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PLATELET-MEDIATED PLASMINOGEN PROCESSING PRODUCES ANGIOSTATINS: AN IMMUNOCHEMICAL STUDY

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The study of reciprocal interactions between the plasminogen/plasmin system and the platelet component of hemostasis is necessary both for understanding the biochemical mechanisms regulating the processes of thrombosis and thrombolysis and for elucidating the role of platelets in angiogenesis.

Aim. The study aimed to investigate the peculiarities of plasminogen processing by cytosolic and plasma membrane-associated proteases of platelets.

Methods. Gel-permeation filtration was used for the isolation of platelets from the donor's blood plasma. Plasminogen was purified from Cohn's fraction III2,3 of human blood plasma by affinity chromatography on lysine-Sepharose. The viability of washed platelets and their response to an agonist were assessed by optical aggregometry. The processing of plasminogen on platelets was induced by stimulating the cells with thrombin (1 NIH/ml) after pre-incubation with 0.25 μM Pg for 30, 60, or 120 min. Plasminogen and its fragments were detected by immunoblot with the use of previously obtained polyclonal antibodies to plasminogen kringles (K1-3 and K5).

Results. It was established that exogenous plasminogen is adsorbed onto the plasma membrane of platelets, converted into the Lys-form, and further fragmented into angiostatins and mini-plasminogen. This indicates the involvement of various platelet proteases in plasminogen cleavage. It was shown that platelets are capable of internalizing exogenous plasminogen in its Glu-form, while formed angiostatins are not internalized by the cells. It has been determined that internalized Glu-plasminogen (0.25 μM) may change its conformation to a Lys-like form within ≥ 120 minutes of incubation with platelets, as immunochemically detected with the use of antibodies against K5 plasminogen fragment.

Conclusion. The obtained results provide new insights into the mechanisms by which platelets may regulate the functioning of the plasminogen/plasmin system. This regulation occurs through their ability to generate plasminogen fragments (angiostatins) and having the potential for internalization and further secretion of the formed angiostatins by both native and activated platelets.

Key words: plasminogen, kringle-containing fragments, angiostatins, platelets, limited proteolysis, immunoblot, antibodies.

The study of interactions between the proteins of the plasminogen/plasmin system and platelets is essential for understanding the biochemical mechanisms regulating thrombosis and thrombolysis processes, as well as clarifying the role of platelets in angiogenesis. Platelets are a rich source of proangiogenic factors. They also store and release angiogenesis inhibitors. Platelets express surface growth factor receptors, which may regulate the process of angiogenesis. Activated platelets serve as procoagulant surfaces amplifying the coagulation reactions [1–3]. Existing literature data on the influence of platelets on the activity of the plasminogen/plasmin system is limited, and

there is currently a lack of information on the role of plasminogen and its fragments in the regulation of platelet functional state. It is known that platelets contain plasminogen-binding sites on their plasma membrane, and the number of these sites significantly increases when cells are stimulated by aggregation agonists [4–6]. The plasminogen molecule has kringle domains, which are responsible for intermolecular interactions [7]. These domains contain lysine-binding sites (LBS) with varying affinities for -aminocarboxylic acids and their analogues [8]. Plasminogen/plasmin molecule proteolysis in the organism leads to the formation of kringle-containing fragments (К 1-3, К 1-4, К 5, etc.) angiostatins that exhibit an anti-angiogenic effect. It has been shown that angiostatins are involved in signaling mechanisms that underlie many normal and pathophysiological processes in the organism, such as cell migration, angiogenesis, metastasis, tissue remodeling, wound healing, axon germination, and others. Thus, the interaction of angiostatins with targets on the plasma membrane of endothelial cells (ATP synthase, integrin αVβ3, c-met receptor of HGF, etc.) leads to suppression of proliferative activity of cells and their ability to move and migrate [9]. For some time, it was believed that angiostatin is produced in the organism by some types of tumors, and indeed, an increase of their generation is observed in tumor growth. However, it has recently been established that angiostatin is found in the organism and under normal conditions, thus being involved in physiological processes. To date, only a few types of cells that are capable of generating angiostatin in the norm are identified, including monocytes and macrophages. The elucidation of regulation of the platelet functioning by the plasminogen/plasmin system is one of the priority areas of our research. To determine the role of platelets in angiogenesis, we are investigating their ability to generate plasminogen fragments angiostatins, internalize and secrete of formed angiostatins by native and activated platelets [10]. Preliminary data [not published] indicate the interaction of isolated plasminogen fragments K1-3 and K5 with the surface of platelets and their accumulation by cells. Angiostatins were detected using western blot analysis with antibodies to K1-3 and K5. After pre-incubation of K1-3 and K5 with platelets, the fragments were found on isolated plasma membranes but were absent in cell lysates (cytosol). Antibodies to K1-3 revealed plasminogen and a 51 kDa angiostatin-like fragment on membranes and in lysates. In contrast, antibodies to K5 revealed mini-plasminogen on membranes and micro-plasminogen in the platelet cytosol, indicating the possibility of endogenous plasminogen conversion by platelets.

Thus, this study aimed to investigate time-dependent characteristics of plasminogen processing in the presence of platelet plasma membranes and cytosol by immunoblot with the use of antibodies against K1-3 and K5.

Material and Methods

Plasminogen was obtained from Cohn's fraction $III_{2,3}$ of human blood plasma (Kyiv City Blood Center, Ukraine) by affinity chromatography on lysine-Sepharose (Sigma, USA) in the presence of the protease inhibitor Contrycal (AWD, Germany) [11]. Plasminogen kringle fragments (K1-3 and K5) for rabbit immunization were prepared as described elsewhere [12, 13]. Intact platelets were obtained from donor blood plasma containing an anticoagulant in a 9:1 ratio (0.1 M sodium citrate, 0.08 M citric acid, 0.11 M glucose) using a standard method [14]. The viability of washed platelets and their response to an agonist were evaluated using optical aggregometry analysis [15]. The platelet count in each sample, according to the aggregometer, was 480,000 per μl. Aggregation was monitored for 5 minutes from the moment of addition of thrombin solution (1.0 NIH units/ml) using an optical aggregometer (SOLAR AT-02, RB) with the software package "Aggregometer 2.01" [16]. The degree of aggregation was no less than 35%. Platelets were activated by 1.0 NIH units/ml of thrombin (Sigma Aldrich, USA).

The processing of plasminogen by agonist-stimulated platelets was studied as follows. Plasminogen at a concentration of 0.25 μM was added to a suspension of isolated platelets (4.8×10^8 cells/ml, V = 0.1 ml) and incubated at 37 °C for 0, 30, 60, and 120 min, with "0 min of incubation" corresponding to the absence of plasminogen in the medium with the cells. Cells were then pelleted by centrifugation at 160 *g* for 20 min at room temperature, and the pellet was washed twice with 20 mM HEPES buffer (pH 6.8) containing 137 mM NaCl, 4 mM KCl, 0.2 mM $MgCl_2$, and 0.2% glucose, to remove unbound plasminogen. The washed platelet pellet was then resuspended in 0.1 ml of the same HEPES buffer additionally containing 0.2% bovine serum albumin per 4.8×10^7 cells. Thrombin (0.1 NIH units/ml) was added,

followed by incubation for 5 minutes at 37 °C. After thrombin-induced activation, the cells were pelleted by centrifugation at 160 *g* for 20 min at room temperature. The platelet pellet was washed twice with 0.1 ml of the same HEPES buffer and centrifuged under the same conditions. To study plasminogen processing, platelets were lysed with 0.1 ml of cooled hypotonic 0.2% NaCl solution. The suspension was incubated for 30 minutes at 4 °C with constant mixing. Plasma membranes were obtained from the suspension by centrifugation at 15,000 g for 10 min at 4 °C, as described elsewhere [17]. The supernatant contained the cytosolic fraction, while the pellet contained the membrane fraction, including subcellular components such as mitochondria. For control, experiments with non-stimulated platelets were performed in the same quantity and subjected to the same incubation procedure, washing, and centrifugation steps as thrombin-activated cells.

To the pellet of obtained washed plasma membranes, 50 µl of sample buffer containing 0.1% SDS was added as described earlier [17], concentrated to 0.025 ml, and then 0.025 ml of double sample buffer containing 0.2% SDS was added. The resultant supernatant, containing cytosolic fraction, was focused to 0.025 ml, and then 0.025 ml of 2× sample buffer was added. Concentration was performed using a SpeedVac vacuum mini-centrifuge (ThermoFisher Scientific, USA) in AQUA mode for 5 h at a temperature of 30 °C. Samples for electrophoresis were prepared so that the sample loaded on each track corresponded to 4.8×10^7 cells.

Tris-tricine electrophoresis of platelet samples was performed in a 10% polyacrylamide gel (PAAG) in the presence of 0.1% sodium dodecyl sulfate (SDS) [18]. Polyclonal IgG antibodies to human plasminogen kringle fragments K1-3 and K5 for immunoblot assay were produced as described earlier [19, 20]. Immunoblot was performed according to the previously described method with some minor modifications [21]. Briefly, protein transfer from PAAG to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biothech, Sweden) was carried out in a transfer-buffer solution (48 mM Tris, 39 mM glycine, 0.037% SDS, and 20% methanol) for 2 hours at a current of 0.8 mA per 1 cm^2 of a membrane. After transfer, membranes were immersed in a 4% solution of skimmed milk powder in PBS for 15 hours at 22–24 °C and probed with polyclonal antibodies (IgG 5 µg/ml) against kringle fragments K1-3 or K5 in tris-buffered saline containing 0.05% Triton X-100 (TBST) for 2 hours at 37 °C. Membranes then were washed five times with TBSP and incubated with appropriate secondary antibodies Goat Anti-Rabbit IgG–HRP conjugate (Sigma Aldrich, USA), 1:2,000 diluted in TBST, for 60 minutes at 37 °C. Unbound antibodies were washed out with TBST, and then membranes were immersed into 0.05 M potassium phosphate buffer (pH 6.0) containing 0.04% hydrogen peroxide and 0.05% 4-chloro-1-naphthol (Sigma Aldrich, USA). The reaction was visually monitored and stopped by washing the membranes with distilled water. An alternative method for blot development was enhanced chemiluminescence (ECL) when 0.25 M luminol in DMSO, 0.09 M p-coumaric acid in DMSO, 0.1 M Tris-HCl (pH 8.5), and 0.0035% H_2O_2 were used. Membrane autoradiography was performed on X-ray films (Konica Minolta, Japan). Depending on the intensity of the signal of chemiluminescence, exposure time on the film ranged from 3 s to 10 min. Immunoreactive bands on the film were developed using commercially available developer and fixer solutions (ONIKO, Kyiv, Ukraine) [22]. The data were collected from three independent replicates.

Results and Discussion

As seen from immunoblot depicted in Fig. 1, upon stimulation of platelets pre-incubated with 0.25 µM plasminogen by thrombin (1 NIH/ml), antibodies to K1-3 detected membrane-bound plasminogen, a variety of its low-molecular-weight fragments ranging from 40 to 70 kDa, among which a 51 kDa angiostatin-like fragment, and undefined high-molecular-weight bands that include plasminogen as well (tracks 2-5). It is noticeable that the quantity of the detected low-molecular-weight fragments is gradually increased, indicating the fragmentation of membrane-associated plasminogen. Additionally, in the cytosolic fraction of the cells (tracks 6-9), increasing amounts of plasminogen, the 51 kDa angiostatin-like fragment, and a series of high-molecular-weight fragments, including plasminogen, were detected over time. This suggests the fragmentation of exogenous Glu-plasminogen by various metalloproteinases [23, 24] and the involvement of the proenzyme in intracellular complexes with cytosolic proteins.

Results of immunoblots presented in Figs. 2 and 3 show that upon stimulation of platelets pre-incubated with 0.25 µM plasminogen by thrombin (1 NIH/

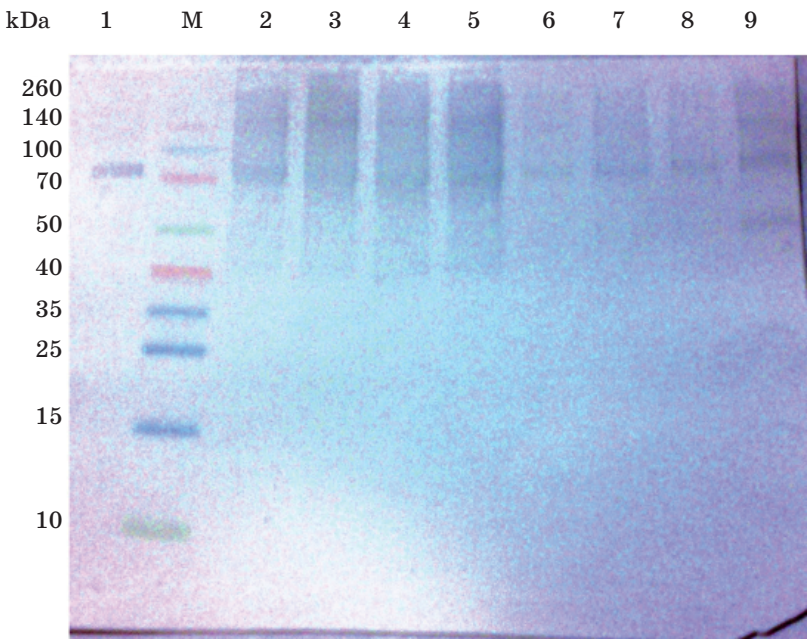

Fig. 1. **Time-dependent profile of plasminogen processing on platelet membranes (2–5) or in cell lysates (6–9) induced with 1 NIH/ml thrombin after pre-incubation with 0.25 μM Pg for 1 — intact plasminogen; 2, 6 — 0 min; 3, 7 — 30 min; 4, 8 — 60 min; 5, 9 — 120 min**
Typical blotogram obtained with polyclonal antibodies to K1-3

ml), antibodies to K5 detect absorbed plasminogen in the Lys-form, a 260 kDa complex containing Lys-plasminogen, and a 38 kDa plasminogen fragment corresponding to mini-plasminogen on the membranes. Notably, the quantity of these fragments remains relatively constant over time of incubation.

In the cytosolic fraction of platelets, antibodies to K5 detect a trace amount of the 260 kDa complex containing Lys-plasminogen up to 60 min of incubation with 0.25 μM plasminogen. By 120 min of incubation, Lys-plasminogen, a significant amount of the 260 kDa complex, and a trace amount of the 140 kDa complex containing Lys-plasminogen are observed. Since previous studies have shown that exogenous plasminogen can be internalized exclusively as a Glu-form, we assume that Glu-plasminogen undergoes conformational changes in the platelet cytosol during the incubation period. Platelet-derived factors, which induce conformational changes in plasminogen molecules, and

It is worth noting that the blot obtained with polyclonal antibodies to K5 using the ECL method appeared to be more illustrative (Fig. 3) than the blot obtained by the routine procedure shown in Fig. 2 due to the higher sensitivity of the immuno-development method. Therefore, in subsequent experiments, we applied the ECL method for immunoreactive band visualization.

As seen in the blotograms presented in Fig. 4, after pre-incubation of 0.25 μM plasminogen with platelets without further stimulation by an agonist, antibodies to K1-3 detect membrane-bound plasminogen, a variety of its low-molecular-weight fragments ranging from 35 to 70 kDa, including a 51 kDa angiostatin-like fragment and the kringle fragment K1-3, and a blurred high-molecular-weight fragments ranged from 140 to 260 kDa, including plasminogen (tracks 2–5). Notably, at "0 minutes," K1-3 is absent on the membranes, but by "60 minutes" of incubation, it appears and its quantity increases, however then decreases by "120 minutes" of incubation. The quantity of the 51 kDa angiostatin-like fragment and other mentioned low-molecular-weight fragments increases over time. These data indicate the fragmentation of Glu-plasminogen associated with the plasma membrane of platelets.

In the cytosolic fraction of platelets, plasminogen is absent at "0 minutes", but high-molecular-weight fragments from 110 to 260 kDa, recognized by antibodies to K1-3, are present. In other cytosol samples (tracks 7–9), increasing amounts of plasminogen, the 51 kDa angiostatin-like fragment, and multiple high-molecular-weight fragments,

including plasminogen, are detected over time. These observations suggest the fragmentation of exogenous Glu-plasminogen and the involvement of the proenzyme in intracellular complexes with cytosolic proteins in both native and activated platelets.

The results of the immunoblotting assay presented in Fig. 5 demonstrate that, following pre-incubation of 0.25 μM plasminogen with platelets without further stimulation by the agonist, antibodies to K5 recognize the presence of only the 260 kDa complex at "0 minutes" in the plasma membrane samples. Lately, absorbed plasminogen in the Lys-form, an abundancy of high-molecular-weight fragments ranging from 140 to 260 kDa that include Lys-plasminogen, and trace amounts of 38 kDa and 51 kDa plasminogen fragments

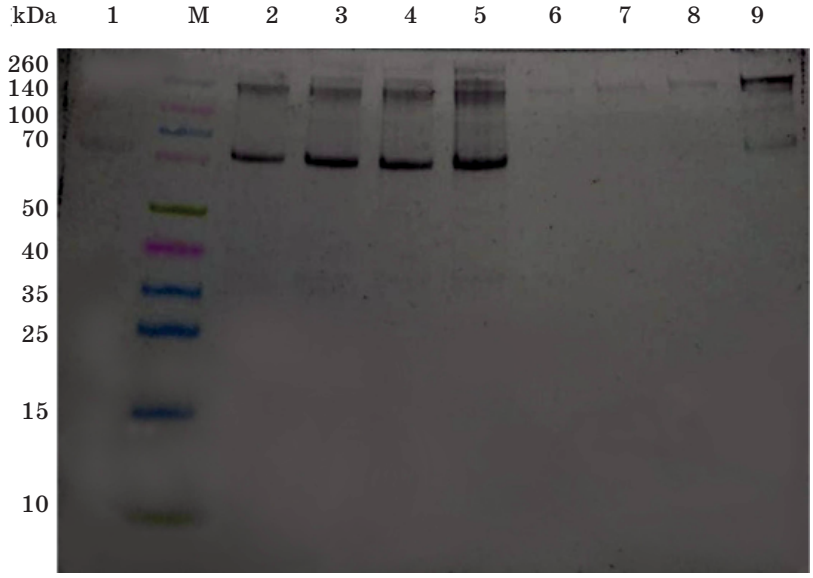

Fig. 2. **Time-dependent profile of plasminogen processing on platelet membranes (2–5) or in cell lysates (6–9) induced with 1 NIH/ml thrombin after pre-incubation with 0.25 μM Pg for 1 — intact plasminogen; 2, 6 — 0 min; 3, 7 — 30 min; 4, 8 — 60 min; 5, 9 — 120 min**
Typical blotogram obtained with polyclonal antibodies to K5

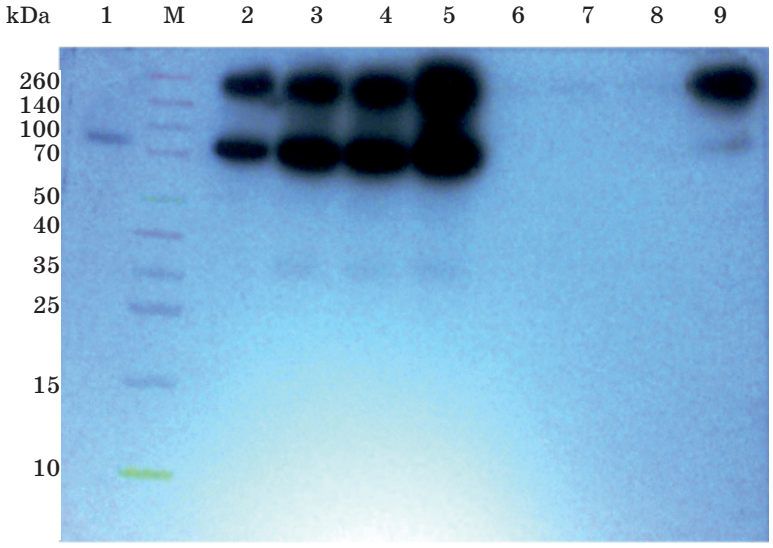

Fig. 3. **Time-dependent profile of plasminogen processing on platelet membranes (2–5) or in cell lysates (6–9) induced with 1 NIH/ml thrombin after pre-incubation with 0.25 μM Pg for 1 — intact plasminogen; 2, 6 — 0 min; 3, 7 — 30 min; 4, 8 — 60 min; 5, 9 — 120 min**
Typical blotogram obtained with polyclonal antibodies to K5 (the same blotogram as presented in Fig. 2 but developed by ECL)

corresponding to mini-plasminogen and an angiostatin-like fragment appear on the membranes. The quantity of these fragments increased until "60 minutes," but they disappeared at "120 minutes," which may indicate their further digestion.

In the cytosolic fraction of platelets, antibodies to K5 detect the 260 kDa complex containing Lys-plasminogen up to 60 minutes of incubation with 0.25 μM plasminogen. By 120 minutes, Lys-plasminogen, a significant amount of the 260 kDa complex, a trace amount of the 140 kDa complex containing Lys-plasminogen, and trace amounts of the 51 kDa angiostatin-like fragment are observed. Since previous studies have shown that exogenous plasminogen is internalized exclusively in the Glu-form, we conclude that

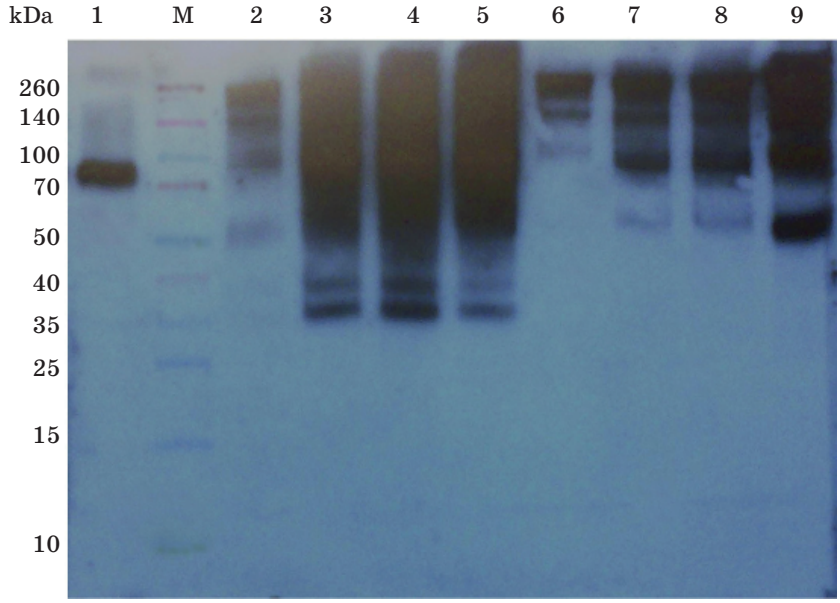

Fig. 4. **Time-dependent profile of plasminogen processing on platelet membranes (2–5) or in cell lysates (6–9) induced on non-stimulated cells after pre-incubation with 0.25 μM Pg for 1 — intact plasminogen; 2, 6 — 0 min; 3, 7 — 30 min; 4, 8 — 60 min; 5, 9 — 120 min**
Typical blotogram obtained with polyclonal antibodies to K1-3 and developed by ECL

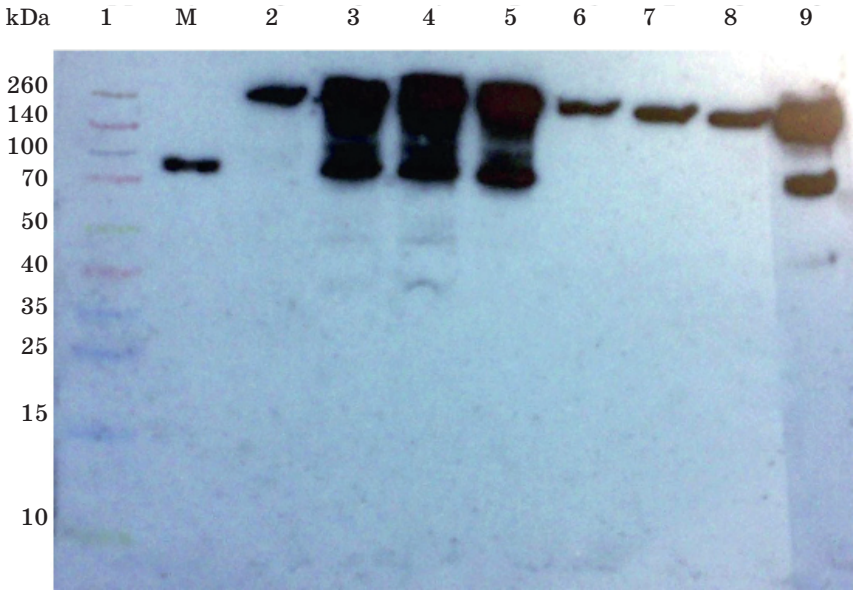

Fig. 5. **Time-dependent profile of plasminogen processing on platelet membranes (2–5) or in cell lysates (6–9) induced on non-stimulated cells after pre-incubation with 0.25 μM Pg for 1 — intact plasminogen; 2, 6 — 0 min; 3, 7 — 30 min; 4, 8 — 60 min; 5, 9 — 120 min**
Typical blotogram obtained with polyclonal antibodies to K5 and developed by ECL

Glu-plasminogen undergoes conformational changes in the cytosol in a time-dependent manner due to the action of still undiscovered factors.

Thus, using immunoblotting with polyclonal monospecific antibodies to plasminogen kringle fragments K1-3 and K5, we studied time-dependent features of plasminogen processing in the presence of either platelet plasma membranes or platelet cytosol. We demonstrated that exogenous plasminogen could serve as a source of angiostatin production on the surface of platelets. Zymogen is internalized by platelets in the Glu-form upon mechanism still unexplored. Plasminogen bound on the plasma membrane is converted into the Lys-form and fragmented into angiostatins and mini-plasminogen, indicating the involvement of various platelet proteases in plasminogen cleavage. However, the resulting fragments are not internalized by the cells.

This study confirms the need for additional studies in this field. The obtained results may have a physiological relevance for several reasons. First, the reciprocal interactions between the plasminogen/plasmin system and platelets are essential for understanding the biochemical mechanisms regulating thrombosis and thrombolysis. These processes are critical in maintaining hemostasis and preventing pathological conditions such as stroke and myocardial infarction. Second, platelets are known to play a role in angiogenesis, the formation of new blood vessels, which is crucial for wound healing and tissue regeneration. The study's results, showing the ability of platelets to process plasminogen and generate angiostatins, highlight a potential mechanism through which platelets can influence angiogenesis and tissue remodeling. Third, the ability to detect specific plasminogen fragments, such as angiostatins, in the plasma membranes and cytosol of platelets suggests that these fragments could serve as biomarkers for platelet activation and function. This could have potential clinical applications in diagnostics and monitoring diseases related to abnormal platelet activity and blood clot formation. Fourth, understanding how platelets internalize and process plasminogen could lead to new therapeutic strategies for managing conditions involving excessive clot formation or inadequate clot breakdown. For example, targeting the pathways involved in plasminogen processing might enhance thrombolytic therapy or prevent thrombotic complications.

Conclusions

We have established that exogenous plasminogen is adsorbed onto the plasma membrane of platelets, converted into Lys-form, and fragmented into angiostatins and mini-plasminogen. This indicates the involvement of various platelet proteases in plasminogen hydrolysis. The formed angiostatins are not internalized by platelets. It has been confirmed that platelets can internalize exogenous plasminogen as a Glu-form. We found that internalized Glu-plasminogen can change its conformation to a Lys-like form (during ≥120 minutes of incubation with 0.25 μM plasminogen), as detected by the antibodies to K5. The obtained results provide new information on the mechanisms by which platelets regulate the functioning of the plasminogen/plasmin system through their ability to generate plasminogen fragments, angiostatins, and the potential for internalization and secretion of the formed angiostatins by both native and activated platelets. Our findings may have important physiological and biomedical applications, providing new insights into the regulatory mechanisms of hemostasis, reparative processes, and wound healing with the involvement of interaction between proteins of the plasminogen/plasmin system and platelets.

Authors' contribution

KLG — conceptualization and design, data collection and analysis, manuscript preparation; YOI — antibody to K1-3 production; KVV — immunoblot assay; GTV — interpretation of results, manuscript preparation.

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Conflicts of Interest

The authors declare no conflicts of interest.

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УТВОРЕННЯ АНГІОСТАТИНІВ У ХОДІ ПРОЦЕСІНГУ ПЛАЗМІНОГЕНУ, ОПОСЕРЕДКОВАНОГО ТРОМБОЦИТАМИ: ІМУНОХІМІЧНЕ ДОСЛІДЖЕННЯ

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Вивчення реципрокних взаємодій між плазміноген/плазміновою системою та тромбоцитарною ланкою гемостазу є необхідним як для розуміння біохімічних механізмів регуляції процесів тромбоутворення й тромболізису, так і для з'ясування ролі тромбоцитів в ангіогенезі.

Метою цього дослідження було дослідити особливості процесінгу плазміногену мембраноасоційованими та цитозольними протеазами тромбоцитів.

Методи. Хроматографічний метод застосовано для ізоляції тромбоцитів із плазми крові. Плазміноген очищали з фракції за Коном $III_{2,3}$ плазми крові людини афінною хроматографією на лізин-сефарозі. Аналіз життєздатності відмитих тромбоцитів та їхні відповіді на дію агоніста проведено оптичною агрегометрією. Процесінг плазміногену на тромбоцитах викликано стимуляцією тромбіном (1 NIH/мл) клітин, попередньо інкубованих з 0,25 мкМ Pg протягом 30 хв; 60 хв та 120 хв. Плазміноген та його кринглвмісні фрагменти на мембранах й у лізатах тромбоцитів детектували методом імуноблоту з використанням отриманих нами раніше поліклональних антитіл до кринглів плазміногену (К1-3 і К5).

Результати. Установлено, що екзогенний плазміноген сорбується на плазматичній мембрані тромбоцитів, перетворюється у Lys-форму та фрагментується до ангіостатинів та міні-плазміногену, що свідчить про залучення різних протеїназ тромбоцитами для гідролізу плазміногену. Утворені ангіостатини клітинами не інтерналізуються. Підтверджено здатність тромбоцитів інтерналізувати екзогенний плазміноген у Glu-формі. Встановлено здатність інтерналізованого Glu-плазміногену в плині часу (≥120хв інкубації з 0,25 мкМ плазміногеном) змінювати конформацію на Lys-подібну, яку виявляють застосовані нами антитіла до К 5.

Висновки. Отримані результати надають нову інформацію про механізми регулювання тромбоцитами функціонування плазміноген/плазмінової системи на прикладі їхньої здатності генерувати фрагменти плазміногену — ангіостатини, можливості інтерналізації та секреції утворених ангіостатинів нативними та активованими тромбоцитами.

Ключові слова: плазміноген, кринглвмісні фрагменти, ангіостатини, тромбоцити, обмежений протеоліз, імуноблот, антитіла.