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PLATELETS ARE ABLE TO CONVERSION OF ENDOGENOUS PLASMINOGEN TO FRAGMENTS AND TO SORT THEM

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Platelets play an important role in the process of tumor angiogenesis. The onset of tumor angiogenesis involves a net change in balance between angiogenesis stimulators and inhibitors in favor of the former. 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].

The elucidation of regulation of the platelet functioning by plasminogen/plasmin system is one of the priority areas of our research. Plasminogen/plasmin molecule proteolysis in the organism leads to the formation of kringle–containing fragments (K 1–3, K 1–4, K 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 cell (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 [2]. For some time it was believed that angiostatine 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.

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.

Materials and methods. Plasminogen was obtained from Cohn fraction $III_{2,3}$ of a human blood plasma ("Kiev municipal blood center", Ukraine) on the Lys–Sepharose in the presence of protease inhibitor kontrikal ("AWD", Germany) [3]. Mini–Pg was obtained by the limited hydrolysis of Pg with pancreatic elastase (3.4.21.36) ("Sigma", USA) [3]. Degradation of mini–Pg was performed with pepsin (3.4.23.1), the proteolytic hydrolase from pig gastric mucosa, ("Sigma", USA) [3]. All of the used chemical reagents were qualified not less than "chemical pure".

Methods used were: proteolytic fragmentation methods, affinity chromatography, ultrafiltration, gel filtration, ion–exchange chromatography, ELISA, Western blot analysis, electrophoresis in polyacrylamide gel, immunization, etc.

Statistical processing of experimental data was performed using Prism 5 software (GraphPad Software Inc., USA). Only the experimental results with the error not exceeding 5 % ($P < 0.05$) are included in the work. Blot results presented in the figures are typical for repeated experiments (at least three repetitions).

Results and discussion. To have an instrument to our assay we developed a method for producing a functionally active fragment of human plasminogen kringle 5 using AH–Sepharose. Proposed method includes the following stages: hydrolysis of plasminogen with elastase, separating the mini–plasminogen from kringle fragments 1–3 and 4 on the Lys–Sepharose, mini–plasminogen hydrolysis with pepsin, affinity chromatography on AH–Sepharose, analytical

electrophoresis in polyacrylamide gel [3]. It was obtained electrophoretically pure fragment of human plasminogen kringle 5, while the ability of kringle 5 to bind specifically with the AH–Sepharose demonstrates its functional activity with respect to the ligands of high and low molecular weight. Weight yield was 4.1%, which corresponds to 27.3% of the theoretically possible.

Then our aim was to obtain the polyclonal antibodies against to fragments of human plasminogen kringle 5 and kringle 1–3, received in the obtaining process of kringle 5. The following approaches were used: immunization of rabbits against the kringle 5 and kringle 1–3, receiving of high titer immune serum, synthesis of affinity sorbent based on the kringle 5 and kringle 1–3 for selection of monospecific antibodies, chromatography on synthesized K5–Sepharose and K1–3–Sepharose, ELISA, analytical electrophoresis in polyacrylamide gel, immunoblotting assay [4, 5].

For the obtained affinity sorbents, the ligand binding to BrCN–Sepharose was no less than 90 %. The received column had capacity per ligand no less than 75 nmol per 1g of BrCN–Sepharose, and capacity for binding antibodies of 1.0 ÷ 1.5 nmol per 1g of BrCN–Sepharose. The content of the obtained immunoglobulins in preparations evaluated by electrophoresis was no less than 95 %, the output of polyclonal antibodies was no less than 1 mg per 1 ml of immune serum.

Further immunochemical properties of obtained antibodies were studied by ELISA and immunoblotting assay. It has been found that polyclonal antibodies against to kringle 5 and kringle 1–3 are monospecific and have a high affinity to their epitopes (the dissociation constants of the value order 10^{-10} M). So, such antibodies were found to be applicable in our research.

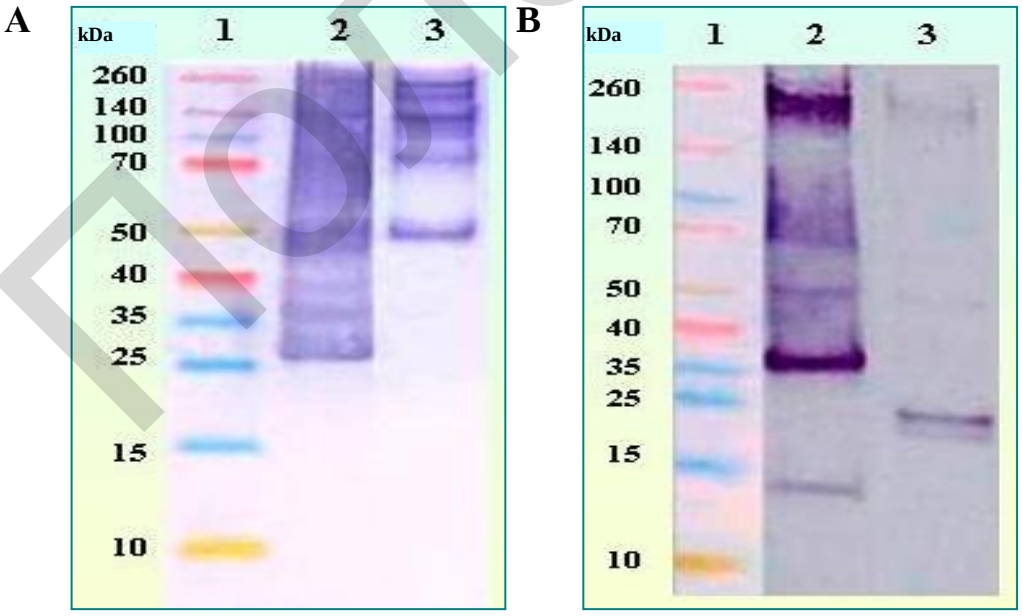

Figure 1 – Proteins detected by the means of polABs specific to K 1–3 (A) or K 5 (B) on the membranes (2) and in the lysates (3) of the platelets; 1 – molecular weight markers. Platelets were preincubated with K 1–3 (A) or K 5 (B).

We obtained preliminary data on the interaction of isolated K 1–3 and K 5 plasminogen fragments (angiostatins) with the platelet surface. Detection of angiostatins was carried out by western blotting assay using polyclonal antibodies (polABs) monospecific to K 1–3 and K 5, which have been obtained by us for this purpose. Results concerning fragments of plasminogen found on the membranes and in the lysates of the platelets by the means of polABs specific to K 1–3 (A) or K 5 (B). are presented in Figure 1. After previous incubation of platelets with K 1–3 and K 5, the fragments were detected in isolated plasma membranes and absent in cell lysates. The antibodies to K 1–3 revealed plasminogen and 51 kDa angiostatin–like fragment on membranes and in lysates, whereas antibodies to K 5 revealed miniplasminogen on membranes, and a microplasminogen in the inner medium of the cells, indicating the ability of platelets to conversion of endogenous plasminogen and to sorting plasminogen fragments.

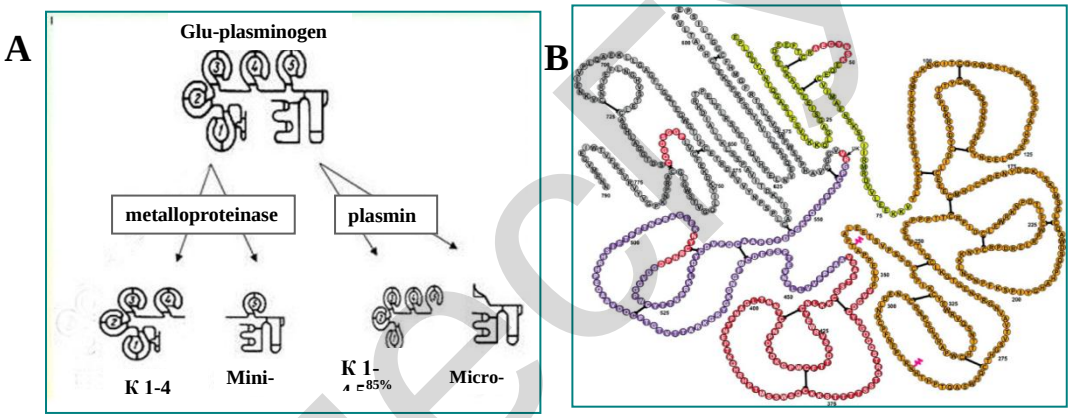

Figure 2 – Scheme of possible proteolytic cleavage of plasminogen molecules (A). Scheme of plasminogen molecules (B).

Conclusions. There are binding sites for plasminogen kringles 1–3 and 5 on the surface of platelets. K 1–3 and K 5 are bound to the surface membranes but do not pass inside the cell. Plasminogen is detected in the inner medium of the platelets and it may undergo proteolytic fragmentation. This data was obtained by the means of polABs specific to K 1–3 or K 5.

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