskeletal proteins on the cell surface has been reported in several other studies [19, 20]. However, the mechanisms of actin export on the cell surface are still obscure and it must be determined how actin, which
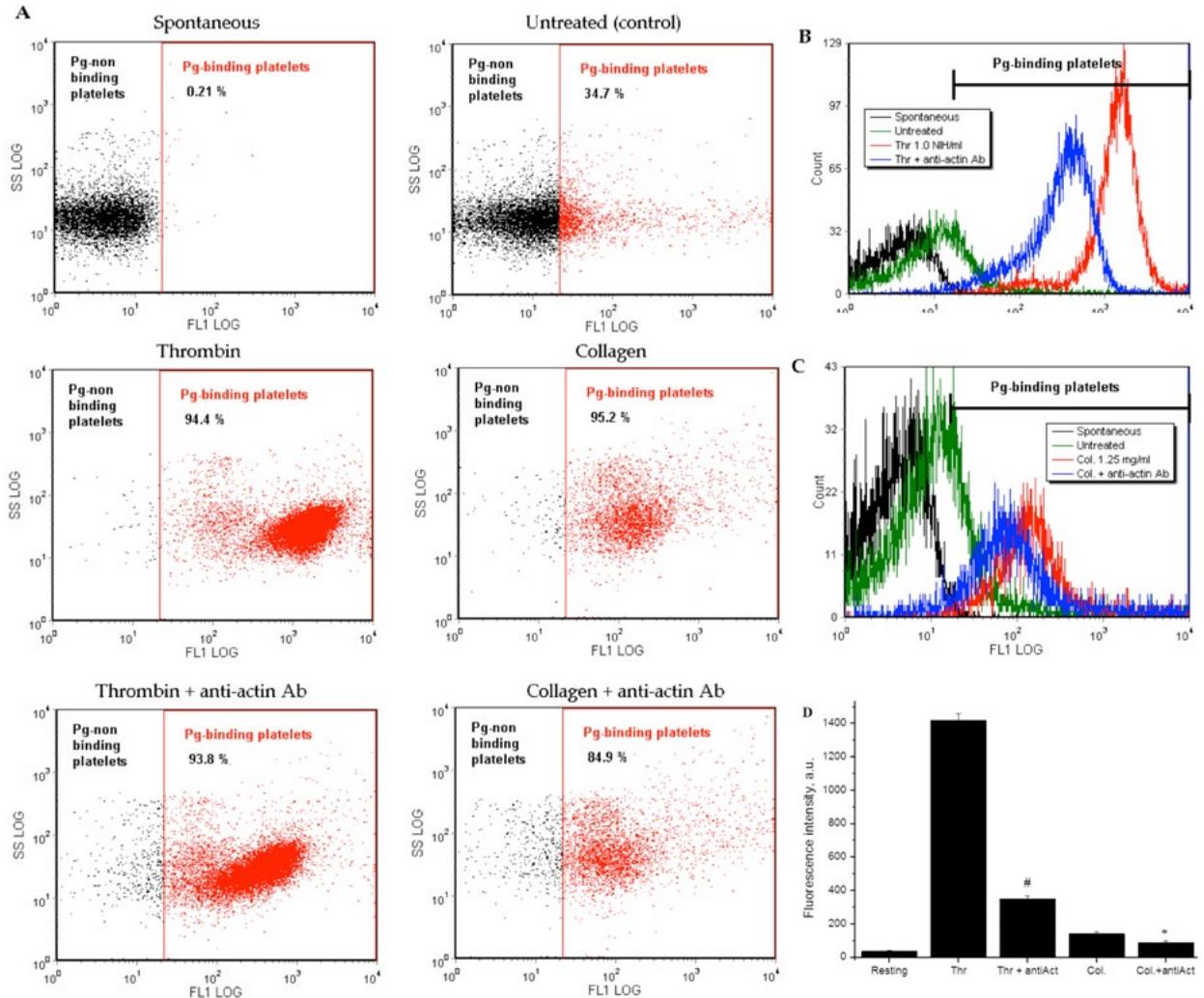
Fig. 2. Flow cytometric analysis of plasminogen binding with platelet surface: *A* – representative dot plots indicating inhibitory effects of anti-actin antibodies on Pg-FITC binding by activated platelets; B and *C* – representative flow cytometry histograms of Pg-FITC showing effects of pre-incubation with anti-actin antibodies on Pg-FITC fluorescence intensity pattern for platelets activated by thrombin or collagen, respectively; *D* – values of signal intensities captured from FITC-positive platelets reflecting actin exposition in plasminogen binding (# – $P < 0.001$ compared to thrombin-stimulated platelets, * – $P < 0.05$ compared to collagen-exposed platelets).

lacks a classical signal sequence, moves to the cell surface and anchors to the plasma membrane. The authors [21] hypothesize the existence of specific mechanisms of actin transport in a complex with other proteins acting as

carriers. For example, gelsolin being actin-binding protein is considered as a plausible candidate for the role of actin transporter because it can penetrate plasma due to a special signal peptide. Interestingly, the actin exposi-

tion on the surface of some cancer cells is thought to be an attribute of their apoptotic changes, in which actin has been shown to play an important role. For instance, the cells of medullary breast carcinoma expose surface actin, which is accumulated in the region of apoptotic blebs [22]. It is well-known that the outside-in agonist-mediated signaling triggers actin cytoskeleton reorganization in platelets, and actin appearance on the platelet surface may be associated with the lipid rafts formation, membrane blebbing and exocytosis [23].

Actin is a 42 kDa structurally highly conserved protein that occurs in all eukaryotic cells. Human platelet actin is represented by β- and γ-isoforms, like in most non-muscle cells [25]. All isoforms are very similar at the sequence level and therefore all of them have been shown to bind plasminogen. The residues Lys61, 68 and 113, which are highly conservative and occur in all actin isoforms, are responsible for the plasminogen recognition. The residue Lys61 is the most important for the interaction with plasminogen because it is situated on the surface of the actin globule. Moreover, proteolytic processing at the C-terminus of actin resulting in the Lys373 exposure increases affinity of plasminogen-actin binding and stimulates the cell-dependent plasminogen activation. In turn, the Lys-binding sites of plasminogen-plasmin kringle domains are involved in interaction with actin that can be inhibited by lysine and its analogues [10, 13].

Plasminogen as a plasmin precursor plays the major role in blood clot fibrinolysis. However, the current researches emphasize that plasminogen is involved in the regulation of the functional state of various cell types affecting different cellular components and signal pathways [25]. Our previous data have shown that the partially truncated Lys-form of plasminogen is able to interrupt the cytoskeleton remodelling, to affect the platelet degranulation, and to inhibit the agonist-induced platelet aggregation [26, 27]. Native (Glu-) plasminogen facilitates phosphatidylserine exposure on the surface of thrombin- or collagen-activated human platelets, thus promoting the formation of the platelet procoagulant surface [28]. Based on the data of Miles and others [13], it can be assumed that the cell-surface forms of actin may be involved in the pericellular plasminogen activation to plasmin as a binding site for proenzyme. The potential of platelets to enhance fibrinolysis by localizing plasminogen in the vicinity of a fibrin clot is in agreement with this observation [6]. Indeed, in the membrane-associated state, plasminogen acquires open conformation that is more readily converted into plasmin, which is able to further modulate the platelet response to thrombin and other aggregating agents that is influenced by its concentration [29]. Participation of surface-expressed actin in the plasminogen activation is additionally supported by the data demonstrating inhibitory effects of specific anti-actin antibodies on the cell-dependent plasminogen activation [13]. On the other hand, the activated platelets have been shown to secret and/or generate *de novo* proteolytically-derived plasminogen fragments, referred to as angiostatins, due to plasmin autoproteolysis. The mechanism of actin-mediated angiostatin formation can represent the way, in which platelets may regulate reparative angiogenesis, thus counteracting pro-angiogenic stimuli [30].

Interestingly, actin is not the only cytoskeletal protein found in such an unusual location. It has been observed previously that the activated platelets presented one more cytoskeletal protein, vimentin, the major component of intermediate filaments. Vimentin exposed upon the platelet activation may serve to localize complexes [vitronectin – plasminogen activator inhibitor type I (PAI-I)] on the platelet surface. In summary, our research is one of the attempts performed to uncover the functional link existing between the platelet cytoskeleton and the plasminogen/plasmin system. We are currently undertaking studies to examine whether the interaction between actin and plasminogen on the platelet surface may, at least partially, contribute to the regulation of processes related to platelet physiology.

Conclusions

Herein, we provide for the first time direct evidence that surface-exposed actin is responsible for increased plasminogen binding with the surface of activated platelets. It can be summarized that the levels of surface-exposed actin may reflect the functional competence of platelets in plasminogen binding.

Acknowledgement

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Роль експонованого актину у зв'язуванні плазміногену на мембрані агоніст-активованих тромбоцитів: цитометричне дослідження

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Мета. З'ясувати здатність актину, що експонується на поверхні тромбоцитів, активованих різними агоністами, зв'язувати плазміноген. **Методи.** Відмиті тромбоцити людини отримували за допомогою гель-фільтрації на Сефарозі-2В. Кількісну оцінку експонування актину на зовнішній поверхні інтактних тромбоцитів та тромбоцитів, активованих тромбіном або колагеном, проводили із застосуванням протокової цитометрії. Зв'язування флуоресцентного кон'югату плазміноген-FITC (Pg-FITC) з тромбоцитами, обробленими антитілами проти актину або неімунними IgG (контроль), аналізували цитометрично. **Результати.** Вплив тромбіну та колагену призводить до появи на зовнішньому боці плазматичної мембрани тромбоцитів актину, кількість якого залежала від концентрації агоністів. Попередня обробка активованих тромбоцитів специфічними антитілами проти актину значно запобігала зв'язуванню Pg-FITC з поверхнею тромбоцитів.

Висновки. Результати представленого дослідження вказують на залучення експонованого актину до зв'язування плазміногену на поверхні тромбоцитів, стимульованими різними агоністами.

К л ю ч о в і   с л о в а: тромбоцити, експонований актин, плазміноген, протокова цитометрія

Роль экспонированного актина в связывании плазминогена на мембране агонист-активированных тромбоцитов: цитометрическое исследование

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Цель. Выяснить способность актина, который экспонируется на поверхности тромбоцитов, активированных различными агонистами, связывать плазминоген. **Методы.** Отмытые тромбоциты человека получали с помощью гель-фильтрации на Сефарозе-2В. Количественную оценку экспонирования актина на внешней поверхности интактных тромбоцитов и тромбоцитов, активированных тромбином или коллагеном, проводили с использованием проточной цитометрии. Связывание флуоресцентного конъюгата плазминоген-FITC (Pg-FITC) с тромбоцитами, обработанными антителами против актина или неимунными IgG (контроль), анализировали цитометрически. **Результаты.** Влияние тромбина и коллагена приводило к появлению на внешней стороне плазматической мембраны тромбоцитов актина, количество которого зависело от концентрации агонистов. Предварительная обработка активированных тромбоцитов специфическими антителами против актина в значительной степени предотвращала связывание Pg-FITC с поверхностью тромбоцитов. **Выводы.** Результаты представленного исследования указывают на вовлечение экспонированного актина в связывание плазминогена на поверхности тромбоцитов, стимулированных различными агонистами.

К л ю ч е в ы е   с л о в а: тромбоциты, экспонированный актин, плазминоген, проточная цитометрия