

## AGGREGATION OF PLATELETS, PROLIFERATION OF ENDOTHELIAL CELLS AND MOTILITY OF CANCER CELLS ARE MEDIATED BY THE B $\beta$ 1(15)-42 RESIDUE OF FIBRIN(OGEN)

Y. M. STOHNII<sup>1</sup>, M. V. RYZHYKOVA<sup>1</sup>, A. V. REBRIEV<sup>1</sup>,  
M. D. KUCHMA<sup>2</sup>, R. Y. MARUNYCH<sup>1</sup>, V. O. CHERNYSHENKO<sup>1</sup>✉,  
V. A. SHABLI<sup>2</sup>, N. M. LYPOVA<sup>3</sup>, O. Yu. SLOMINSKYI<sup>1</sup>,  
L. V. GARMANCHUK<sup>4</sup>, T. M. PLATONOVA<sup>1</sup>, S. V. KOMISARENKO<sup>1</sup>

<sup>1</sup>Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv, Ukraine;

<sup>2</sup>Institute of Cell Therapy, Kyiv, Ukraine;

<sup>3</sup>University of Louisville, USA;

<sup>4</sup>ESC "Institute of Biology and Medicine", Taras Shevchenko National University of Kyiv, Ukraine;

✉e-mail: [bio.cherv@gmail.com](mailto:bio.cherv@gmail.com)

**Received:** 23 December 2019; **Accepted:** 27 March 2020

The fibrinogen molecule contains multiple binding motifs for different types of cellular receptors, acting as a molecular link between coagulation and cell adhesion. In this study we generated a truncated form of the fibrinogen molecule lacking the B $\beta$ 1-42 sequence by site-specific proteolysis and evaluated the role of the fragment in adhesive capabilities of platelets, endothelial and cancer cells. Fibrinogen with the removed B $\beta$ 1-42 sequence and fibrin without the B $\beta$ 15-42 fragment (desB $\beta$ 1-42 fibrinogen and desAB $\beta$ 15-42 fibrin) were obtained by proteolysis using the specific protease from the venom of *Echis multisquamatis*. The cleaved fragment was purified by HPLC and was identified using MALDI-TOF. ADP- and collagen-induced aggregation of washed platelets in the presence of fibrinogen desB $\beta$ 1-42 was studied using an aggregometer. Proliferation of mice aortic endothelial cells (MAEC) and human umbilical vein endothelial cells (HUVEC) was studied using the fibrin desAB $\beta$ 15-42 as the scaffold. Cell viability was quantified by the MTT test (MAEC). Generation time was calculated for the estimation of proliferative activity of HUVEC. Lung cancer cell line H1299 was used to evaluate cancer cell motility in vitro using the scratch assay. Direct comparison of cellular behavior in the presence of truncated vs native forms demonstrated attenuated cell adhesion in the presence of fibrinogen desB $\beta$ 1-42 and fibrin desB $\beta$ 15-42. The platelet aggregation rate was only slightly decreased in the presence of fibrinogen desB $\beta$ 1-42 but resulted in 15-20% disaggregation of adhered platelets. We also observed the substantial decrease of generation time of HUVEC and inhibition of viability of MAEC cells grown on scaffolds of a desAB $\beta$ 15-42 matrix. Finally, desB $\beta$ 1-42 modulated the motility of H1299 cells in vitro and suppressed the wound healing by 20% compared to the full-length fibrinogen. We postulate that fragment 1-42 of the B $\beta$ N-domain of fibrinogen is not sufficient for platelet aggregation, however it may contribute to platelet clot formation in later stages. At the same time, this fragment may be important for establishing proper cell-to-cell contacts and cell viability of endothelial cells. Also, 1-42 amino acid fragment of the B $\beta$ N-domain supported the migration of cancer cells suggesting that interactions of fibrinogen with cancer cells could be a target for anticancer therapy. The B $\beta$ 1-42 fragment of fibrinogen contributes to efficient intracellular interactions of different types of cells, including platelets, endothelial cells and cancer cells.

**Key words:** fibrinogen, adhesion, cell migration, endothelium, cell proliferation, platelets.

## Introduction

Fibrinogen is a large glycoprotein composed of three pairs of polypeptide chains bound by disulfide bonds into the complex structure represented by two peripheral D-regions, central E-region and flexible  $\alpha$ C-region [1]. The amino acid composition of the whole fibrinogen molecule is A $\alpha$ 1-610, B $\beta$ 1-461 and  $\gamma$ 1-411. Each chain is presented in duplicate.

Because of its size and high concentration in blood plasma (2-3 mg/ml) fibrinogen can modulate various physiological processes apart from blood coagulation. In particular it can interact with various biological surfaces, binding non-specifically to glycocalyx and specifically to biological membranes mainly through integrin receptors. The fibrinogen molecule contains several active sites that can interact with cellular integrins, most of them were studied as ligands of platelet integrin IIbIIIa. The first of these are the C-terminal polypeptides of both  $\gamma$ -chains ( $\gamma$ 400-411) and RGD-residues of the A $\alpha$ -chains ( $\alpha$ 95-98,  $\alpha$ 572-574) [2, 3]. Another site that is important in fibrinogen interactions with IIbIIIa is the fragment  $\gamma$ 312-324 that is hidden in the fibrinogen molecule and exposed after its conversion to fibrin [4]. An important feature of fibrinogen binding to platelets is the role of platelet activation in this process. Fibrinogen cannot bind to non-activated platelets and interacts only with stimulated cells [5].

Among other parts of the fibrinogen molecule, the B $\beta$ N-domains (B $\beta$ 1-60) are supposed to interact with platelets [6]. In particular, peptides that are structural analogues of B $\beta$ 15-42 residues of fibrinogen were shown to inhibit platelet aggregation [7]. These residues are also important for the spreading of platelets [8] but their role in platelet adhesion and aggregation is rather controversial.

At the same time, B $\beta$ 15-42 residues of B $\beta$ N-domains can directly bind to VE-cadherin of endothelial cells (EC) [9, 10]. This finding was confirmed in several studies and is supposed to be an important mechanism of the stabilization of the endothelial monolayer during inflammation [11, 12]. Another site of EC interactions with fibrinogen are very low-density lipoprotein receptors (VLDLR).

They also occur through B $\beta$ 15-42 residues but have a rather regulatory role not contributing to the stability of interactions [13, 14].

EC as well as numerous other cells were shown to interact with the fibrinogen residue  $\gamma$ 117-133 through intracellular adhesion molecule type 1 (ICAM-1) receptors [15]. Among other cells interacting with fibrinogen in an ICAM-1 dependent manner are leucocytes [16] and various types of cancer cells.

Fibrinogen is a component of the extracellular matrix and can regulate cancerogenesis and metastasis [17]. Binding to fibrinogen through ICAM-1 was shown for several types of cancer cells [18, 19]. Several studies indicate a regulatory role of this kind of interaction [20].

Here we would like to emphasize that different parts of the fibrinogen molecule can interact with cellular receptors more or less specifically, sometimes regulating the activity of different types of cells. Identification of the sites of fibrinogen that are important for molecular recognition provides not only information about mechanisms of cellular adhesion, but can also aid in the detection of targets for modulation of such important biological processes as thrombosis, atherogenesis or cancerogenesis.

In this work we focused on B $\beta$ N-domains of fibrinogen by studying their role in intracellular interactions of platelets, EC and cancer cells (lung cancer line H1299). To achieve this goal we removed the B $\beta$ 1-42 peptide of the fibrinogen molecule using a specific protease and studied the changes in behavior of cells that interacted with this truncated form of fibrinogen. We studied aggregation of platelets, proliferation of EC and migration of cancer cells.

## Materials and Methods

*Fibrinogen.* Fibrinogen was purified from blood plasma of healthy donors by the method of Belitser & Varetska, its purity and clottability were controlled as previously described [21]. The procedure of blood collection was performed under the license of National Military Medical Clinical Center "Main Military Clinical Hospital" (Kyiv, Ukraine).

---

**Abbreviations:** EC – endothelial cells; VLDLR – very low density lipoprotein receptors; ICAM-1 – intracellular adhesion molecule type 1; HUVEC – human umbilical vein endothelial cells; MAEC – mouse aortic endothelial cells; TBS – 0.05 M Tris-HCl buffer of pH 7.4 with 0.13 M NaCl; ADP – adenosine diphosphate; MTT – 3-(4, 5-diethylthiazoly-2-yl)-2,5-diphenyltetrazolium bromide; FBS – fetal bovine serum, BSA – bovine serum albumin; DMEM – Dulbecco's Modified Eagle's medium; FGF – fibroblast growth factor.

*Monomeric fibrin desA and desAB.* Fibrin desA and desAB were prepared from human fibrinogen. Fibrin polymerization was initiated by 0.75 NIH/ml thrombin-like enzyme from the venom of *Agkistrodon halys halys* for fibrin desA and 0.5 NIH/ml thrombin for fibrin desAB [22]. In the case of fibrin desAB, 0.1 ml of 40 mM monoiodoacetic acid was added to the sample to avoid fibrin cross-linking. Each mixture was incubated for 30 min at 37 °C. The fully formed clot was removed from the incubation mixture by a glass stick, washed and re-dissolved in 0.125% acetic acid.

The truncated form of monomeric fibrin desA was prepared from human fibrinogen that was truncated by protease. Protease from the venom of *Echis multisquamatis* (0.01 mg/ml) was added to fibrinogen (15 mg/ml) and incubated in 0.05 M Tris-HCl buffer of pH 7.4 with 0.13 M NaCl (TBS) for 45 min at 25 °C. Fibrin polymerization was induced by 0.5 NIH/ml thrombin and prepared as described above for fibrin desAB.

*Washed platelets.* Venous blood of healthy volunteers ( $n = 5$ ) who had not taken any medication for 7 days was collected by venipuncture of the basilic vein using a 19G sterile needle. Blood was collected into sterile plastic 10 ml tubes where it was mixed immediately with 38 g/l sodium citrate (9 parts blood to 1 part sodium citrate). Blood was spun down at 160 g for 30 min at 25 °C. Platelet-rich plasma was collected and spun down again at 300 g for 15 min. The pellet of platelets was re-suspended in 0.004 M HEPES, 0.137 M NaCl, 0.0027 M KCl, 0.001 M MgCl<sub>2</sub>, 0.0056 mM glucose, 0.003 M NaH<sub>2</sub>PO<sub>4</sub>, 0.35 mg/ml bovine serum albumin (BSA), pH 7.4 to obtain a homogenous suspension of washed platelets as previously recommended [23].

Patients signed informed consent prior to blood sampling. This study was approved by the Ethics Committee of Palladin Institute of Biochemistry (03.09.2015, N7).

*Human umbilical vein endothelial cells (HUVEC).* Umbilical cord sampling was performed after the delivery. All birthing mothers signed informed consent prior to delivery. All manipulations were conducted under the license of the Ministry of Health of Ukraine given to the Institute of cell therapy, Kyiv, Ukraine. The umbilical vein was washed and poured with 0.1% solution of collagenase in PBS and incubated for 30 min at 37 °C. The obtained suspension was spun down and cultivated in Dulbecco's Modified Eagle's medium (DMEM) with 15% fetal

bovine serum (FBS, Gibco, Dublin, Ireland) 5 mM HEPES, 2 mM L-glutamine, and 20 ng/ml fibroblast growth factor (FGF, Biochrom, Cambridge, UK) in Petri dishes. After 2 passages HUVEC were seeded on plates covered with fibrin scaffolds or untreated plates (controls). Cells were cultivated during 6 passages. To confirm that we obtained endothelial-derived cells we used flow-cytometry with specific labeled antibodies (Becton Dickinson, Franklin Lakes, USA): anti-CD34 APC, anti-CD90 FITC, anti-CD45 APC-Cy7, anti-CD105 PerCP-Cy 5.5, anti-CD73 PE, and anti-CD31 PE. Analysis was performed on the laser flow-cytometer BD FACSAria (Becton Dickinson) using the software FACSDiva 6.1.2. (Becton Dickinson) Immunophenotyping demonstrated that cells grown on all studied plates belonged to the HUVEC type having a phenotype CD31+CD105+CD73+CD90-CD34-CD45.

*Mouse aortic endothelial cells (MAEC).* MAEC (ThermoFisher Scientific, Waltham, USA) were grown on 96-well plates covered with fibrin scaffolds as described above, in Iscove's modified Dulbecco's medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% FBS and gentamicin (50 µg/ml). All cells were incubated at 37 °C in 5% CO<sub>2</sub>.

*Cancer cell culture.* The lung cancer H1299 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). H1299 cells were cultured in RPMI medium (Invitrogen Waltham, USA) supplemented with 10% FBS (Clontech, Kyoto, Japan) and 50 µg/ml gentamicin (Life Technologies Carlsbad, USA). Cells were incubated at 37 °C with 5% CO<sub>2</sub>.

*Chemicals and reagents.* Adenosine diphosphate (ADP), dimethyl sulfoxide, MTT reagent, benzamidine, thrombin, and Superdex G-75 were purchased from Sigma-Aldrich. Collagen was purchased from RenaU (Kharkiv, Ukraine). Chromogenic substrate S2238 and C18 reversed phase HPLC columns were purchased from ThermoFisher Scientific. Anti-CD34 APC, anti-CD90 FITC, anti-CD45 APC-Cy7, anti-CD105 PerCP-Cy 5.5, anti-CD73 PE, and anti-CD31 PE were purchased from Becton Dickinson. All other reagents were of chemical grade provided by local suppliers.

*SDS-PAGE.* The molecular weights and purity of proteins were determined by SDS-PAGE using 10% or 12% gels according to Laemmli [24]. Hydrolysis products of fibrinogen and fibrin by fibrinogenase were also analysed by SDS-PAGE under reducing conditions.

**FPLC chromatography.** The mixture of fibrinogen (15 mg/ml) and protease (0.01 mg/ml) was incubated for 45 min in 0.05 M Tris-HCl buffer of pH 7.4 with 0.13 M NaCl (TBS) at room temperature. The reaction of hydrolysis was then stopped by addition of benzamidine at a final concentration 0.016 M. The mixture was gel-filtered through a Superdex G-75 column using the FPLC system ÄKTA (GE Healthcare, Pharmacia, Pittsburgh, USA). The column volume was 60 ml, with a flow rate of 2 ml/min. Fractions that contained proteins or benzamidine were detected at 280 nm using the Spectrophotometer Optizen-POP (Optizen, Daejeon, Korea).

**Proteinase activity detection.** Activity of proteinase in the volume eluted from the Superdex G-75 column was detected using synthetic chromogenic substrate S2238 – H-D-Phe-Pip-Arg-pNA [25]. An aliquot of each fraction (100 µl) was added to the well of a 96-well plate and mixed with 0.1 mM of S2238 in 0.05 M Tris-HCl buffer of pH 7.4 containing 0.13 M NaCl. Hydrolytic activity was continuously monitored at 405 nm [26].

**HPLC chromatography.** The chromatographic system Agilent 1100 (Agilent, Santa Clara, USA) was used for analysis of peptide fractions collected from the Superdex G-75 column with the C18 reversed phase HPLC column (ThermoFisher Scientific) at a pressure of 140 bar and flow rate of 1.5 ml per min. During 80 min, we used an increasing gradient of acetonitrile from 0 to 100% against buffered saline (0.15 M Tris HCl, pH 6.5, 0.13 M NaCl).

**MALDI-TOF.** MALDI-TOF analysis of purified peptide derived from fibrinogen was performed using a Voyager-DE (Applied Biosystems, Waltham, USA). H<sup>+</sup>-matrix ionization of polypeptides with sinapine acid (Sigma-Aldrich) was used. Results were analyzed by Data Explorer 4.0.0.0 (Applied Biosystems) [27].

**Aggregometry.** Platelet aggregation measurements were based on changes in the turbidity of human platelet-rich plasma [28]. Studies were performed on the Aggregometer Solar AP2110 (SOLAR, Minsk, Belorussia) according to the recommendations of the manufacturer. Washed platelets (200 µl) were added into the sample tube, mixed with 50 µl of fibrinogen (9 mg/ml) and activated by addition of 25 µl CaCl<sub>2</sub> (0.025 M) and 25 µl of platelet agonist ADP (25 µM). Aggregation was registered for 5 min at 37 °C.

**Calculation of generation time.** For the characterization of culture growth we calculated the time

of generation according to the formula: “Generation time” = “Time of culture growth”/“duplication of cells”, where “duplication of cells” =  $\ln(\text{“number of collected cells”}/\text{“number of seeded cells”})/\ln 2$ .

**MTT test.** Cell viability was measured by 3-(4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated in 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-Aldrich) at 37°C for 4 h followed by solubilization with dimethyl sulfoxide (Sigma-Aldrich). The absorbance at 540 nm was measured with a microplate reader [29].

**Scratch wound-healing test.** The scratch wound-healing assay was applied as previously described [30]. It is a widely used approach for estimating cell migration, and is especially useful for the study of migration of cancer cells [31]. Briefly,  $4 \times 10^5$  cells were seeded into 24-well cell culture plates and allowed to grow to form a confluent monolayer (24 h). On the next day, the cells were serum-starved in RPMI medium containing 0.5% FBS for 24 h prior to treatment. The monolayer was gently scratched with a sterile pipette tip to create a “scratch” 1 cm long. The wells were washed 2 times with 1 ml of the growth medium to remove the debris and smooth the edge of the scratch. Lyophilized fibrinogen was reconstituted in 50 mM Tris, 130 mM NaCl, pH 7.5 prior to the treatment. Fresh medium 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. The wound area was measured and the number of migrated cells were counted using ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA). The number of cells that migrated into a zone of scratching was normalized to the vehicle-treated wells (no fibrinogen).

**Statistical analysis.** Results are presented as means ± standard deviation, calculated using Microsoft Excel (Microsoft Corp., Redmond, USA). Data were considered significant for  $P < 0.05$ .

## Results

**Preparation of fibrinogen desBβ1-42.** Proteolytic enzyme from the venom of *Echis multisquamatis* was previously described as the fibrinogenase

targeted to N-terminal portions of B $\beta$ -chains of human fibrinogen [32]. We incubated pure human fibrinogen (15 mg/ml) with protease (0.01 mg/ml) in TBS at room temperature and detected the compositions of polypeptide chains of fibrinogen using SDS-PAGE. After 45 min 90% of B $\beta$ -chains were cleaved and we detected the larger part of the remnant chain in the same zone as the  $\gamma$ -chain of fibrinogen (Fig. 1, A). This composition of mixture and time of incubation was selected for preparative hydrolysis. The reaction was terminated by the addition of 0.016 M of benzamidine (an inhibitor of serine proteases) and inhibited the activity of the protease we used [33].

After incubation and addition of inhibitor, the mixture was fractionated using size-exclusion chromatography on a Superdex G-75 column. As shown in Fig. 1, B, we obtained a huge fraction of truncated fibrinogen (fraction 5) and detected the elution of benzamidine by its absorbtion at 280 nm (fraction 6). We also used chromogenic substrate assay to detect the presence of proteinase in the fractions. The content of proteinase was too small for detection using optical density measurement but was enough for the detection of specific enzyme activity. Fractions from 60 ml to 80 ml of eluted volume contained different amounts of protease (fraction E). We collected whole eluted volume between fraction 5 and fraction 6 in an attempt to collect the fraction of peptides cleaved from fibrinogen by protease (fraction 7).

The collected fraction 7 was then further purified using HPLC-chromatography on a C-18 column. The major polypeptide peak was eluted by an increasing gradient of acetonitrile and collected for mass spectrometry (Fig. 2, A).

The performed MALDI-TOF analysis allowed us to detect the peak with the mass to charge ratio 4589 that corresponded to the polypeptide with molecular weight 4589 Da (Fig. 2, B). According to the software program "Peptide Mass Calculator" (<http://www.peptidesynthetic.co.uk/tools/>), the peptide with the molecular weight corresponded to the peptide "s qgvndneegffsarghrpldkkreeapslrpappppisgg-yr", which was formed after cleavage of the peptide bond B $\beta$ R42-A43. Thus, we confirmed that by using the limited proteolysis of fibrinogen by proteinase from the venom of *Echis multisquamatis*, we obtained the truncated form of fibrinogen – desB $\beta$ 1-42 that was used in further studies.

*Study of platelet aggregation.* Platelet aggregation is strongly dependent on fibrinogen content and can be impossible without fibrinogen. To study the

peculiarities of platelet aggregation in the presence of control fibrinogen or its truncated form desB $\beta$ 1-42 we obtained the washed human platelets re-suspended in HEPES buffer. To control samples of platelet suspensions we added 1.5 mg/ml of control fibrinogen, to the studied samples an equal amount of fibrinogen desB $\beta$ 1-42 was added. Platelet suspensions were incubated with fibrinogen in an aggregometer tube for 2 min and then platelets were activated by the addition of ADP or collagen as the most common agonists that act in a different manner. ADP is the agonist for P2Y1 and P2Y12 receptors that directly induce aggregation of platelets [34]. Collagen acts through the integrin receptor GPVI and induces platelet degranulation with following aggregation that occurs mainly under the granules constituent action [35].

We demonstrated that in both cases the initial rate of platelet aggregation did not differ (Fig. 3). ADP-stimulated aggregation was completely normal and achieved a maximal rate of 35% in the presence of native fibrinogen and in the presence of fibrinogen desB $\beta$ 1-42. However constant mixing of sample in the tube for aggregometry led to the dissociation of platelet aggregates and disaggregation of platelets in the sample with the truncated form of fibrinogen (Fig. 3, A).

We observed the same trace in the case of collagen-induced platelet aggregation (Fig. 3, B). Here we observed not only disaggregation of platelets following maximal aggregation, but also a decreasing of the maximal aggregation rate on 10%. Thus, we can conclude that the residue B $\beta$ 1-42 of the fibrinogen molecule is not important for platelet aggregation but can increase the stiffness of the fibrin-platelet clot whether by forming additional points of contact between platelet receptors and fibrinogen or by stabilizing the structure of fibrinogen in the clot.

*Study of proliferation of EC.* Another important cellular component of the hemostasis involved in thrombus formation is EC. Fibrinogen is an important factor that mediates binding of other cells to EC, and also provides a scaffold for smooth muscle cells and EC during vessel tissue repair [36].

Here we studied two types of endothelial cells – primary HUVEC culture and immortalized PAE cell culture. Both types of cell were grown on the scaffolds formed with monomeric fibrin derived from control or truncated fibrinogen. For this the surface of the culture plate with a 35 mm diameter was covered by PBS. Then an equal volume of fibrino-

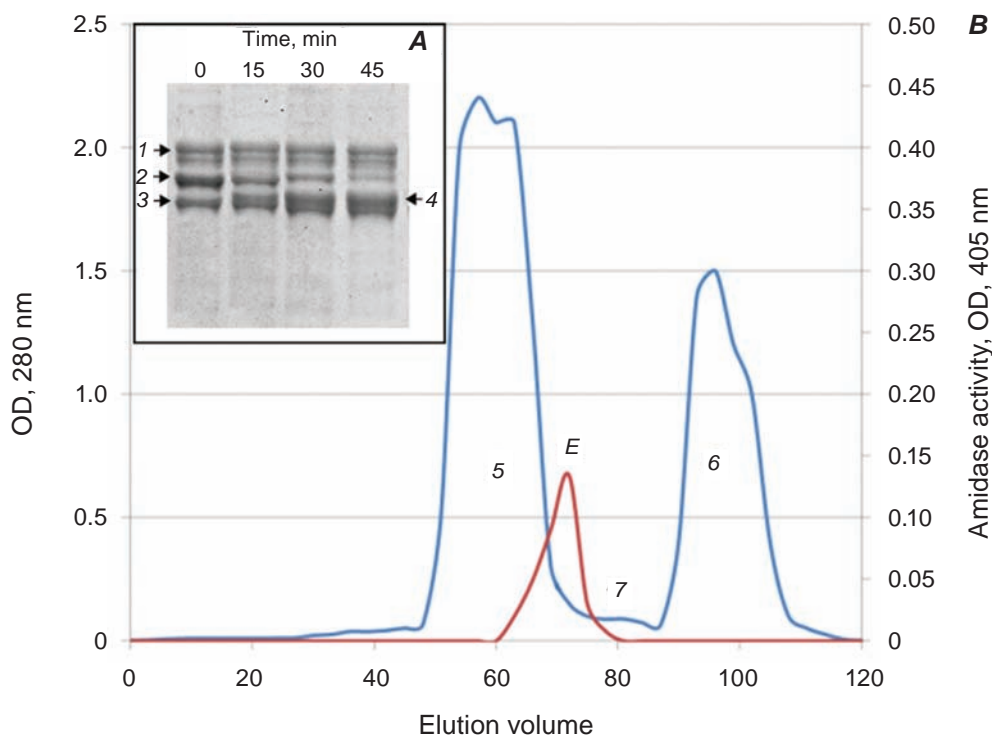


Fig. 1. Fibrinogen (15 mg/ml) digested by 0.010 mg/ml of fibrinogenase from the venom of *Echis multisquamatis*. **A** – SDS-PAGE of fibrinogen digested by fibrinogenase from the venom of *Echis multisquamatis* before and after 15, 30 and 45 min of digestion. 1, 2, 3 –  $\alpha$ -,  $\beta$ - and  $\gamma$ -chain of fibrinogen, respectively. 4 – digested  $\beta$ -chain of fibrinogen. **B** – Elution of fibrinogen from Superdex G-75 column after incubation with the enzyme. Fractions: 5 – truncated fibrinogen; 6 – benzamidine; 7 – peptide zone; E – enzyme; OD – optical density

gen solution in 0.125% acetic acid was added to the plate to reach a final concentration of 0.386 mg/ml. Monomeric fibrin is insoluble at neutral pH so it was oligomerized and adhered to the surface of plate at the final concentration of 35  $\mu$ g/ml.

In our studies we compared the generation time of HUVEC calculated for the different passages on the scaffolds formed with fibrin desAB (peptide composition  $\alpha$ 17-610; B $\beta$ 15-461;  $\gamma$ 1-411), fibrin desA ( $\alpha$ 17-610; B $\beta$ 1-461;  $\gamma$ 1-411) and fibrin desAB $\beta$ 15-42 ( $\alpha$ 17-610; B $\beta$ 43-461;  $\gamma$ 1-411).

It was demonstrated that the generation time during the cultivation on fibrin desA and desAB was constant during the entire study. At the same time, cultivation on fibrin desAB $\beta$ 15-42 led to the prolongation of generation time that indicated slower duplication of cells and less efficient growth of the culture of HUVEC (Fig. 4, A). These observations were obvious in the images obtained using inverted microscopy (Fig. 4, B).

Another model we used for studying the effect of the fibrinogen B $\beta$ N-domain on EC growth was MAEC. Cells were cultivated in a 96-well plate, and

the surface of the wells were modified with fibrin as described above. The vitality of MAEC was estimated using the MTT test. The substantial decrease of viability of MAEC grown on the scaffolds formed by fibrin desAB $\beta$ 15-42 was observed (Fig. 5).

Thus, in two independent systems we demonstrated that the absence of the B $\beta$ N-domain prominently affected the ability of fibrin to support EC proliferation. The increasing of the observed effect from passage to passage in the case of HUVEC can indicate some regulatory role of the interaction of the B $\beta$ 15-42 fragment with EC, possibly occurring through recently described interactions with VLDLR. Both HUVEC and MAEC cell lines confirmed the exceptional role of the B $\beta$ 15-42 fragment in fibrin(ogen) binding to EC.

*Study of motility of cancer cells.* Having sites of interactions with cancer cells on its surface, fibrinogen can stimulate cancerogenesis, as well as promote tumor growth and invasion [37]. Using our model, we estimated the role of the B $\beta$ 1-42 fragment of fibrinogen in the motility of the lung cancer cell line H1299.

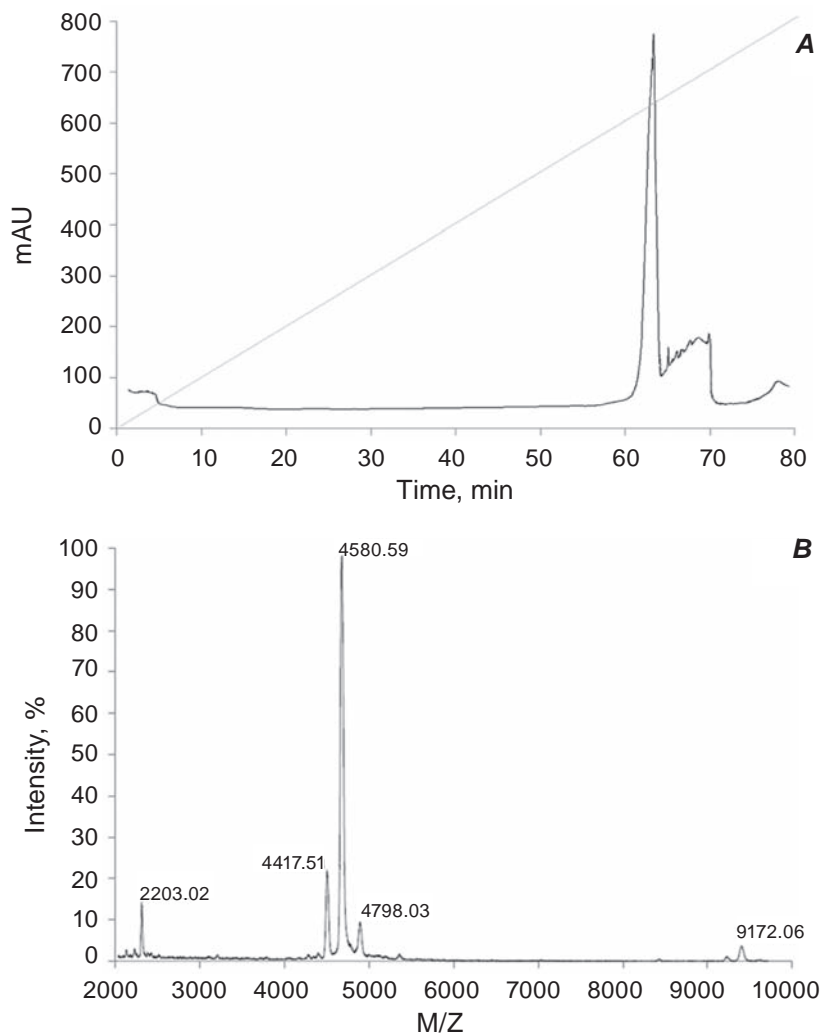


Fig. 2. Protease-digested peptide. **A** – Purification of protease-digested peptide using HPLC-chromatography on a C-18 column. The elution was performed using a linear gradient of acetonitrile from 0 to 100% against buffered saline. **B** – MALDI-TOF spectra of peptide digested from fibrinogen by protease and purified using size-exclusion chromatography on a Superdex G-75 column followed by HPLC on phenyl-sepharose. M/Z – mass to charge ratio

For these experiments we prepared monolayers of H1299 cells, generated scratches and observed cell migration over time in the presence of control or truncated fibrinogen (0.05 mg/ml). As shown in Fig. 9, desB $\beta$ 1-42 fibrinogen modulates the motility of H1299 cells in vitro. This effect can also be observed in Fig. 6.

By this preliminary experiment we demonstrated fibrinogen lacking the B $\beta$ 1-42 region was a less effective scaffold for cancer cell proliferation. Thus, this fragment is possibly important for fibrinogen binding to cancer cell receptors. It is still unclear whether it can interact with CAM-receptors as the  $\gamma$ 117-133 residue of fibrinogen. It would also

be important to study the direct effect of the B $\beta$ 1-42 fragment on the proliferation of cancer cells. Using the limited proteolysis technique we obtained a very useful model for testing on additional cancer cell lines. On the other hand, other receptors that can bind fibrinogen must be looked for more precisely on the surface of cancer cells, as interactions of fibrinogen with cancer cells could be a target for anticancer therapy.

### Discussion

Using protease from *Echis multisquamatis* venom we obtained fibrinogen lacking the B $\beta$ 1-42 fragment and compared it to the native uncleaved

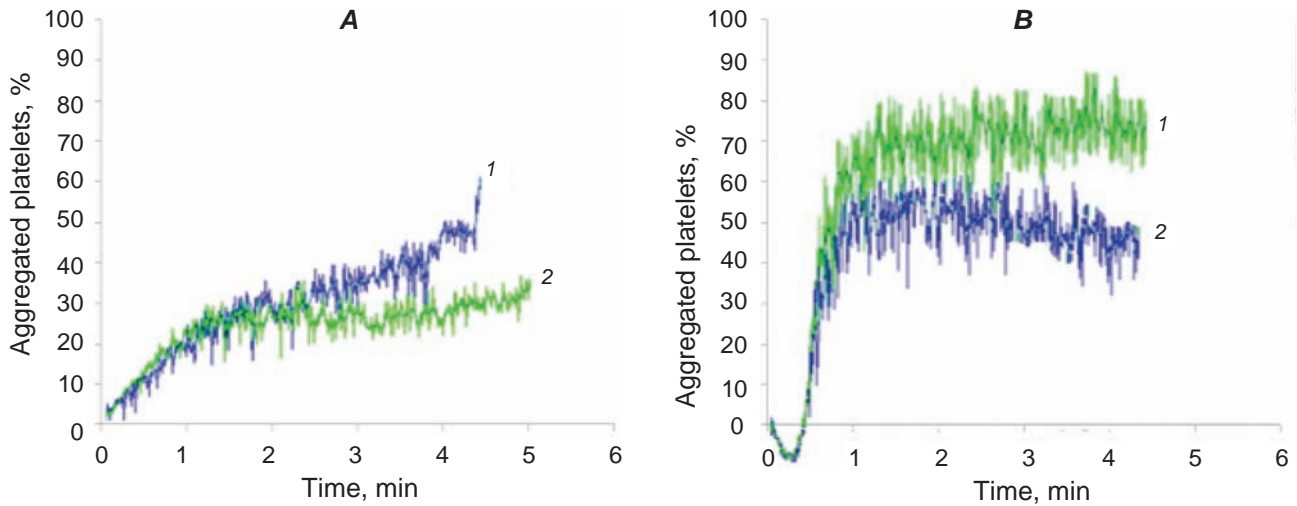


Fig. 3. Aggregation of washed platelets activated by (A) 2 μM of ADP or (B) 2.5 mg/ml of collagen in the presence of (1) native fibrinogen or (2) fibrinogen desBβ1-42. Curves of typical experiments, n = 5

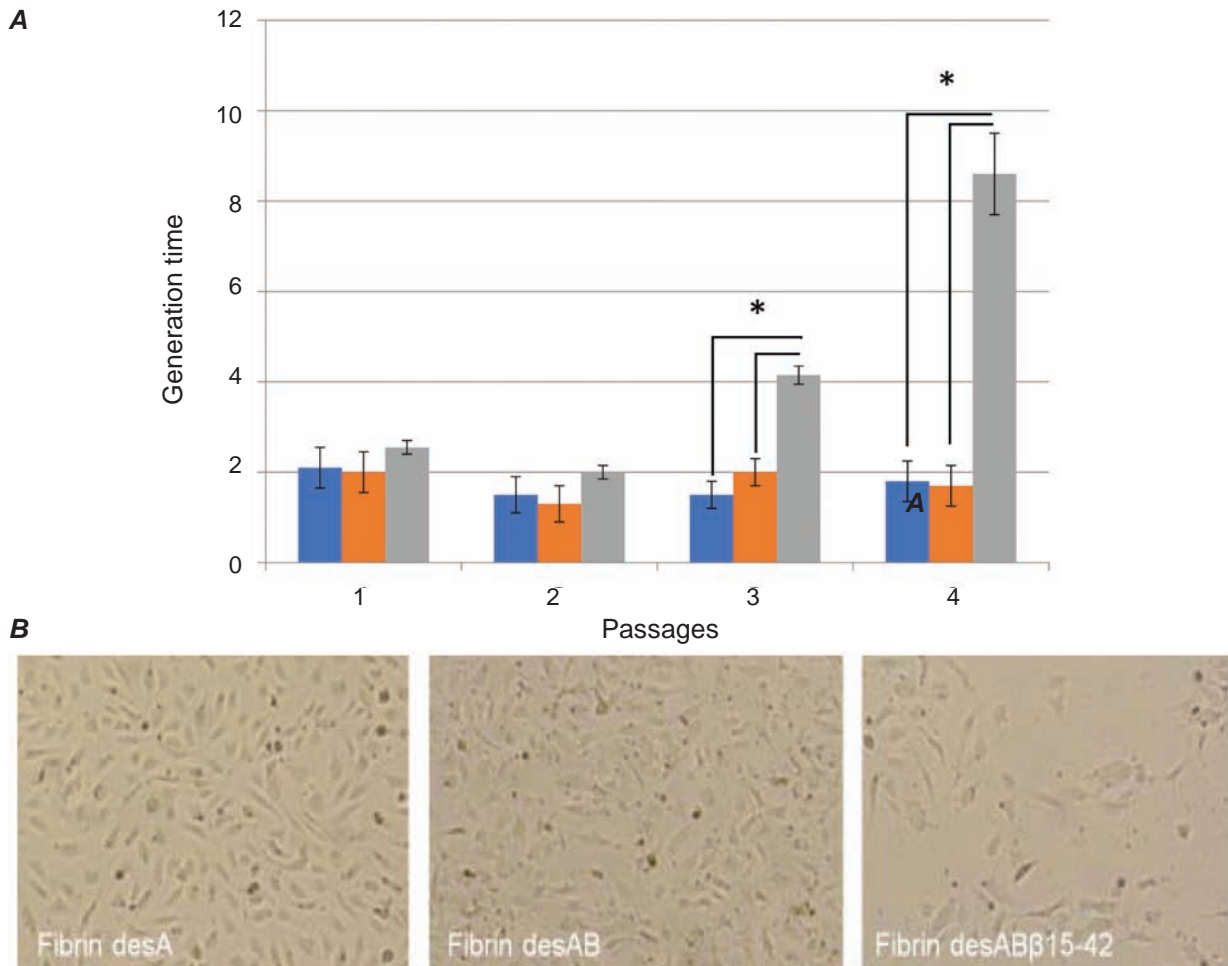


Fig. 4. A – Generation time of endothelial cells cultivated on the scaffolds formed by fibrin desA (blue bars), desAB (orange bars) and desABβ15-42 (grey bars). 1-4 – number of the cell passages. Result is significant for  $P < 0.05$ . B – Typical view of endothelial cells cultivated on the scaffolds formed by fibrin desA, desAB and desABβ15-42 (7<sup>th</sup> passage)



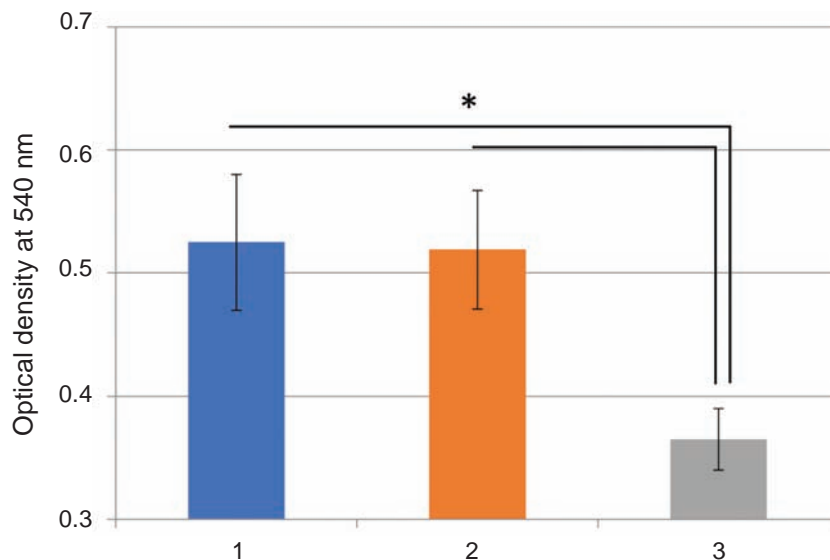


Fig. 5. Vitality of mouse aorta endothelial cells (MAEC) on the scaffolds formed by (1) fibrin desA, (2) desAB, and (3) desABβ15-42. Result is significant for  $P < 0.05$

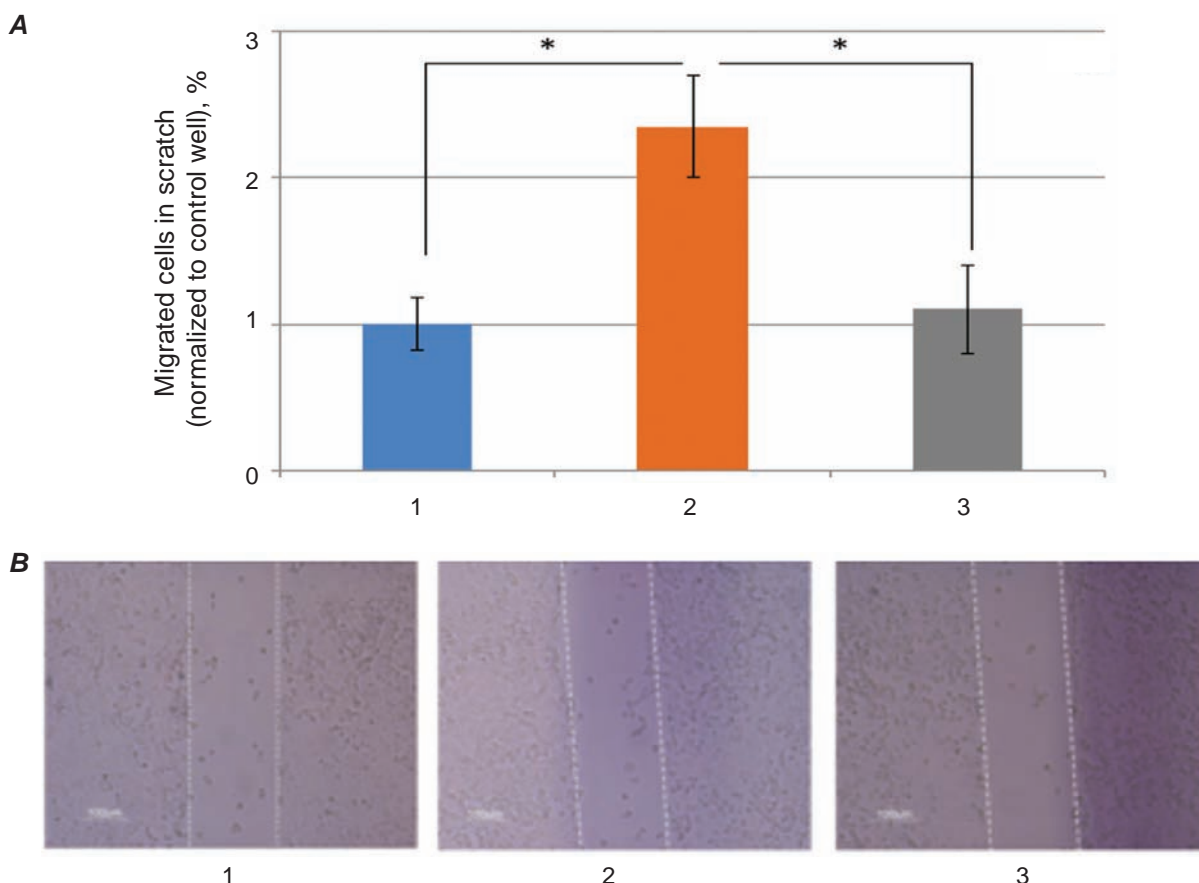


Fig. 6. H1299 cells in vitro in the presence of (2) native and (3) desBβ1-42 fibrinogen 1 – control sample without addition of fibrinogen. **A** – Motility of H1299 cells. Result is significant for  $P < 0.05$ . **B** – Typical experiment in scratch wound-healing test: migration of H1299 cells into the scratched zone in the presence of native or desBβ1-42 fibrinogen. Zones of the original scratch are marked by white lines

molecule in studies of aggregation of platelets, proliferation of EC and migration of cancer cells. The efficacy of all these processes were decreased in the presence of desB $\beta$ 1-42 fibrinogen in comparison to the native one. The findings allowed us to conclude that the B $\beta$ 1(15)-42 fragment of fibrin(ogen) contributed to the efficient interaction with platelets, EC and cancer cells.

However, these observations were rather indirect. We studied the processes in the presence of fibrinogen without the B $\beta$ 1-42 fragment, observing the effects of cell interactions with the rest of the molecule. These results must be assessed in combination with the results that were obtained using more direct methods. To probe the role of distinct parts of the residue B $\beta$ 1-42 in physiological processes one may use recombinant technology to obtain fibrinogen with amino-acid substitutions [38, 39], and monoclonal antibodies [40, 41] or peptides [42, 43] to block some part of the residues.

Our approach based on limited proteolysis has some advantages. In particular we are working with the delicately cropped native fibrinogen molecule that has its structure, glycosylation and conformation preserved; this is impossible when using recombinant analogues. Also, monoclonal antibodies or their Fab-fragments, even polypeptides, can cause non-specific spatial obstacles for intramolecular interactions. Using truncated forms of fibrinogen allowed us to avoid these limitations.

Taken together, from our data and the data obtained by other approaches we can assume the B $\beta$ N-domain of fibrinogen is an important center of fibrin(ogen) interactions with cellular receptors that can act by stabilizing the structure of the molecule (as in the case of platelet aggregation) as well as interacting directly with cellular receptors (as in the case of EC). Being also obligatory for fibrin polymerization, the B $\beta$ N-domain can be a target of therapeutics that would decrease not only thrombus formation, but also decrease fibrin(ogen) adhesion to endothelium or prevent metastasis of cancer cells.

*Conflict of interest.* Authors have completed the Unified Conflicts of Interest form at [http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi\\_disclosure.pdf](http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf) and declare no conflict of interest.

*Acknowledgement.* We thank for Cedars-Sinai Medical Center's International Research and Innovation in Medicine Program, the Association for Re-

gional Cooperation in the Fields of Health, Science and Technology (RECOOP HST Association) for their support of our organization as a participating Cedars-Sinai Medical Center – RECOOP Research Centers (CRRC).

## АГРЕГАЦІЯ ТРОМБОЦИТІВ, ПРОЛІФЕРАЦІЯ ЕНДОТЕЛІОЦИТІВ І МІГРАЦІЯ РАКОВИХ КЛІТИН ОПОСЕРЕДКОВАНІ В $\beta$ 1(15)-42 ФРАГМЕНТОМ ФІБРИНОГЕНУ

Є. М. Стогній<sup>1</sup>, М. В. Рижикова<sup>1</sup>,  
А. В. Ребрів<sup>1</sup>, М. Д. Кучма<sup>2</sup>, Р. Ю. Маруніч<sup>1</sup>,  
В. О. Чернишенко<sup>1</sup>✉, В. А. Шаблій<sup>2</sup>,  
Н. М. Липова<sup>3</sup>, О. Ю. Сломінський<sup>1</sup>,  
Л. В. Гарманчук<sup>4</sup>, Т. М. Платонова<sup>1</sup>,  
С. В. Комісаренко<sup>1</sup>

<sup>1</sup>Інститут біохімії ім. О.В. Палладіна  
НАН України, Київ, Україна;

<sup>2</sup>Інститут клітинної терапії, Київ, Україна;

<sup>3</sup>Університет Луїсвілла, США;

<sup>4</sup>ІНЦ “Інститут біології та медицини”,  
Київський національний університет  
імені Тараса Шевченка, Україна;

✉e-mail: bio.cherv@gmail.com

Молекула фібриногену містить численні ділянки зв'язування для різних типів клітинних рецепторів і виконує роль сполучної ланки між системою зсідання крові та клітинною адгезією. У цій статті описано отримання форми фібриногену, позбавлену фрагменту В $\beta$ 1-42 за допомогою спрямованого протеолізу, для вивчення ролі цієї послідовності у адгезивних властивостях тромбоцитів, ендотеліоцитів і ракових клітин. Фібриноген та фібрин, позбавлені В $\beta$ 1-42 та В $\beta$ 15-42 фрагментів відповідно (des $\beta$ 1-42 фібриноген і desAB $\beta$ 15-42 фібрин), отримано за допомогою протеїнази з отрути *Echis multisquamatis*. Відщеплений фрагмент отримували за допомогою HPLC та ідентифікували з використанням MALDI-TOF. ADP- і колаген-індуковану агрегацію тромбоцитів за приступності фібриногену desB $\beta$ 1-42 вивчали за допомогою агрегометра. Проліферацію клітин аорти миші (МАЕС) і клітин пуповинної вени людини (HUVES) вивчали з використанням фібрину desAB $\beta$ 15-42 як матриці. Вживаність клітин МАЕС оцінювали з використанням МТТ-тесту. Для оцінки проліферативної активності HUVES розраховували час подвоєння.

Міграцію клітин раку легенів H1299 вивчали за допомогою *in vitro* тесту подряпини. Пряме порівняння поведінки клітин за присутності нативної та частково гідролізованої форм показало порушення процесів клітинної адгезії за присутності фібриногену desB $\beta$ 1-42 та фібрину desB $\beta$ 15-42. Ступінь агрегації тромбоцитів незначно знижувався за присутності фібриногену desB $\beta$ 1-42, однак було виявлено дезагрегацію тромбоцитів на рівні 15-20%. Ми також виявили значне зниження інтенсивності поділу клітин HUVEC та інгібування виживаності клітин лінії МАЕС вирощених на матриці з desAB $\beta$ 15-42 фібрину. Крім того, фібриноген desB $\beta$ 1-42 модулював рухливість клітин лінії H1299 *in vitro* і знижував інтенсивність “заростання подряпини” до 20% порівняно з повнорозмірним фібриногеном. Показано, що фрагмент 1-42 B $\beta$ N-домени молекули фібриногену не є необхідним для агрегації тромбоцитів, однак вносить вклад у формування фібриновотромбоцитарного тромбу на пізніших стадіях. У той же час, цей фрагмент може бути важливим для забезпечення міцних міжклітинних контактів та виживаності ендотеліоцитів. Також амінокислотна послідовність 1-42 B $\beta$ N-домени підтримує міграцію ракових клітин, що дозволяє розглядати взаємодію з фібриногеном як потенційну мішень протиракової терапії. Фрагмент B $\beta$ 1-42 молекули фібриногену вносить вклад у ефективність міжклітинних взаємодій різних типів клітин, включаючи тромбоцити, ендотеліоцити і ракові клітини.

**Ключові слова:** фібриноген, адгезія, міграція клітин, ендотелій, проліферція клітин, тромбоцити.

### References

1. Medved L, Weisel JW. Recommendations for nomenclature on fibrinogen and fibrin. *J Thromb Haemost.* 2009; 7(2): 355-359.
2. Fuss C, Palmaz JC, Sprague EA. Fibrinogen: structure, function, and surface interactions. *J Vasc Interv Radiol.* 2001; 12(6): 677-682.
3. Weisel JW, Litvinov RI. Fibrin formation, structure and properties. *Subcell Biochem.* 2017; 82: 405-456.
4. Remjin JA, Ijsseldijk MJV, De Groot PG. Role of the fibrinogen  $\gamma$ -chain sequence  $\gamma$ 316-322 in platelet-mediated clot retraction. *J Thromb Haemost.* 2003; 1(10): 2245-2246.
5. Hantgan RR, Stahle MC, Lord ST. Dynamic regulation of fibrinogen: integrin  $\alpha$ IIb $\beta$ 3 binding. *Biochemistry.* 2010; 49(43): 9217-9225.
6. Chen CS, Chou S-H, Thiagarajan P. Fibrin(ogen) peptide B $\beta$  15-42 inhibits platelet aggregation and fibrinogen binding to activated platelets. *Biochemistry.* 1988; 27(16): 6121-6126.
7. Adelman B, Gennings C, Strony J, Hanners E. Synergistic inhibition of platelet aggregation by fibrinogen-related peptides. *Circ Res.* 1990; 67(4): 941-947.
8. Hamaguchi M, Bunce LA, Sporn LA, Francis CW. Spreading of platelets on fibrin mediated by the amino terminus of the  $\beta$  chain including peptide  $\beta$  15-42. *Blood.* 1993; 81(9): 2348-2356.
9. Bach TL, Barsigian C, Yaen CH, Martinez J. Endothelial cell VE-cadherin functions as a receptor for the beta15-42 sequence of fibrin. *J Biol Chem.* 1998; 273(46): 30719-30728.
10. Martinez J, Ferber A, Bach TL, Yaen CH. Interaction of fibrin with VE-cadherin. *Ann N Y Acad Sci.* 2001; 936: 386-405.
11. Yakovlev S, Medved L. Interaction of fibrin(ogen) with endothelial cell receptor VE-Cadherin: localization of the fibrin-binding site within the third extracellular VE-cadherin domain. *Biochemistry.* 2009; 48(23): 5171-5179.
12. Yakovlev S, Gao Y, Cao C, Chen L, Strickland DK, Zhang L, Medved L. Interaction of fibrin with VE-cadherin and anti-inflammatory effect of fibrin-derived fragments. *J Thromb Haemost.* 2011; 9(9): 1847-1855.
13. Yakovlev S, Mikhailenko I, Cao C, Zhang L, Strickland DK, Medved L. Identification of VLDLR as a Novel Endothelial Cell Receptor for Fibrin That Modulates Fibrin-Dependent Transendothelial Migration of Leukocytes. *Blood.* 2012; 119(2): 637-644.
14. Yakovlev S, Medved L. Interaction of fibrin with the very low density lipoprotein receptor: further characterization and localization of the fibrin-binding site. *Biochemistry.* 2015; 54(30): 4751-4761.
15. Garnacho C, Serrano D, Muro S. A fibrinogen-derived peptide provides intercellular adhesion molecule-1-specific targeting and intraendothelial transport of polymer nanocarriers in human cell cultures and mice. *J Pharmacol Exp Ther.* 2012; 340(3): 638-647.
16. Duperray A, Languino LR, Plescia J, McDowall A, Hogg N, Craig AG, Berendt AR,

- Altieri DC. Molecular identification of a novel fibrinogen binding site on the first domain of ICAM-1 regulating leukocyte-endothelium bridging. *J Biol Chem.* 1997; 272(1): 435-441.
17. Simpson-Haidaris PJ, Rybarczyk B. Tumors and fibrinogen. The role of fibrinogen as an extracellular matrix protein. *Ann N Y Acad Sci.* 2001; 936: 406-425.
  18. Roche Y, Pasquier D, Rambeaud JJ, Seigneurin D, Duperray A. Fibrinogen mediates bladder cancer cell migration in an ICAM-1-dependent pathway. *Thromb Haemost.* 2003; 89(6): 1089-1097.
  19. Lin Y-C, Shun C-T, Wu M-S, Chen C-C. A novel anticancer effect of thalidomide: Inhibition of intercellular adhesion molecule-1 mediated cell invasion and metastasis through suppression of nuclear factor-KB. *Clin Cancer Res.* 2006; 12(23): 7165-7173.
  20. Bonan S, Albregues J, Grasset E, Kuzet SE, Nottet N, Bourget I, Bertero T, Mari B, Meneguzzi G, Gaggioli C. Membrane-bound ICAM-1 contributes to the onset of proinvasive tumor stroma by controlling acto-myosin contractility in carcinoma-associated fibroblasts. *Oncotarget.* 2017; 8(1): 1304-1320.
  21. Belitser VA, Varetska TV, Manjakov VPh. On the model for the fibrinogen molecule. Consecutive stages of fibrin polymerization. *Thromb Res.* 1973; 2(6): 567-577.
  22. Solov'ev DA, Ugarova TP. Isolation and Characteristics of Alpha-Specific Thrombin-Like Enzymes From Venoms of the Common Pit Viper (*Agkistrodon Halys Halys*) and the Eastern Pit Viper (The Central Asian Subspecies *Agkistrodon Halys Blomhoffii*). *Biokhimiia.* 1993; 58(8): 1221-1233. (In Russian).
  23. Chernyshenko V, Shteinberg K, Lugovska N, Ryzhykova M, Platonova T, Korolova D, Lugovskoy E. Preparation of highly-concentrated autologous platelet-rich plasma for biomedical use. *Ukr Biochem J.* 2019; 91(2): 19-27.
  24. Laemmli UK. Cleavage of structural proteins during the assembly of the Head of bacteriophage T4. *Nature.* 1970; 227(5259): 680-685.
  25. Chernyshenko VO. Limited proteolysis of fibrinogen by fibrinogenase from *Echis multisquamatis* venom. *Protein J.* 2015; 34(2): 103-104.
  26. Gershkovich AA, Kibirev VK. Chromogenic and fluorogenic peptide substrates of proteolytic enzymes. *Bioorg Khim.* 1988; 14(11): 1461-1488. (In Russian).
  27. Chapman JR, Ed., Mass Spectrometry of Proteins and Peptides. Humana Press. Totowa, New Jersey. 2000, 538 p.
  28. Cattaneo M, Cerletti C, Harrison P, Hayward CP, Kenny D, Nugent D, Nurden P, Rao AK, Schmaier AH, Watson SP, Lussana F, Pugliano MT, Michelson AD. Recommendations for the standardization of light transmission aggregometry: A consensus of the working party from the Platelet Physiology Subcommittee of SSC/ISTH. *J Thromb Haemost.* 2013; 11(6): 1183-1189.
  29. Gao W, Chen SR, Wu MY, Gao K, Li YL, Wang HY, Li CY, Li H. Methylprednisolone exerts neuroprotective effects by regulating autophagy and apoptosis. *Neural Regen Res.* 2016; 11(5): 823-828.
  30. Liang C, Park A, Guan J. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc.* 2007; 2(2): 329-333.
  31. Mouritzen VM, Jenssen H. Optimized scratch assay for in vitro testing of cell migration with an automated optical camera. *J Vis Exp.* 2018;(138): e57691.
  32. Chernyshenko V, Platonova T, Makogonenko Y, Rebriev A, Mikhalevska L, Chernyshenko T, Komisarenko S. Fibrin(ogen)olytic and platelet modulating activity of a novel protease from the *Echis multisquamatis* snake venom. *Biochimie.* 2014; 105: 76-83.
  33. Chernyshenko VO, Myasnikova MP, Platonova TM, Lougovskoi EV, Makogonenko EM. Purification and biochemical characterisation of fibrinogenase from *Echis multisquamatis* venom. *Biotechnology.* 2010; 3(1): 27-34. (In Ukrainian).
  34. Zhou L, Schmaier AH. Platelet aggregation testing in platelet-rich plasma description of procedures with the aim to develop standards in the field. *Am J Clin Pathol.* 2005; 123(2): 172-183.
  35. Bryckaert M, Rosa J-P, Denis CV, Lenting PJ. Of von Willebrand factor and platelets. *Cell Mol Life Sci.* 2015; 72(2): 307-326.
  36. Brown AC, Barker TH. Fibrin-based biomaterials: Modulation of macroscopic properties through rational design at the molecular level. *Acta Biomater.* 2014; 10(4): 1502-1514.

37. Kwaan HC, Lindholm PF. Fibrin and fibrinolysis in cancer. *Semin Thromb Hemost.* 2019; 45(4): 413-422.
38. Moen JL, Gorkun OV, Weisel JW, Lord ST. Recombinant BbetaArg14His fibrinogen implies participation of N-terminus of Bbeta chain in desAA fibrin polymerisation. *Blood.* 2003. 102(7): 2466-2471.
39. Soya K, Terasawa F, Okumura N. Fibrinopeptide A release is necessary for effective B:b interactions in polymerisation of variant fibrinogens with impaired A:a interactions. *Thromb Haemost.* 2013; 109(2): 221-228.
40. Lugovskoi EV, Makogonenko EM, Chudnovets VS, Derzskaya SG, Gogolinsikaja GK, Kolesnikova IN, Bukhanevich AM, Komisarenko SV. The study of fibrin polymerisation with monoclonal antibodies. *Biomed Sci.* 1991; 2: 249-296.
41. Morris TA, Marsh JJ, Fagnani R, Hagan M, Moser KM. Degree of polymer organization decreases the binding of a monoclonal antibody raised against the beta-chain amino terminus of fibrin. *Thromb Haemost.* 1997; 77(4): 704-709.
42. Lugovskoy EV, Gritsenko PG, Kapustianenko LG, Kolesnikova IN, Chernishov VI, Komisarenko SV. Functional role of B $\beta$ -chain N-terminal fragment in the fibrin polymerization process. *FEBS J.* 2007; 274(17): 4540-4549.
43. Doolittle RF, Pandi L. Probing the beta-chain hole of fibrinogen with synthetic peptides that differ at their amino termini. *Biochemistry.* 2007; 46(35): 10033-10038.