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Functional properties of individual sub-domains of the fibrin(ogen) αC-domains

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ABSTRACT

Background: Fibrinogen is a large polyfunctional plasma protein consisting of a number of structural and functional domains. Among them, two αC-domains, each formed by the amino acid residues Aα392–610, are involved in fibrin polymerization, activation of fibrinolysis, platelet aggregation, and interaction with different cell types. Previous study revealed that each fibrinogen αC-domain consists of the N-terminal and C-terminal sub-domains. The major objections of the present study were to test functional role of these sub-domains in the above mentioned processes.

Methods: To achieve these objections, we used specific proteases to prepare two truncated forms of fibrinogen, fibrinogen desAα505–610 and fibrinogen desAα414–610, missing their N-terminal and both N- and C-terminal sub-domains, respectively.

Results: Our study with these truncated forms using turbidity measurements and electron microscopy revealed that the N- and C-terminal subdomains both contribute to protofibril formation and their lateral aggregation into fibers during fibrin polymerization process. These two sub-domains also contributed to platelet aggregation with the N-terminal sub-domains playing a more significant role in this process. At the same time, the C-terminal sub-domains make the major contribution to the plasminogen activation process. Further, our experiments revealed that the C-terminal sub-domains are involved in endothelial cell viability and migration of cancer cells.

Conclusions: Thus, the results obtained establish the functional role of individual sub-domains of the αC-domains in fibrin polymerization, activation of fibrinolytic system, platelet aggregation, and cellular interactions.

General significance: The present study expands our understanding of the functional role of individual fibrinogen domains and their specific portions in various fibrin(ogen)-dependent processes.

1. Introduction

Fibrinogen is a large glycoprotein consisting of three pairs of polypeptide chains, Aα, Bβ, and γ [1]. Blood vessel injury initiates the blood clotting cascade resulting in conversion of fibrinogen to fibrin which spontaneously polymerizes to form the core of blood clots that prevent blood loss [1,2]. Besides of its blood clotting function, fibrinogen as a multifunctional protein involved in various processes including platelet aggregation [3–5], migration and proliferation of endothelial cells [6,7], leucocyte migration [8], and regulation of vessel tonus [9], and endothelial permeability [10].

Structurally, fibrinogen consists of the central E-region, two

List of abbreviations: ADP, adenosine diphosphate; BSA, bovine serum albumin; FBS, fetal bovine serum; HRP, Horseradish Peroxidase; mAb, monoclonal antibody; MAEC, mouse aortic endothelial cells; MALDI-TOF, matrix assisted laser desorption ionization time of flight mass-spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RPMI, Roswell park memorial institute medium; SDS, sodium dodecyl sulfate; SPR, surface plasmon resonance; TEMED, tetramethyl ethylenediamine; tPA, tissue-type plasminogen activator.

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peripheral D regions, and two αC regions formed by the 221–610 portions of the Aα chains. Each αC region includes the unordered αC-connector (amino acid residues Aα221–391) and the compact αC-domain formed by the Aα392–610 residues [11]. During fibrin polymerization, the αC regions mainly participate in lateral association of protofibrils [12,13,2] and subsequently in activation of fibrinolysis [14]. This portion of the molecule also contains the RGD motif (amino acid residues Aα572–574) which is known to bind integrin receptors on platelets and endothelial cells [15,16]. Such reactivity and various interactions of the αC regions suggest that distinct portions of these regions contain different binding sites that participate in protein-protein and protein-cellular interactions. Thus, it is important to identify which portions of the αC region are responsible for its specific functions.

In the present study, we focused on the role of different portions of fibrin(ogen) αC-domains in fibrin polymerization, activation of fibrinolytic system, platelet aggregation, endothelial cells proliferation, and cancer cells migration. In our experiments, we utilized two highly selective proteases prepared from the cultural liquid of *Bacillus thuringiensis* var. *israelensis* IMV B-7465 and from the venom of snake *Gloydius halys halys*. These proteases allowed us to obtain truncated forms of fibrin(ogen) lacking the Aα414-610 and Aα505-610 portions of their αC regions. Comparison of the properties of fibrin(ogen) desAα505-610 and desAα414-601 with those of the parent molecule allowed us to elucidate the role of the Aα414-504 and Aα505-610 portions of the αC-domains in the above mentioned processes.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Molecular weight markers were from Thermo Fisher Scientific (Waltham, USA). Superdex G 75, SDS, and β-mercaptoethnol were from Bio-Rad Laboratories (Hercules, USA). Acrylamide, bis-acrylamide, TEMED, Thrombin (50 NIH units/ml), ADP, carboxypeptidase B, 4-Nitrophenyl phosphate disodium salt, Blue-Sepharose, NaCl, HEPES, KCl, $MgCl_2$, NaH_2PO_4, BSA, SDS, PBS tablets, $CaCl_2$, modified Dulbecco's medium, fetal bovine serum (FBS), gentamicin, 3-(4, 5-diethylthiazoly-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent), dimethyl sulfoxide (DMSO) were from Sigma-Aldrich (St. Louis, USA). Recombinant tPA 'Actilyse' was from Boehringer Ingelheim (Ingelheim am Rhein, Germany) and chromogenic substrate S2251 (Val-Leu-Lys-p-nitroanilide) was from Siemens (Munich, Germany).

2.1.2. Proteases

Proteolytic enzyme from the venom of *G. halys halys* was purified by the method developed previously [17]. This serine protease was described as the fibrinogen-specific enzyme that preferentially cleaves the Aα413-414 peptide bond. After 90 min of incubation of fibrinogen (10 mg/ml) with this enzyme (0.02 mg/ml) at 37 °C, most of the Aα chains were hydrolyzed while the Bβ and γ chains remained intact, as reported previously [18].

Proteolytic enzyme from the cultural liquid of *Bacillus thuringiensis* was purified by the method developed previously [19]. This serine protease is specific towards peptide bond Aα504-505. After 90 min of incubation of fibrinogen (10 mg/ml) with this enzyme (0.02 mg/ml) at 37 °C, most of the Aα chains were hydrolyzed while the Bβ and γ chains remained intact [20].

2.1.3. Antibodies

Mouse monoclonal antibody II-5C (anti-Aα20-78) and 2D-2A (anti-Bβ12-25) used in the present study were prepared as described earlier [21,22]. Polyclonal rabbit antibody against human Glu-plasminogen and against recombinant tissue-type plasminogen activator were also prepared as described earlier [23,24]. All antibodies were produced in immunology department as well as in protein structure and functions department of Palladin Instutute of biochemistry of NAS of Ukraine and were used in 1:1000 dilution. Anti-Rabbit IgG (whole molecule) Alkaline Phosphatase conjugated antibody produced in goat was purchased from Sigma-Aldrich (St. Louis, USA).

2.1.4. Proteins

Fibrinogen was purified from the human blood plasma according to the method described earlier [25]. Glu-Plasminogen was obtained from the human blood plasma by affinity chromatography on Lys-Sepharose as described previously [26]. Thrombin-like enzyme Ancistron, which removes from fibrinogen only fibrinopeptides A, was purified from the venom of *G. halys halys* using Blue-Sepharose (Sigma-Aldrich, St. Louis, USA) according to the previously described method [27].

2.1.5. Platelets

Venous blood of healthy volunteers ($n = 5$) who had not taken any medication for 7 days was collected by venipuncture of basilic vein using 19 G sterile needle. Blood was collected into sterile plastic 10 ml tubes. Blood was spinned down at 160 g for 30 min at 25 °C. Platelet rich plasma was collected and spinned down again at 300 g for 15 min. Pellet of platelets was re-suspended in 0.004 M HEPES, pH 7.4, 0.137 M NaCl, 0.0027 M KCl, 0.001 M $MgCl_2$, 0.0056 mM glucose, 0.003 M NaH_2PO_4, and 0.35 mg/ml BSA to obtain the homogenous suspension of washed platelets, as recommended earlier [28]. Patients signed informed consent prior to blood sampling. This study was approved by the Ethics Committee of the Palladin Institute of Biochemistry (09.09.2021, N12).

2.1.6. Cell lines

HeLa (human cervix epitheloid carcinoma, ECACC 93021013) were obtained from European Collection of Authenticated Cell Cultures.

Murine aortic endothelial cell line (MAEC) [29] were purchased by National Bank of Cell Lines and Tumor Strains of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of National Academy of Sciences of Ukraine (IEPOR, NAS of Ukraine).

2.2. Methods

2.2.1. Preparation of fibrinogen desAα505-610 and fibrinogen desAα414-610

Fibrinogen hydrolysis was performed by incubation with proteases from the cultural liquid of *Bacillus thuringiensis* var. *israelensis* IMV B-7465 and from the venom of snake *Gloydius halys halys* for 60 and 30 min, respectively, at enzyme:substrate ratio of 1:300 for both enzymes. The reaction was performed in 0.05 M tris-HCl buffer, pH 7.4, containing 0.13 M NaCl, at 37 °C, and was terminated by adding benzamidine solution to a final concentration of 0.0016 M. The incubation mixture after hydrolysis was fractionated on Superdex 75 column using FPLC-system Akta Prime equilibrated with the same buffer. Truncated forms of fibrinogen, fibrinogen desAα414-610 and fibrinogen desAα505-610, were then characterized using SDS-PAGE and Western blotting. For the MALDI-TOF analysis, fractions eluted from Superdex 75, which contained cleaved peptides, were concentrated using the centrifuge microconcentrators Amicon Ultra 3 K (MilliporeSigma, Burlington, USA).

Both truncated forms of fibrinogen were used for preparation of monomeric fibrins desAB-desAα414–610 and desAB-desAα505–610. These forms were incubated with 0.15 NIH units of thrombin per 1 mg of fibrinogen to convert fibrinogen to fibrin. After 30 min of incubation at 37 °C, fibrin clots were removed, washed with 0.15 M NaCl solution, and then dissolved in 0.125% acetic acid as described in [30]. All samples of truncated fibrinogen and fibrin were frozen in liquid nitrogen and lyophilized using LyoQuest (Telstar, Terrassa, Spain). The lyophilized samples of fibrinogen and fibrin were stored at +4 °C and dissolved before experiments in 0.05 M tris-HCl buffer, pH 7.4, or in 0.125% acetic acid, respectively.

2.2.2. SDS-PAGE and Western blotting

The purity of truncated fibrin(ogen) samples and their molecular masses were determined by SDS-PAGE using 10% or 12% gels according to Laemmli. The products of hydrolysis of fibrinogen and fibrin by fibrinogenase were also analyzed by SDS-PAGE under reducing conditions. The purified proteins were further transferred to a nitrocellulose membrane to specify the bands by immunoprobing. The membrane was blocked with 5% milk in PBS for 1 h, incubated with a mouse monoclonal antibody II−5C (for the Aα chain detection) or antibody 2D-2A (for the Bβ chain detection) for another hour and then developed with a secondary HRP-labelled goat anti-mouse antibody. The bands were visualized using 0.001 M 4-chloro-1-naphtol solution in 0.05 M Tris, pH 7.5, and 0.3% H_2O_2.

2.2.3. MALDI-TOF analysis

Mass spectrometry analysis was performed on a MALDI-TOF spectrometer Voyager DE PRO (Applied Biosystems, USA). H^+-matrix ionization was performed by laser irradiation. The concentration of Sinapinic acid (Sigma, USA) in the matrix reagent was 1 mg/ml. The reagent was dissolved in a solution containing equal volumes of acetonitrile (Sigma, USA) and 1% aqueous trifluoroacetic acid (Sigma, USA). The spectra obtained were processed by Data Explorer 4.0.0.0 (Applied Biosystems) [31].

2.2.4. Turbidity measurements

The process of fibrin polymerization was monitored by measuring the change in turbidity at 350 nm during thrombin-induced polymerization of fibrin using spectrophotometer POP (Optizen, Daejeon, Korea) [32]. The samples of native or truncated fibrinogens were dissolved in 0.05 M Tris buffer, pH 7.5, containing 0.13 M NaCl, and 10^{-3} M $CaCl_2$ to final concentration of 0.1 mg/ml). Polymerization was initiated by addition of thrombin (0.25 NIH units/ml).

2.2.5. Surface plasmon resonance

Studies were performed using 'Plasmon 6′ device developed at the V. E. Lashkarev Institute of Semiconductor Physics (Kyiv, Ukraine). The gold surface of a chip was covered by the self-assembled layer formed by 11-mercaptoundecanoic acid and 6-mercapto-1-hexanol [33]. Human fibrinogen was immobilized covalently onto the functionalized chip using standard amino group coupling chemistry. Native or truncated forms of fibrinogen at 0.01 mg/ml were applied to the chip in 0.05 M Tris buffer, pH 7.5, with 0.13 M NaCl. Protein-protein interactions were registered as the change in Response.

2.2.6. Electron microscopy

Polymerization of native fibrin and its truncated forms was studied by transmission electron microscopy of negatively contrasted samples using H-600 Transmission Electron Microscope (Hitachi, Tokyo, Japan) at 75 kV; 1% water solution of uranyl acetate (Merck, Germany) was used as a negative contrast. For sample preparation, 0.32 mg/ml fibrinogen in 0.05 M ammonium formiate buffer, pH 7.9, with 0.025 M $CaCl_2$ was placed in sterile glass tubes. Fibrin polymerization was initiated by adding thrombin to a final concentration of 0.25 NIH units/ml. After 180 s, aliquots were taken from the polymerization mixture. Each aliquot was diluted to a final fibrinogen concentration of 0.07 mg/ml, and 0.01 ml probes of fibrinogen solution were transferred to a carbon lattice which was treated with a 1% uranyl acetate solution after 2 min. Electron microscopic images were obtained at magnification of 20,000–50,000 as described before [34].

2.2.7. Plasminogen activation assay

Activation of Glu-plasminogen by tissue-type plasminogen activator was studied on native fibrin, as well as on desA-desAα414–504 and desA-desAα505–610 truncated forms of fibrin. To prepare these forms, we used thrombin-like enzyme Ancistron. Incubation mixture contained fibrinogen (0.2175 μM), Ancistron (0.01 mg/ml), Glu-plasminogen (0–0.11 μM), tissue-type plasminogen activator (3 IU/ml), and chromogenic substrate S2251 (0.3 mM). The reaction was performed in 0.05 M tris-HCl buffer, pH 7.4, containing 0.15 M NaCl at 37 °C. The resulting plasmin activity was monitored using chromogenic substrate S2251. Generation of p-nitroaniline was monitored using Multiscan FC (ThermoFisher, Waltham, USA). Native fibrinogen and its truncated forms were incubated with carboxypeptidase B for 15 min at ambient temperature at 1:50 mass ratio for the removal of the C-terminal Lysine residues.

2.2.8. Aggregometry

Platelet aggregation measurements were based on the changes in the turbidity of human PRP [35]. The measurements were performed on Aggregometer Solar AP2110 (Belorussia) according to manufacturer's recommendations. Washed platelets (0.2 ml) were added to the sample tube, mixed with 0.05 ml of fibrinogen (9 mg/ml) and were activated by the addition of 0.025 ml $CaCl_2$ (0.025 M) and 0.025 ml of platelet agonist ADP (25 μM). Aggregation was registered for 5 min at 37 °C.

2.2.9. Study of endothelial cells viability

Immortalized line of endothelial cells MAEC were cultivated in Iscove's modified Dulbecco's medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% FBS and gentamicin (50 μg/ml). Cells were incubated at 37 °C in 5% CO_2. Cell viability was measured by 3-(4, 5-diethylthiazoly-2-yl)−2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated on 96-well plates and were incubated with 100 μl of complete medium containing 1 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)−2,5-Diphenyltetrazolium Bromide (Sigma) at 37 °C for 4 h followed by solubilization with dimethyl sulfoxide (Sigma). The absorbance at 540 nm was measured with Multiscan FC (ThermoFisher, Waltham, USA) [36].

2.2.10. Scratch test

The scratch wound-healing assay was applied as described previously [37]. This assay is widely used for estimating cell migration and is especially useful for the study of migration of cancer cells [38]. Briefly, 4×10^5 cells were seeded onto 24-well cell culture plates and allowed to grow to form a confluent monolayer (24 h). Next day, the cells were serum-starved in RPMI containing 0.5% FBS for 24 h prior to treatment. The monolayer was gently scratched with a sterile pipette tip to create a 1 cm long "scratch". The wells were washed 2 times with 1 ml of the growth media to remove the debris and smooth the edge of the scratch. Lyophilized fibrinogen was reconstituted in 50 mM Tris, pH 7.5, with 130 mM NaCl prior to treatment. Fresh media containing 50 μg/ml of control or truncated fibrinogen was added to cells and cells were incubated in fibrinogen-containing media for 2 h. After 2 h, media was replaced with RPMI + 0.5% FBS and cells were incubated for up to 24 h. The images of scratches were acquired at 0, 12, and 24 h post treatment. Wound area was measured, and number of migrated cells were counted using ImageJ software (NIH). The number of cells migrated into a zone of scratching was normalized to vehicle-treated wells (no fibrinogen).

2.2.11. Molecular modeling of αC-domain structure

The structure of the human fibrinogen αC-domain was obtained from https://faculty.uml.edu//vbarsegov/research/fibrin.html#the-structures-of-fibrin-oligomers-and-protofibrils. The structure of the αC-domain (amino acid residues Aα392-610) was constructed by the authors with the Modeller software using sequence homology with the structure of the bovine αC-domain (PDB code: 2JOR). The αC-domain models were used as initial structures in all-atom MD simulations. Energy minimization of the αC-domain was performed using the steepest descent algorithm, heated αC-domains to 300 K, and equilibrated the obtained structures for 1 μsec. Then the structures of the αC-domains were randomly selected from the last 500-ns portion of equilibrium simulations and incorporated into the structure of protofibrils. In coarse-grained modeling, the structures of the αC-domains were stabilized by

native contacts identified with Modeller [39].

2.2.12. Statistics

One-Way ANOVA was used to compare the means of independent groups in order to determine significant difference. Results are presented as means ± standard deviation calculated using Microsoft Excel (Microsoft, Redmond, USA). Data were considered significant when $P < 0.05$.

3. Results

3.1. Characterization of truncated forms of fibrinogen

The truncated forms of fibrinogen were obtained using proteases from the venom of *G. halys halys* and from the culture medium of *B. thuringiensis*. They were characterized by SDS-PAGE and Western blotting. SDS-PAGE analysis revealed that both forms had truncated Aα chains with the molecular masses of 54 and 43 kDa for fibrinogen desAα505-610 and fibrinogen desAα414-610, respectively (Fig. 1A and B). Western blot analysis using monoclonal antibody (mAb) II-5C specific to the fibrinogen Aα chain residues 20–78 [21] revealed that Aα chain truncation occurs at its C-terminal part (Fig. 1C and D). In addition, Western blot analysis with mAb 2D-2A specific to the fibrinogen Bβ chain residues 12–25 [22] confirmed that this chain was intact in both forms of fibrinogen (Fig. 1E).

Further, MALDI-TOF analysis of the purified products of fibrinogen's hydrolysis by the snake venom protease yielded a peak with *M/Z* ratio of 21,109. According to the Peptide Mass Calculator, this product of hydrolysis corresponds to the Aα414-610 peptide. MALDI-TOF analysis of the purified products of fibrinogen's hydrolysis by the protease from the bacterial culture medium revealed a peak with *M/Z* ratio of 11,441 which corresponds to the Aα505-610 peptide. Thus, this analysis further confirmed that the above mentioned proteases allowed us to obtain truncated forms of fibrinogen missing the Aα414-610 and Aα505-610 portions, i.e., fibrinogen desAα414-610 and fibrinogen desAα505-610, respectively [18,20]. In one of these forms, desAα414-610, practically the entire αC-domains were missing while the other form, desAα505-610, contained only about half of each αC-domain. These fibrinogen forms have been used in our subsequent study.

3.2. Effect of the fibrinogen Aα chain truncations on fibrin polymerization

To evaluate the role of the αC-domains in fibrin polymerization process, we measured changes in turbidity of fibrinogen solutions during thrombin-induced conversion of fibrinogen to fibrin. This method allowed us to evaluate the time which is required for protofibril formation (lag-stage), the rate of lateral association of protofibrils into fibers (exponential phase), and the final turbidity of the clot which characterizes the structure of polymerized fibrin. Both truncated fibrins, desAB-desAα505-610 and desAB-desAα414-610, demonstrated longer lag-stage in comparison to the native fibrin desAB tested in the same conditions (Fig. 2). Despite of this prominent delay in lateral aggregation of protofibrils, the rate of polymerization and the final turbidity of fibrin clots formed by the truncated fibrinogen forms were higher than those observed for native fibrin.

To further evaluate the effects observed with the truncated fibrin forms, we analyzed the structure of fibrin polymers using electron microscopy. Samples for electron microscopy were collected 300 s after addition of thrombin to native fibrinogen and its truncated forms in conditions used for turbidity measurements. As shown in Fig. 3A–C, both truncated fibrinogens formed thinner fibers than native fibrinogen. However, their fibrin clots were denser (Fig. 3 D–F). This may explain higher final turbidity of fibrin clots formed by truncated forms of fibrinogen observed in the turbidity study.

To evaluate the role of the αC regions in the intermolecular interactions, we selected fibrinogen instead of fibrin. While polymerization sites A and B are not available in the fibrinogen molecule, its αC regions can be involved in fibrinogen-to-fibrinogen interactions [40]. To study αC-αC interactions, we immobilized native fibrinogen on the surface of a sensor chip and tested its interaction with added native fibrinogen (control) and fibrinogens desAα505-610 and desAα414-610 using SPR (Fig. 4). The experiments revealed that the removal of the Aα505-610 portion from the αC regions decreased the ability of fibrinogen to bind to immobilized fibrinogen through αC-αC interactions by about 30% when compared to control. The loss of the Aα414–504 portion further decreased such ability by approximately 70% compared to control. These findings further confirm the importance of the αC-domains in the intermolecular fibrinogen-fibrinogen interactions that was reported earlier [41].

3.3. Effect of the fibrinogen Aα chain truncations on plasminogen activation

It is known that in fibrinolytic process fibrin is required for efficient activation of plasminogen by its activator, tissue-type plasminogen activator (tPA). To evaluate the involvement of the Aα414-504 and Aα505-610 portions of the αC-domain in fibrinolysis, we tested generation of active plasmin by tPA in the presence of truncated forms of fibrinogen. The Aα chains of fibrinogen desAα505-610 do not contain C-terminal Lys residues, which can support plasminogen activation [42],

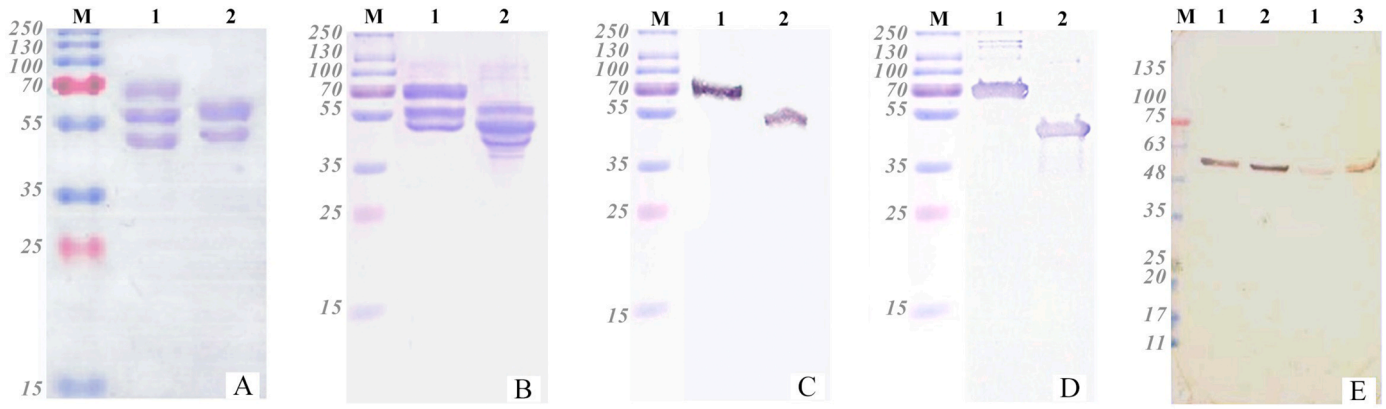

Fig. 1. SDS-PAGE and Western blot analyses of the truncated forms of fibrinogen. [A and B] SDS-PAGE analysis of fibrinogen desAα505–610 and fibrinogen desAα414–610, respectively. [C and D] Western blot analysis of fibrinogen desAα505–610 and fibrinogen desAα414–610, respectively, using mAb II-5C specific to the Aα chain residues 20–78. [E] Western-blot analysis of fibrinogen desAα505–610 and fibrinogen desAα414–610 using mAb 2D-2A specific to the Bβ chain residues 12–25. 1 – native fibrinogen; 2 - fibrinogen desAα505–610; 3 – fibrinogen desAα414–610; M – molecular mass markers.

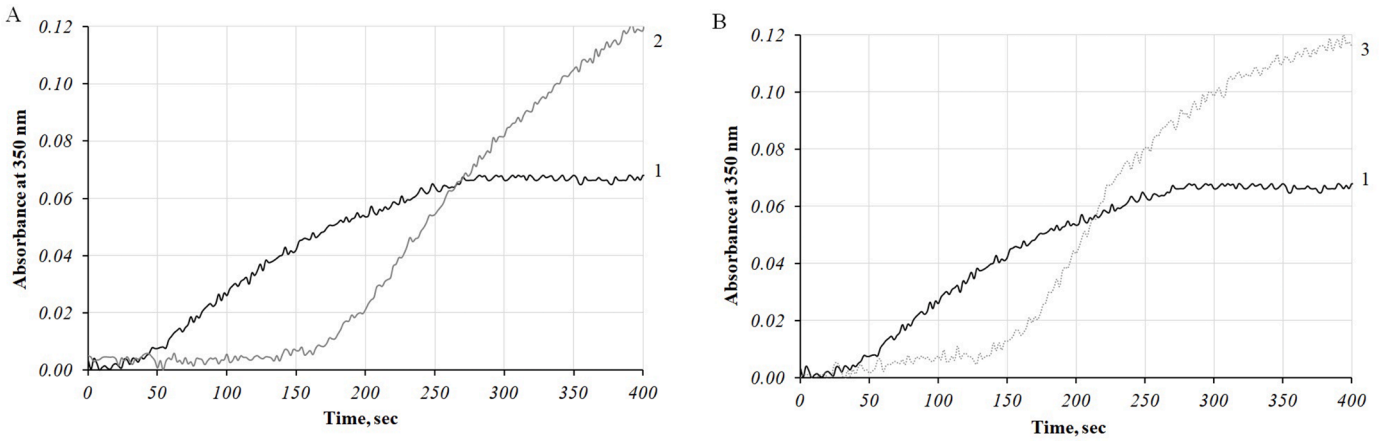

Fig. 2. The change in turbidity during thrombin-induced polymerization of native and truncated forms of fibrin. [A] Turbidity curves showing polymerization of fibrin desAB-desAα505–610 vs native fibrin desAB. [B] Turbidity curve showing polymerization of fibrin desAB-desAα414–610 vs native fibrin desAB. Data are representative of typical experiments ($n = 5$). 1 – native fibrin; 2 - fibrin desAB-desAα505–610; 3 – fibrin desAB-desAα414–610.

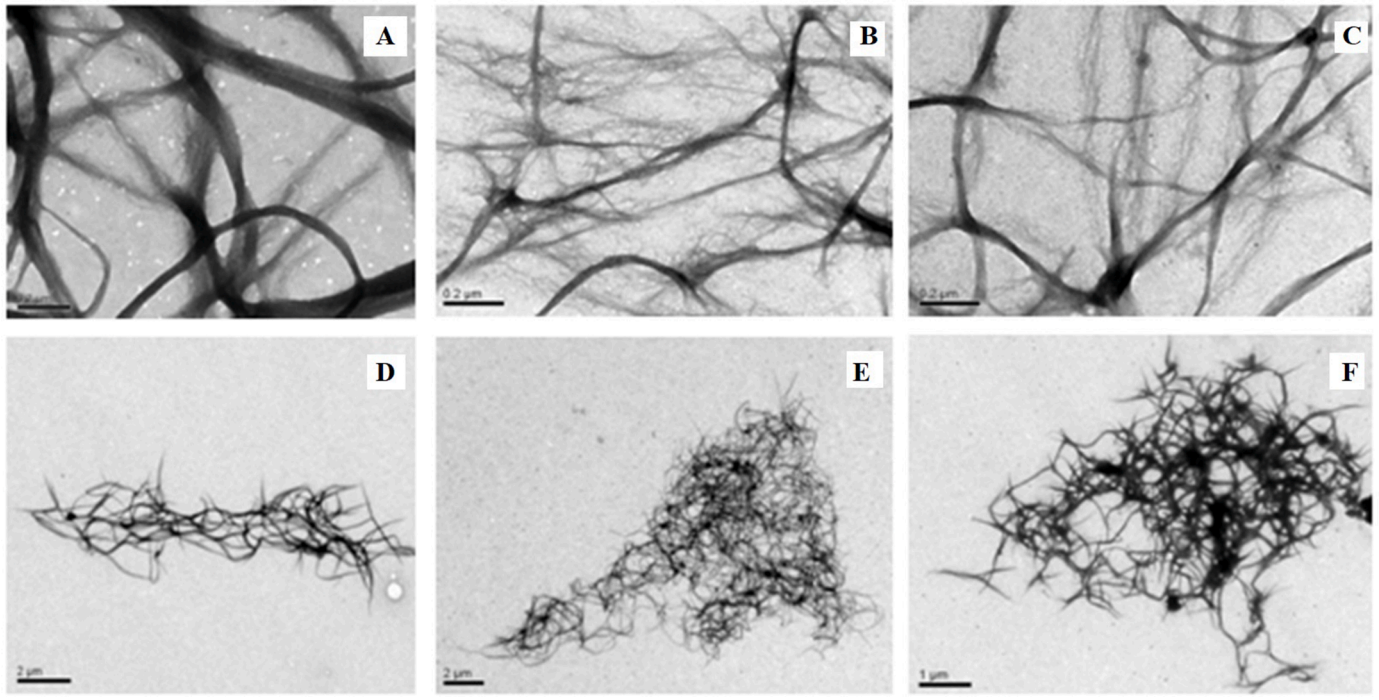

Fig. 3. Electron microscopy of fibrin clots formed by thrombin-induced polymerization of native and truncated forms of fibrin obtained at the different magnification. [A and D] Polymerized native fibrin desAB. [B and E] Polymerized fibrin desAB-desAα505–610. [C and F] Polymerized fibrin desAB-desAα414–610. Data represent typical experiments ($n = 5$). Scale bars: 0.2 μm in panels A-C and 2 μm in panels D-F.

while the Aα chains of fibrinogen desAα414-610 have such residues. To remove these Lys residues, fibrinogen desAα414-610 was treated with carboxypeptidase B. Fibrin desA and its truncated forms, desA-desAα414-610 and desA-desAα505-610, were generated *ex tempore* using thrombin-like enzyme Ancistron. Generation of active plasmin was monitored using specific chromogenic substrate S2251. Both truncated forms of fibrin exhibited lower ability to stimulate activation of plasminogen (0.044 μM) by tPA (3 IU/ml) compared to native fibrin desA (Fig. 5A). In the range of plasminogen concentrations close to saturation, this difference reached 20% (Fig. 5B). Importantly, the rates of plasmin generation on both truncated forms of fibrin were very close. This observation suggests that plasminogen- and tPA-binding sites involved in activation of fibrinolysis are located within the Aα505-610 portion of the αC-domain.

3.4. *Platelet aggregation in the presence of fibrinogens desAα505-610 and desAα414-610*

Since interaction of fibrinogen with platelet receptor $\alpha_{IIb}\beta_3$ plays an important role in platelet aggregation [43], we studied ADP-induced aggregation of washed human platelets in the presence of native and truncated forms of fibrinogen. In a typical experiment, we added fibrinogen or its truncated forms (final concentration of 1.5 mg/ml) to suspension of washed platelets in HEPES buffer. After 2 min of incubation under stirring, platelets were activated by the addition of ADP to a final concentration of 12.5 μM. The experiments revealed that platelet aggregation rate in the presence of fibrinogen desAα505-610 was decreased by 15 ± 5% compared to that in the presence of native fibrinogen; the speed of aggregation was practically the same for both

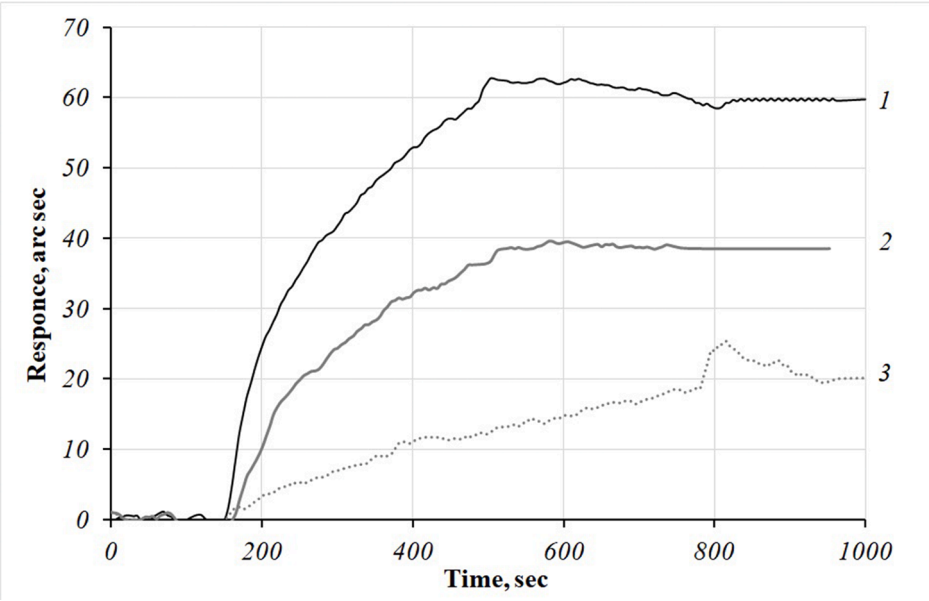

Fig. 4. Surface Plasmon resonance-detected interaction of immobilized native fibrinogen with fibrinogen desAα505–610 and fibrinogen desAα414–610. Data are representative of typical experiments ($n = 5$). 1 – native fibrinogen; 2 - fibrinogen desAα505–610; 3 – fibrinogen desAα414–610.

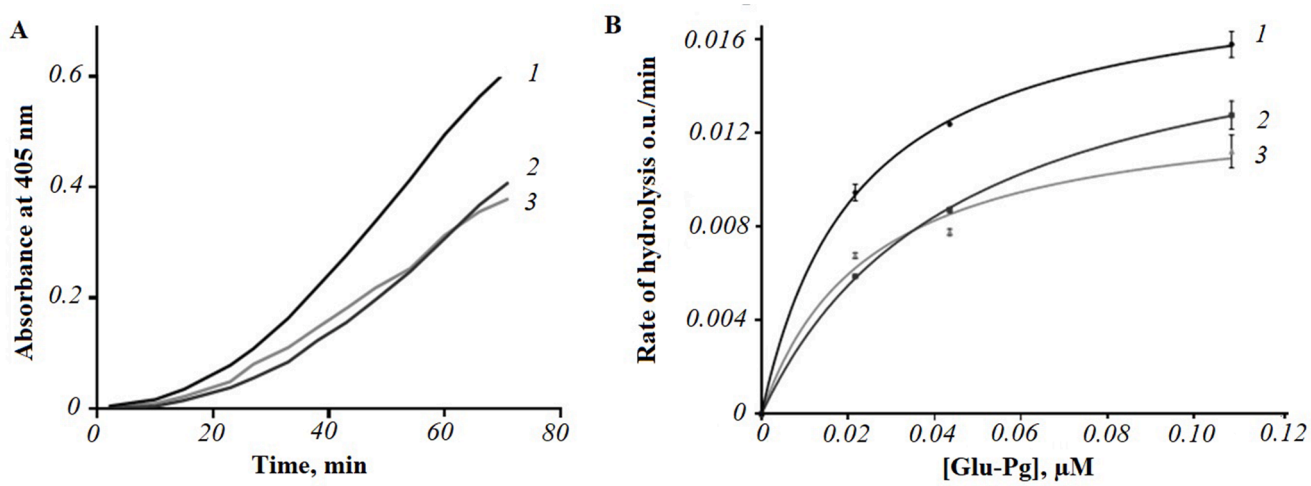

Fig. 5. Generation of plasmin from Glu-plasminogen by tissue-type plasminogen activator in the presence of fibrin desA (curves 1), fibrin desA-desAα414–610 (curves 2), and fibrin desA-desAα505–610 (curves 3). [A] Typical kinetic curves of plasminogen activation in the presence of fibrin and its truncated forms. Data are representative of typical experiments ($n = 5$). [B] The rate of hydrolysis of chromogenic plasmin-specific substrate S2251 at different concentrations of plasminogen (0-0.11 μM) and constant concentration of tPA (3 IU/ml) in the presence of fibrin and its truncated forms.

fibrinogens (Fig. 6A). At the same time, the loss of the Aα414-504 portions in fibrinogen desAα414-610 resulted in a significant reduction of its ability to support platelet aggregation. As shown in Fig. 6B, the rate of platelet aggregation decreased by 50 ± 9% and the speed of the process declined by 35 ± 7% compared to control fibrinogen. These findings suggest that the loss of Aα414-504 and Aα505-610 portions of the αC-domains in truncated fibrinogens reduced their ability to support platelet aggregation with the Aα414-504 portion to be more critical than the Aα505-610 one.

3.5. *Viability of endothelial cells grown on scaffolds of truncated variants of fibrin*

The involvement of fibrinogen and fibrin in the migration and proliferation of endothelial cells, as well as in the vascularization process [44,45], prompted us to compare the viability of endothelial cells grown on scaffolds of truncated forms of fibrin with that of native fibrin. Different forms of fibrin, fibrin desAB, fibrin desAB-desAα505–610, and fibrin desAB-desAα414–610, were obtained from fibrinogen and its truncated forms by incubation with thrombin with subsequent dissolution of fibrin clots in 0.125% acetic acid as described in Materials and methods. These forms of fibrin were adsorbed to the wells of 96-well cultural plates at the final amount of 40 μg/cm^2. After aspiration and washing wells with PBS, cell suspension was applied to the wells and cultivated as described in Methods section. Mouse aorta endothelial cells (MAEC) were chosen for these experiments. MTT-test was used for the analysis of cell viability after their cultivation on scaffolds formed by the truncated forms of fibrin in comparison to native fibrin. The experiments revealed that the loss of both C-terminal portions of the fibrinogen Aα chains substantially decreased the viability of MAEC grown on fibrin scaffolds (Fig. 7). No difference between fibrin desAB-desAα505–610 and fibrin desAB-desAα414–610 was found. These results suggest that

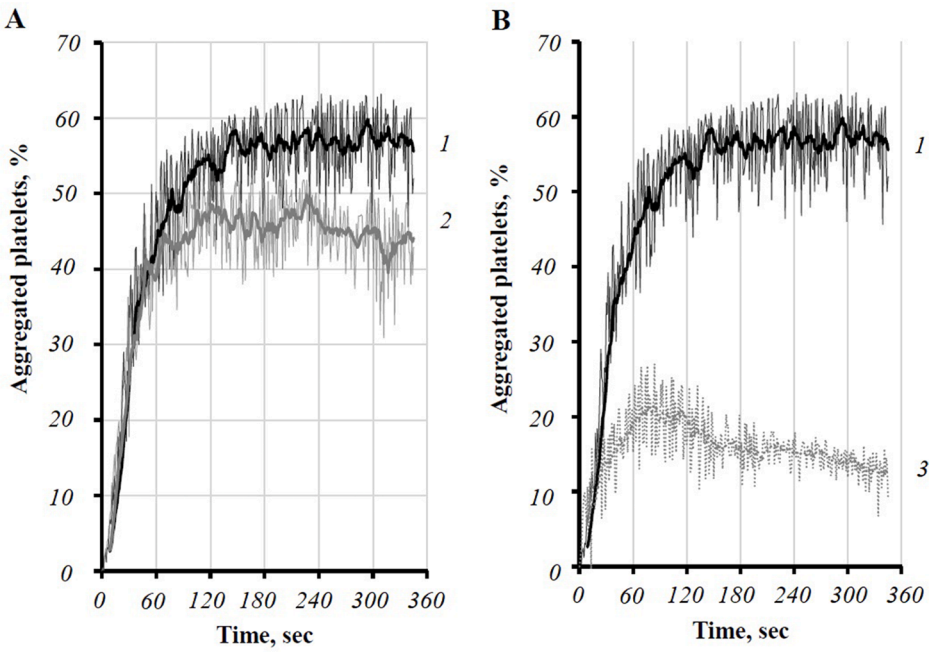

Fig. 6. Typical aggregation curves of washed platelets activated by ADP in the presence of native and truncated forms of fibrinogen. [A] Aggregation of washed platelets activated by ADP (12.5 μM) in the presence of native fibrinogen vs fibrinogen desAα505–610 (1.5 mg/ml). [B] Aggregation of washed platelets activated by ADP (12.5 μM) in the presence of native fibrinogen vs fibrinogen desAα414–610 (1.5 mg/ml). Data are representative of typical experiments ($n = 5$). 1 – native fibrinogen; 2 - fibrinogen desAα505–610; 3 – fibrinogen desAα414–610.

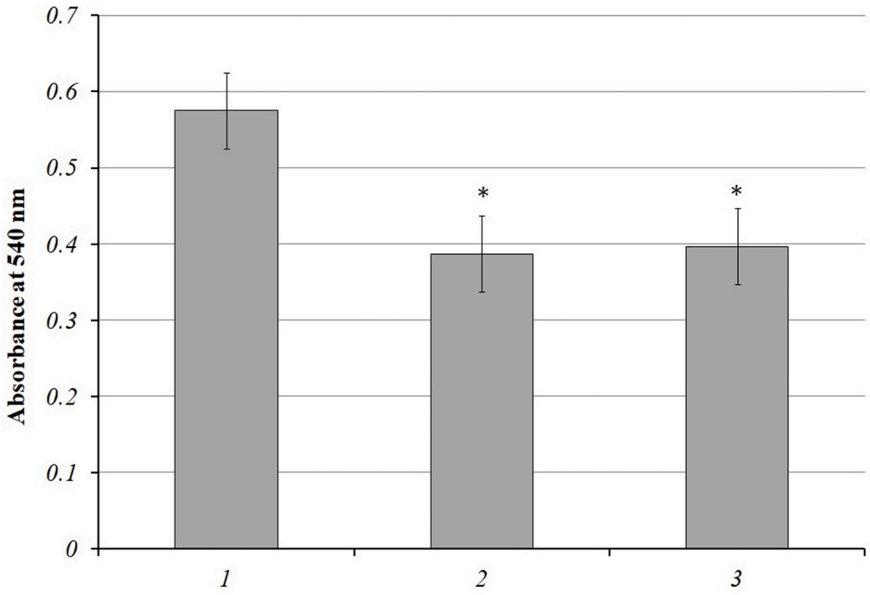

Fig. 7. Viability of mouse aorta endothelial cells (MAEC) cultivated on fibrin scaffolds according to the results of MTT-test. Cells were cultivated on fibrin desAB (1), desAB-desAα505–610 (2), and fibrin desAB-desAα414–610 (3). *$P \leq 0.05$ ($n = 8$).

interaction fibrin with endothelial cells is mediated mainly by the Aα505–610 portions of its αC-domains.

3.6. Migration of HeLa cells on truncated variants of fibrinogen

Having the sites for interaction with cancer cells and being the component of intracellular matrix, fibrinogen supports tumor growth and invasion [46]. To investigate the role of the Aα414–610 and Aα505–610 portions missing in the truncated forms of fibrinogen in the process of cancer cells migration, we utilized scratch-test. After forming the monolayer of HeLa cancer cells, we made a scratch with pipette tip and added to the culture media different forms of fibrinogen at the final concentration of 0.05 mg/ml. Cell migration to the wounded zone was observed at 12 and 24 h (Fig. 8). For the quantitative analysis of series of images ($n = 5$), we used ImageJ software. We calculated the number of cells migrated to the scratch-zone at 24 h after scratch. The results of quantification are presented in Fig. 9. It was found that truncated forms of fibrinogen supported cell migration less effectively compared to native fibrinogen. The calculated 10–15% impairment of the migration was statistically significant and correlated well with the data of MAEC viability impairment. As in the viability test on endothelial cells, we detected no difference between cell migration on fibrinogens lacking the Aα505-610 or Aα414-610 portions. These results suggest the greater importance of the Aα505-610 portions of the αC-domains for the interactions with endothelial cells.

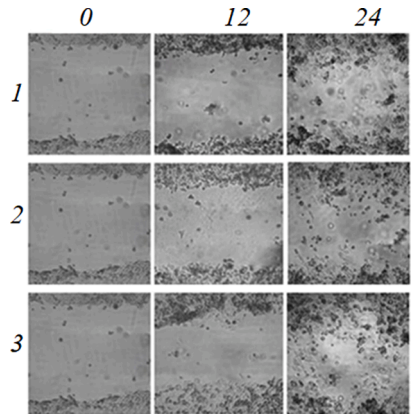

Fig. 8. Migration of HeLa cells to the scratch zone in the presence of native fibrinogen or its truncated forms after 0, 12, and 24 h of incubation. Magnification 1 × 40. 1 – native fibrinogen; 2 - fibrinogen desAα505–610; 3 – fibrinogen desAα414–610. Data are representative of typical experiments ($n = 5$).

4. Discussion

The use of proteases from the cultural liquid of *Bacillus thuringiensis* and from the venom of *G. halys halys* allowed us to obtain two truncated forms of fibrinogen, desAα505–610 and desAα414–610. It was suggested earlier that fibrinogen αC-domain (amino acid residues Aα392–610) consists of two compact sub-domains, N-terminal sub-domain and C-terminal one, formed by the N- and C-terminal halves of this domain, respectively [47]. While the structure of the N-terminal sub-domain has been solved [47,48] that of the N-terminal sub-domain was predicted to contain β-sheet structures [47]. We have constructed the structure of the complete αC-domain using approach described in Methods section. The structure revealed two distinct sub-domains with the cleavage site for the above mentioned proteases at the beginning of the N-terminal sub-domain and approximately in between the N- and C-terminal sub-domains (Fig. 10). Accordingly, fibrinogen desAα505–610 contained the N-terminal sub-domains while in fibrinogen desAα414–610 both sub-domains of the αC-domains were missing. Thus, these truncated forms of fibrinogen represent convenient models for studying functional properties of individual sub-domains of the αC-domains in fibrin(ogen)-mediated processes.

First, we tested the role of the N- and C-terminal sub-domains in fibrin polymerization process. Turbidity measurements revealed a significant delay in lag-stage for both truncated fibrinogens compared to the native one indicating that both sub-domains of the αC-domain contribute significantly to the process of protofibril formation at the early stage of fibrin polymerization. Further, electron microscopy study revealed that both truncated forms had thinner fibers compared to native fibrin highlighting a significant role of the αC-domains in lateral aggregation of protofibrils at the stage of fibrin fiber formation. These results are in a good agreement with the previous observations with other truncated variants of fibrinogen that have been recently reviewed [40].

Our SPR experiments revealed that native fibrinogen and its truncated forms bound to immobilized fibrinogen although binding of the truncated fibrinogens was less pronounced (Fig. 4). As was shown earlier, fibrinogen in solution can bind to adsorbed fibrinogen through the intermolecular interaction between their αC-domains [39]. It was also shown that formation of αC polymers in fibrin occurs mainly through intermolecular interactions between the N-terminal sub-domains of the αC-domains and between their C-terminal sub-domains and the αC-connectors [40,49]. Thus, the fact that fibrinogen desAα505–610 containing the N-terminal sub-domains binds to immobilized fibrinogen can be easily explained by the involvement of these sub-domains in the αC-αC interaction. As to fibrinogen desAα414–610, its interaction with immobilized fibrinogen was less pronounced than that of fibrinogen desAα505–610. Taking into account that this truncated fibrinogen devoid of both αC-domains but still contains the αC-connectors, its binding to fibrinogen most probably occurs through the interaction of its αC-connectors with the C-terminal sub-domains of immobilized fibrinogen. Thus this finding provides further experimental evidences for the proposed molecular mechanism of αC polymer formation in fibrin [40,48].

It was previously shown that the plasmin- and tPA-binding sites are cryptic in fibrinogen and are exposed in fibrin due to conformational changes during formation of fibrin polymers [50–52]. It is also known

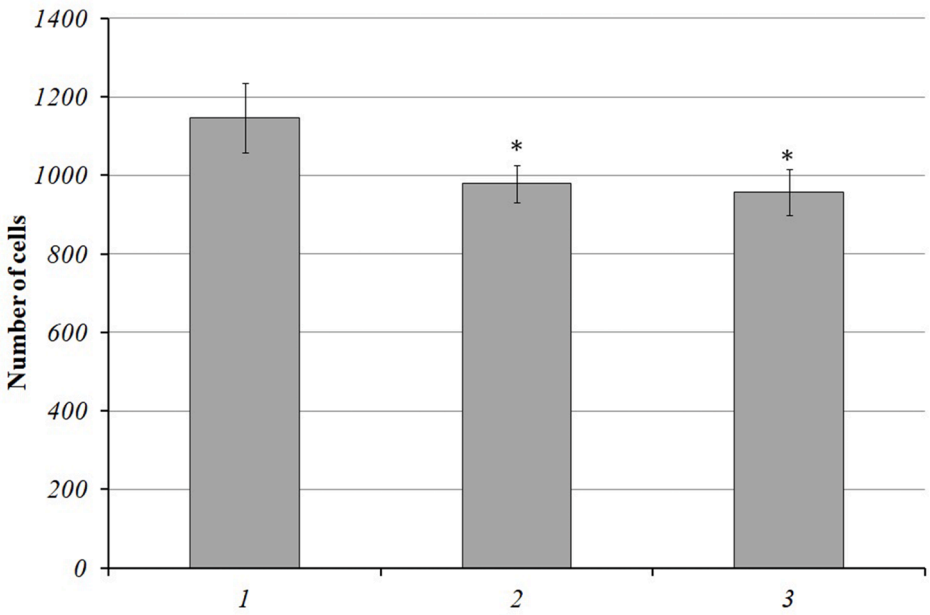

Fig. 9. Number of HeLa cells migrated to the scratch zone in wound-healing test in the presence of native fibrinogen or its truncated forms after 0, 12, and 24 h of incubation. Calculations were performed using ImageJ software. 1 – native fibrinogen; 2 - fibrinogen desAα505–610; 3 – fibrinogen desAα414–610. *$P \leq 0.05$ ($n = 8$).

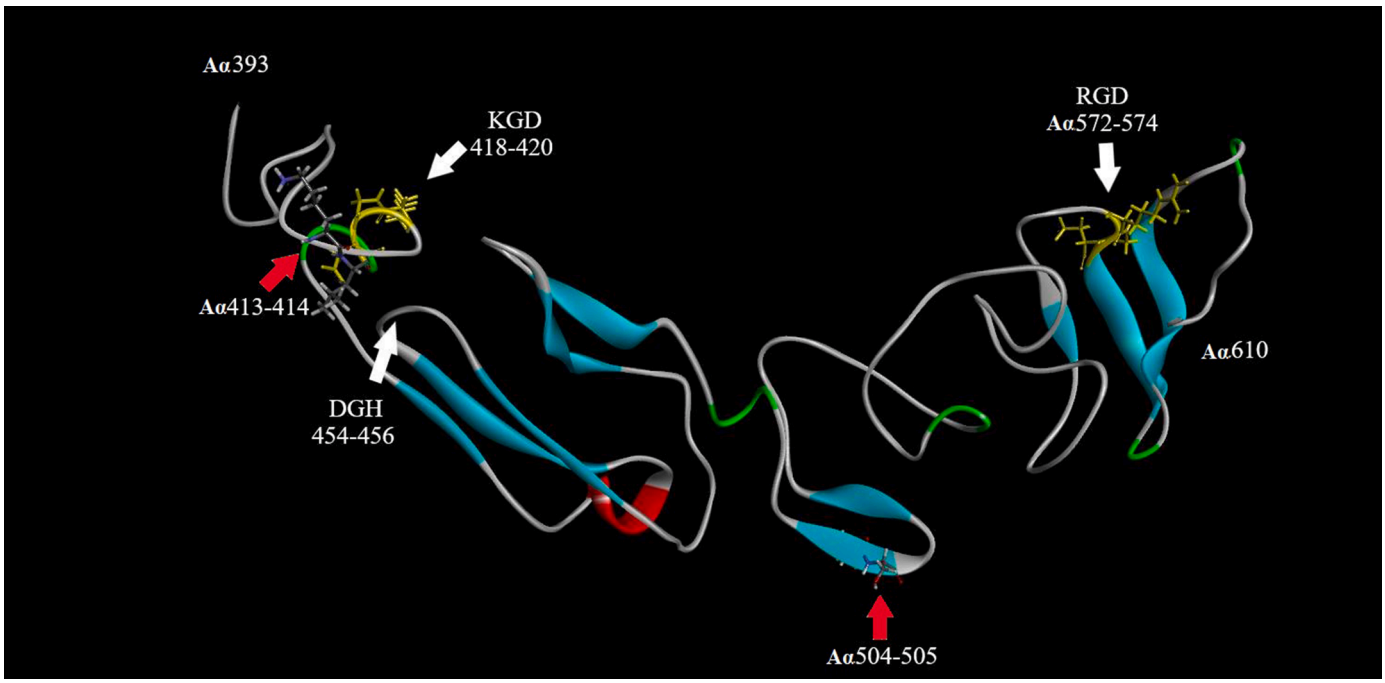

Fig. 10. Structure of the fibrinogen αC-domain constructed as described in the Methods section. Red arrows indicate cleavage sites by proteases from the cultural liquid of *Bacillus thuringiensis* (Aα504–505) and from the venom of snake *Gloydius halys* (Aα413–414). Potential sites of platelet receptors binding (Lys-Gly-Asp, Arg-Gly-Asp, and Asp-Gly-His) are shown by white arrows; β-sheets and α-helix are shown by blue and red ribbons, respectively.

that the major binding sites for plasminogen and tPA are located in the D regions of fibrin [51] and the additional binding sites for these components of fibrinolytic system are located in the αC-domains [51,53]. To evaluate the involvement of individual sub-domains of the αC-domains in the activation of fibrinolytic system, we studied generation of plasmin activity in the activation complex formed by plasminogen, tPA, and different forms of fibrin. Since both truncated forms of fibrin contained the major tPA- and plasminogen-binding sites, they both stimulated generation of plasmin although to the lesser extent than native fibrin due to the absence of their additional binding sites located in the αC-domains. Importantly, the stimulating effects of both truncated forms were very similar in spite of the absence of the N-terminal sub-domains in fibrinogen desAα414–610. This finding indicates that the tPA- and plasminogen-binding sites are located within the C-terminal sub-domains of the αC-domains.

Another process that strongly depends on fibrinogen is platelet aggregation. Previous studies revealed that fibrinogen γ chain residues γ400–411 and the Aα chain residues Aα95–97 and Aα572–574 containing Arg-Gly-Asp motif play an important role in platelet aggregation [1]. In our study, proteolytic removal of the Aα505–610 portions of the αC-domains, i. e. their C-terminal sub-domains, slightly suppressed the ability of fibrinogen desAα505–610 to support platelet aggregation (Fig. 6A), most likely due to the loss of the Aα572–574 Arg-Gly-Asp motif located in these sub-domains (Fig. 10). At the same time, we found that the ability of fibrinogen desAα414–610 to support platelet aggregation was significantly reduced (Fig. 6B). The most probable explanation for this finding is the following. It is known that Lys-Gly-Asp motif, which is located in the N-terminal sub-domains (Fig. 10), can interact with platelet receptor $\alpha_{IIb}\beta_3$ [54]; therefore, it is most likely that the absence of these sub-domains in fibrinogen desAα414–610 results in the significant reduction of platelet aggregation. Altogether, our experiments indicate that both sub-domains of the αC-domains are involved in platelet aggregation and the N-terminal sub-domains play a more significant role in this process.

Previous studies revealed that fibrin(ogen) interacts with endothelial cells through various receptors including the VLDL receptor, VE-cadherin, integrin receptors αvβ3 and α5β1, etc., and such interactions play an important role in fibrin-induced inflammation, angiogenesis, and other important physiological and pathological processes [40,55–57].. Our study revealed a significant decrease in viability of endothelial cells (MAEC) grown on both truncated forms of fibrin. Namely, the removal of the C-terminal domains from fibrin resulted in a significant decrease of the viability (Fig. 7). Importantly, further removal of the N-terminal sub-domains did not change this effect suggesting that interaction of fibrin with endothelial cells is mediated mainly by the C-terminal domains, most probably through their Arg-Gly-Asp-motif (Fig. 10). Similarly, the C-terminal sub-domains of the αC-domains play the major role in HeLa cell migration observed in our scratch test.

Despite the numerous intermolecular interactions of different sub-domains of αC-domains that were demonstrated, their exact role *in vivo* remains to be addressed [41]. It is important to underline that findings presented in the current work can contribute the understanding of the role of αC-domains in various (patho)physiological conditions accompanied by the disturbance of fibrin clot structure including clotting abnormalities or amyloid deposits [58].

5. Conclusion

In the present study, in which we utilized the truncated fibrin(ogen) forms lacking either its C-terminal sub-domains of the αC-domains or both the N- and C-terminal sub-domains, we distinguished the involvement of these sub-domains in several fibrin(ogen)-dependent processes. Specifically, the results obtained revealed that the N- and C-terminal sub-domains both play an important role in fibrin polymerization. These sub-domains also play an important role in platelet aggregation with the N-terminal sub-domains playing a more significant role in this process. At the same time, the C-terminal sub-domains make the major contribution to the plasminogen activation process, endothelial cell viability, and migration of cancer cells. These findings may contribute to the development of molecular effectors able to regulate such important physiological and pathological processes as blood coagulation, wound healing, and tumorigenesis.

CRediT authorship contribution statement

Y.M.S: manuscript preparation, methodology, preparation of fibrinogen forms, turbidity measurements and aggregometry; T.A.Y: fibrinolysis study; V.V.N: cell cultivation; Y.P.K: SPR study; O.O.H: visualization and data analysis; O.Yu.S: chromatography, protein purification; K.S.S: MTT test, cell migration test; L.V.G: endothelial cells cultivation; L.D.V: preparation of the proteases; A.O.T: fibrinolysis study; V.O.C: methodology, concept of the article, review and editing, supervision.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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